Universidade de Évora - Instituto de Investigação e Formação Avançada

Programa de Doutoramento em Biologia

Tese de Doutoramento

Incorporating host ecology and the environment into infectious disease epidemiology: modelling the transmission risk of animal tuberculosis in a multi-host system

Eduardo Miguel Ramos Ferreira

Orientador(es) | Elsa Leclerc Duarte

Sara M. Santos

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Abstract

Shared infections at the wildlife-livestock interface are a major concern for local and global economies, animal welfare, biodiversity, and public health. In many Mediterranean ecosystems, animal tuberculosis (TB), caused by *Mycobacterium bovis*, an ecovar of *Mycobacterium tuberculosis complex* (MTBC), is maintained by multi-host communities in which cattle and wildlife species establish interaction networks that contribute to *M. bovis* transmission and persistence in the environment. The interplay between host species and the environment can significantly affect pathogen transmission dynamics, yet this remains an overlooked research topic. In this PhD project, we aimed to assess the transmission risk of *M. bovis* in a multi-host system involving wildlife (ungulates and carnivores) and cattle, focusing on the importance of host interactions through an integrated eco-epidemiological perspective. Based on an experimental design within a *Montado* ecosystem, we demonstrate that disease-relevant interactions between wildlife and cattle are mainly indirect, through asynchronous space sharing, and that indirect interactions between wildlife species also occur frequently at shared interfaces. Overall, indirect interactions between sympatric species are determined by different ecological backgrounds, depending on the host and season, with host abundance playing a key role. We also demonstrated the presence of MTBC DNA in the environment, covering a large portion of the study area. Transmission risk analyses – integrating host space use data, environmental variables, and MTBC mapping – confirmed that red deer and wild boar significantly overlap with areas where MTBC occurs, determining the highest-risk areas for MTBC transmission. Additionally, when considering the co-occurrence of hosts (multi-host scenario), we found that one-quarter of the study area harbours conditions enabling high-risk MTBC transmission. Altogether, our results highlight the importance of incorporating host ecology and the environment into infectious disease epidemiology when multiple hosts species are involved, identifying critical areas for pathogen transmission, and providing insights to prioritise monitoring and control strategies.

Keywords

Camera-trapping; Interactions; Multi-host pathogen; Wildlife–cattle interface

Integração da ecologia dos hospedeiros e do ambiente na epidemiologia de doenças infeciosas: modelação do risco de transmissão da tuberculose animal em sistemas multi-hospedeiro

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Resumo

A circulação de agentes infeciosos na interface fauna selvagem–animais de produção tem impactos económicos expressivos, implicações no bem-estar animal, biodiversidade e saúde pública. Em ecossistemas mediterrânicos, a tuberculose animal (TB), causada por _Mycobacterium bovis_, uma ecovariante do complexo _Mycobacterium tuberculosis_ (MTBC), é mantida por comunidades multi-hospedeiro nas quais bovinos e espécies silvestres estabelecem redes de interações que contribuem para a transmissão e persistência de _M. bovis_. A relação entre espécies hospedeiras e o ambiente pode afetar significativamente a dinâmica de transmissão do agente etiológico, tópico que carece de maior investigação. Neste projeto, visámos avaliar o risco de transmissão de _M. bovis_ num sistema multi-hospedeiro envolvendo ungulados e carnívoros silvestres e bovinos, focando a importância das interações entre hospedeiros sob uma perspetiva eco-epidemiológica. No _Montado_, as interações entre bovinos e espécies silvestres registadas foram principalmente indiretas, através do uso assíncrono do espaço. As interações indiretas envolvendo exclusivamente animais silvestres foram também registadas frequentemente em interfaces partilhadas. Verificou-se que as interações indiretas entre espécies simpátricas são determinadas por diferentes contextos ecológicos, dependendo do hospedeiro e da época do ano, desempenhado a abundância de hospedeiros um papel significativo nestes gradientes. Confirmou-se a presença do DNA de MTBC no ambiente, com deteção alargada a grande parte da área de estudo. As análises de risco de transmissão – integrando dados espaciais dos hospedeiros, variáveis ambientais e mapeamento de MTBC – indicam que o veado e o javali são as espécies associadas às áreas de maior risco de transmissão do MTBC. Considerando um cenário multi-hospedeiro, os resultados sugerem que um quarto da área de estudo apresenta condições para um risco elevado de transmissão de MTBC. Estes resultados destacam a importância de incorporar a ecologia dos hospedeiros e o ambiente na caraterização epidemiológica de doenças infeciosas em cenários multi-hospedeiro, identificando áreas críticas para a transmissão de agentes patogénicos.

Palavras-chave

Armadilhagem fotográfica; Interacções; agente patogénico multi-hospedeiro; interface fauna selvagem–animais de produção

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Background

1.1 Infectious disease dynamics in wildlife–livestock interfaces

Livestock has appeared with the domestication of wildlife species by humans thousands of years ago (McMichael 2004). Since then, wildlife has been coexisting with livestock throughout the years in dynamic shared interfaces. Wildlife-livestock interfaces are defined as physical spaces where wildlife and domestic species can overlap in space and time, frequently along with humans (so called wildlife-livestock-human interface), and where they can potentially interact (Hassell et al. 2017, Caron et al. 2021). Such interfaces can be found on every continent on Earth, and, depending on the region and ecosystem, they are composed by many distinct domestic and wildlife species, as well as management systems. Those interfaces are complex and permeable to changes, particularly human-derived changes. The loss of natural environments driven by land-use changes, habitat fragmentation and habitat loss (e.g. agricultural, livestock production and urbanization) to support expanding human population (Venter et al. 2016, White & Razgour 2020, Meurens et al. 2021) promotes human encroachment into wildlife habitats, leading to ecosystems changes, including the dynamic alteration and/or creation of new wildlife-livestock interfaces (Figure 1-1).

Such changes bring livestock (and also humans) into closer proximity to wildlife, thereby enhancing the intensity of epidemiological interactions between species at shared interfaces where communities mix (Gordon 2018). Epidemiological interactions are events during which different species come directly/indirectly into contact with one another and with the potential to spread pathogens (Manlove et al. 2022, Yang et al. 2023). Wildlife and livestock species interact because they share and/or compete for resources (e.g. water and cover). The greater intensity of species interactions increases the risk of pathogen transmission, thus affecting the dynamics and emergence of infectious diseases (Jones et al. 2013, Alexander et al. 2018, White & Razgour 2020).

Figure 1-1. Newly formed wildlife-livestock interfaces resulting from the encroachment of human activities (livestock production in this case) into natural habitats where wildlife populations exist. The plus sign (+) indicates higher likelihood of pathogen spillover and transmission in shared interfaces. Adapted from Karmacharya et al. 2024.

From an epidemiological perspective, the risk of emerging diseases at shared interfaces have never been so real, and this is confirmed by the global spread of animal pandemics, such as avian influenza, African swine fever, rabies, and pathogenic coronaviruses (Jori et al. 2019, Gortázar & de la Fuente 2020). Shared pathogens can impact animal health, production systems, biodiversity, and wildlife management. Indeed, over 20% of global animal production losses are caused by animal diseases (Karmacharya et al. 2024). Furthermore, human well-being is also at risk, as many shared pathogens are zoonotic, meaning they also involve humans in their epidemiology. Indeed, non-human animal-borne infections have the potential to spread to humans. A significant proportion of emerging zoonotic diseases (at

least 70%) are estimated to have a wildlife origin (Jones et al. 2008, 2013, White & Razgour 2020).

The transmission of pathogens from a wildlife source to domestic hosts (and vice-versa) involves complex mechanisms operating within ecological communities, often with many hosts involved in disease dynamics (De Garine-Wichatitsky et al. 2021). These processes are influenced by the characteristics of both hosts and environments at shared interfaces. Different wildlife-livestock interfaces emerge as a function of the extent of agriculture use, livestock production husbandry systems, the composition and structure of natural communities, as well as human influence (Jones et al. 2013, Cravino et al. 2024). Shared wildlife-livestock interfaces can occur with varying degrees of human influence, from landscapes with limited human intervention to highly human-modified landscapes characterised by intensive farming and livestock production, and by increasing interactions with wildlife (Jones et al. 2013). Wildlife-livestock interfaces cover a significant amount of the world's geographic area, and the coexistence of agriculture, livestock and wildlife is frequent (Vicente et al. 2021). On a global scale, extensive research has been conducted in Europe, examining infectious disease across diverse wildlife-livestock interfaces (Wiethoelter et al. 2015, White & Razgour 2020). Within these interfaces, those involving cattle *Bos taurus* have garnered considerable attention, being one of the most studied.

1.2   Wildlife–cattle interfaces in the Iberian Peninsula

The Iberian Peninsula (IP) is characterised by a variety of ecosystems and landscapes unique in Europe due to its geographical location and climate. The region's traditional land uses, particularly those associated with agricultural and forestry systems, are highly diverse. Here, cattle plays an important economic role in human-modified landscapes where cattle production occurs in different intensity regimes, from intensive to extensive (Hocquette et al. 2018, Plieninger et al. 2021). Extensive cattle farming in the southwestern quadrant of the Iberian Peninsula is significantly linked to agroforestry systems, known as *Montado* in Portugal and *Dehesa* in Spain (Pinto-Correia et al. 2011, Freitas et al. 2020). Oak-based agroforestry systems (hereafter called *Montado*) are highly complex structures and often considered high nature value farming systems, covering an area of about 3.5-4.0 M ha of the Iberian Peninsula (Pinto-Correia et al. 2011, 2018), and are included in Annex I of the European union Habitats Directive (92/43/CEE). These systems combine cork (*Quercus*

suber) and/or holm (*Quercus rotundifolia*) forestry – with varying tree densities and shrub cover – and mostly cattle production, often also encompassing other livestock species, such as pigs, sheeps, and goats, and even cereal production for supplementary animal feed (McAdam et al. 2008, Carreira et al. 2023). Given the habitat heterogeneity and multiple vegetation layers, these systems tend to support high levels of biodiversity, and a balance between human activity and biodiversity values is evident (Curveira-Santos et al. 2017, da Silva et al. 2019). As a result, a wider and permeable interface exists, allowing cattle and wildlife to coexist in sympatry, sharing habitats and resources (Figure 1-2).

Figure 1-2. Example of a wildlife–cattle interface in an agroforestry system (*Montado*) located in southern Portugal, where livestock (cattle and domestic pigs) and wildlife (wild boar *Sus scrofa*, on the left size of the picture) share habitats and resources. Photo credits: Eduardo M. Ferreira.

Various environmental and socio-economic factors have been acting as driving forces in wildlife–cattle interfaces in *Montado* landscapes across the Iberian Peninsula (Godinho et al. 2016, Plieninger et al. 2021). Particularly, hunting interests, alongside other factors (e.g. farming practices), have led to a notable overlap between large game hunting and cattle farming, and also to artificialization, a trend now observed in many regions (Kukielka et al. 2013, Carpio et al. 2021, Barroso & Gortázar 2024). Consequently, these interfaces have become more interconnected, resulting in increased species interactions, and ultimately modifying the eco-epidemiology of various animal shared diseases (Gortázar et al. 2016, Ciaravino et al. 2021, Jiménez-Ruiz et al. 2021, Duarte et al. 2024). Numerous diseases

impact cattle production systems in wildlife–cattle interfaces across the Iberian Peninsula. However, animal tuberculosis (TB) stands out as a primary disease concern, epidemiologically interconnecting cattle, wildlife, and environmental factors, not only in the Iberian Peninsula but also in other regions worldwide (Wiethoelter et al. 2015, Caminiti et al. 2016, Gortázar et al. 2016, Pereira et al. 2024a). The control of TB is considered a central component of global one-health strategies (González-Barrio 2022, Zhang et al. 2022, Gortázar et al. 2023). Therefore, it is crucial to study dynamic wildlife–cattle interfaces in agroforestry systems to uncover solutions that cope to our changing environment and mitigate the impacts of TB.

1.3 Animal Tuberculosis

1.3.1 Etiology

Animal tuberculosis is caused by bacterial species belonging to the *Mycobacterium tuberculosis* complex (MTBC) that comprises now 11 recognized species. *Mycobacterium bovis* is the species that displays the broadest spectrum of hosts, infecting cattle and wildlife and the target of most eradication programmes. All MTBC members share 99.9% similarity at the nucleotide level and identical 16S rRNA sequences (Fitzgerald & Kaneene 2013, Pereira et al. 2020). TB is a chronic slow-progressing disease that can affect a wide range of wild mammals, and also humans (Gortázar et al. 2015, Reis et al. 2020a), being one of the most widespread zoonotic diseases worldwide. Indeed, animal TB has been detected in almost every country across the world (Humblet et al. 2009). Other major MTBC pathogenic mycobacteria species include the *M. tuberculosis* – the leading infectious pathogen in the world causing tuberculosis in humans – and *M. caprae*, responsible, to a lesser extent, for TB in animals, namely caprine (Palmer et al. 2012, Zhang et al. 2022).

1.3.2 Tuberculosis in cattle

M. bovis infection and TB are important in animal production systems, as infection can occur in a broad range of domestic mammal species (e.g. goats, sheep, and pigs). Nevertheless, cattle, a multi-purpose livestock as they serve as source of milk, meat, and hides, for example (Felius et al. 2011), are very likely the most economically relevant domestic species affected by the disease on a global scale (Hardstaff et al. 2014, Pesciaroli et al. 2014, Pereira et al.

2020, Reis et al. 2020b). Today there is almost 38 million cattle in Western Europe (worldwide it is estimated ~ 1.5 billion cattle), with beef production reaching 6.8 million tonnes in the European Union, being the world´s third largest producer, after the United States of America (USA) and Brazil (FAOSTAT 2024).

Animal TB has substantial economic implications for the livestock industry and animal welfare since infected cattle may exhibit reduced weight gain and milk production. Also, the negative economic impacts of TB in cattle include premature culling of animals, trade restrictions, rejections at slaughterhouses, and costly eradication plans when mandatory (Zinsstag et al. 2008, Caminiti et al. 2016, Pérez-Morote et al. 2020). Several animal TB free European countries have an epidemiological surveillance programme of the disease implemented. In non-animal TB free European Union (EU) member states, considerable financial costs are associated with eradication programmes, including testing (e.g. performing skin and blood tests) and economic compensation to farmers. For instance, in 2022, the financial package to fund bovine TB eradication programmes in Europe constituted an investment of ~ 12.4 million euros (HaDEA 2023). While some countries may include small ruminants in their eradication programmes, the majority of such initiatives exclusively target cattle. The single intra-dermal comparative tuberculin skin test (SICTT) is the primary screening test for identification *in vivo* of suspected infected animals in most countries, including Portugal. The IFN-γ assay, a blood-based test recommended by the World Organization for Animal Health (WOAH), is a complementary test with higher sensitivity and earlier detection of infected animals (Pereira et al. 2020, Ncube et al. 2022).

Long-term TB control and eradication programmes has allowed some countries in the EU to achieve a disease-free status: in 2022, 17 member states (e.g. Germany, Poland, France) were officially tuberculosis-free, three countries had disease-free zones or provinces (e.g. Portugal, one region [Algarve] and all the Azores islands, except São Miguel) and seven countries had no zones with disease-free status (EFSA & ECDC 2023). TB affects cattle on a global scale (Figure 1-3), and disease prevalences is very heterogenous across continents and regions, depending also on the diagnostic tests used (Ramos et al. 2020). The pooled prevalence of TB in cattle was estimated to be 10.3% (CI 95% [8.8 – 25.9]) in Africa and 13.8% (CI 95% [8.5 – 11.9]) in Asia, with North America (33.6%, CI 95% [25.2 – 43.2] and Europe (17.8%, CI 95% [11.8 – 25.9%]) having higher rates; such differences are likely related to greater cattle densities and more intensive production systems (Ramos et al. 2020).
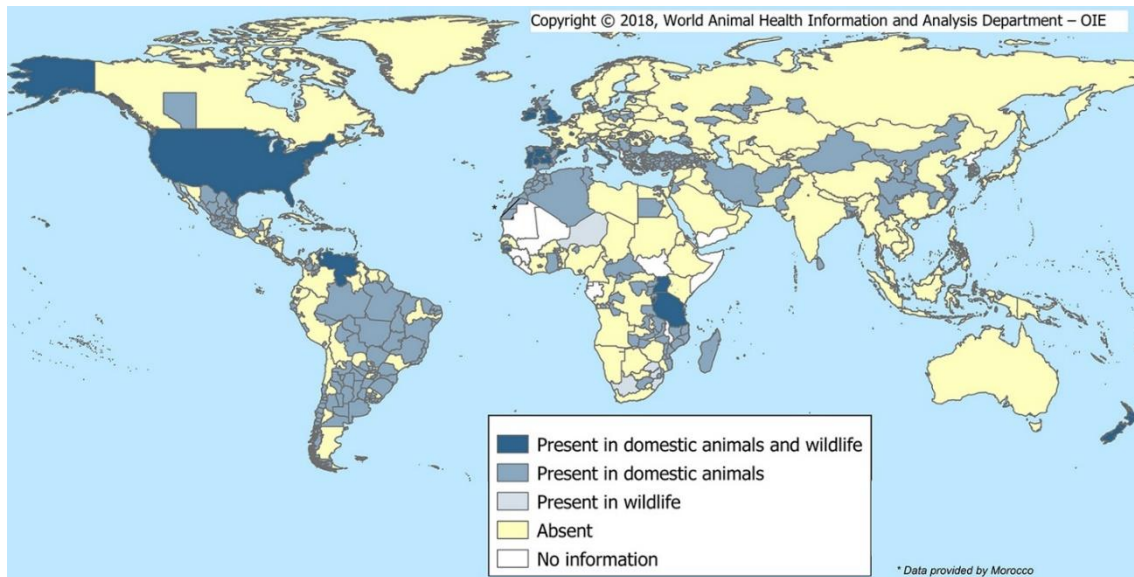
Figure 1-3. Global distribution of animal TB in livestock and wildlife based on 2017 and 2018 epidemiological data. Source: World Animal Health Information System (WAHIS)

The epidemic situation in the EU in 2022 was characterised by an overall 0.61% proportion of cattle herds infected with MTBC, slightly higher compared with the previous year (2021; 0.54%). However, TB prevalence varied widely among member states with approved eradication programmes (EFSA & ECDC 2023). In Portugal, a TB eradication programme has been in place since 1987, funded by the EU since 1992 (Council Decision 92/299/CE) (Cunha et al. 2011). The eradication programme encompasses Portugal mainland and also the autonomous regions of Azores and Madeira (having the SICTT test as the reference diagnostic test). In 2022, the overall herd prevalence of TB in Portugal mainland and Azores remains low (0.65%), below the values of other non-TB free European countries (DGAV 2023, EFSA & ECDC 2023). A marked heterogeneity is observed in TB prevalence across main regions of Portugal mainland, with the Alentejo region having the highest percentage of infected herds (1.83%).

1.3.3 Tuberculosis in wildlife

A wide range of wildlife species can be infected with *M. bovis* in distinct eco-epidemiological scenarios across the world (Palmer et al. 2012, Gortázar et al. 2015, Reis et al. 2020a, Ncube et al. 2022) (Figure 1-3). Specifically, wildlife reservoir hosts are considered to be a main obstacle to cattle eradication programmes worldwide (Palmer 2013, Gortázar et al. 2015, Pereira et al. 2020) due to the occurrence of *M. bovis* infected wildlife and consequently

wildlife-to-cattle transmission. Reservoir hosts are defined as epidemiologically connected populations in which the pathogen can be permanently maintained, and from which infection is transmitted to other susceptible hosts, thus contributing to the geographical expansion of animal TB (Palmer et al. 2012, Palmer 2013). Recognised wildlife reservoir hosts of *M. bovis* include the African buffalo *Syncerus caffer* in South Africa, brushtail possums *Trichosurus vulpecula* in the New Zealand, badger *Meles meles* in the United Kingdom (UK) and Republic of Ireland, white-tailed deer *Odocoileus virginianus* in the USA, and wild boar and red deer *Cervus elaphus* in the Iberian Peninsula (Fitzgerald & Kaneene 2013, Pereira et al. 2020). In Australia, the Asian buffalo *Bubalus arnee* was only considered a reservoir of *M. bovis* during a restricted period of time, as successful eradication of the disease was achieved. Furthermore, *M. bovis* has been reported in other wildlife species (e.g. carnivores and ungulates) that occur in different TB contexts (Delahay et al. 2007, Walter et al. 2013, Matos et al. 2014b, Richomme et al. 2020, Varela-Castro et al. 2021a, Payne et al. 2022).

A significant amount of research on TB epidemiology in wildlife has been conducted throughout the world (Gortázar et al. 2012), with European countries contributing the most to this field, as evidenced by Reis et al. (2020a). Varying spatial and temporal trends in terms of TB prevalence has been documented, depending on the host and eco-epidemiological contexts. For instance, TB prevalence in wild boar can reach up to 23% in Europe, with low incidence rates in Asia. At the country level, TB prevalence in wild boar varied between 31.68% in Spain to 9.49% in France (Reis et al. 2020a). In the Iberian Peninsula, TB is maintained in a multi-host system where *M. bovis* circulate between cattle and wildlife species that share space and resources under extensive husbandry systems (Santos et al. 2012). Besides the red deer and wild boar – considered the most TB-relevant wildlife hosts (Santos et al. 2022) – other species have also been found infected (e.g. badger, red fox *Vulpes vulpes*, fallow deer *Dama dama*) (Santos et al. 2012). Considerable prevalence estimates of TB, for example, in foxes (up to 26.9%) and in badgers (up to 23%) were reported in multi-host TB endemic regions of the Iberian Peninsula (Santos et al. 2012, Matos et al. 2014b, a). Even though these species probably act as spillover hosts, the extent to which those species may be involved in TB epidemiology remains poorly understood and should be further clarified according to specific eco-epidemiological contexts (Santos et al. 2012, Matos et al. 2014b).

Regarding wild boar and red deer, trends in TB prevalence increment in these species have been reported during the 1990s and 2000s years across the Iberian Peninsula (Santos et al. 2012). For instance, in Doñana National Park (Southern Spain), *M. bovis* prevalence increased by 100% in wild boar and by 50% in red deer from 1998-2003 to 2006-2007 (Gortazar et al. 2011). Similarly, in east-central Portugal, some studies recorded high animal TB prevalence values and evidenced an increase in prevalence from 2008-2009 to 2009-2013, with a 35% increase in wild boar and a 300% increase in red deer (Vieira-Pinto et al. 2011, Matos et al. 2014). Given the growing role of wild boar and red deer in TB epidemiology in the last decades in Portugal, in April of 2011, an epidemiological risk area for TB in big game has been established in Portugal (Figure 1-4). Accordingly, from a legal standpoint, it demands a mandatory veterinary meat examination – performed by a credentialed veterinarian – and eventual sample collection for bacteriology and histopathology in the case of big game showing TB compatible lesions (DGAV 2011). Nowadays, the high-risk areas for TB in wildlife in Portugal exhibit spatial structuring, being confined to the east-central region of mainland Portugal, contiguous to Spain with no physical separation (Cunha et al. 2011, Santos et al. 2018). Given that, the disease can be considered as a transboundary disease since wildlife movement (e.g. ungulates) across borders are not infrequent and transmission pathways may extend across both countries. High animal TB prevalences are documented in Portugal, though differences in prevalences values occur across regions (Cunha et al. 2011, Santos et al. 2012). A recent meta-regression and systematic review analyses estimated the pooled TB prevalence at a national scale as 27.5% and 13.3% for the red deer and wild boar, respectively (Reis et al. 2020a). Indeed, recently, Santos et al. (2020) demonstrated that TB burden in wildlife hosts exceeds that of infected cattle in the Iberian Peninsula.

Figure 1-4. Epidemiological risk area for big game (wild boar and red deer) in mainland Portugal. Adapted from DGAV 2011. The five regions of Continental Portugal according to NUTS II are also displayed.

1.3.4 Pathology

M. bovis infection manifests differently in various hosts. In cattle, it is typically present as a chronic, progressive respiratory disease, with lesions commonly found in the respiratory tract and mesenteric lymph nodes depending on the infection route (Menzies & Neill 2000, Cassidy 2006). While inhalation of contaminated aerosols is acknowledged as the primary transmission route, ingestion of contaminated fomites can also lead to infection although generally requiring a higher bacterial load. In countries with active eradication programmes, generalized TB lesions are less common in cattle (Neill et al. 2001) as animals are culled earlier during the infection course. In ungulates like wild boar and red deer, TB lesions are frequently observed in lymph nodes and lungs (Martín-Hernando et al. 2010, Santos et al. 2010, Vieira-Pinto et al. 2011, Palmer 2013). Lesions can affect a specific organ or present a generalized pattern (i.e. lesions in > 1 anatomic region) (Martín-Hernando et al. 2010, Vieira-Pinto et al. 2011). *M. bovis* shedding may occur through multiple sources, including aerosols, saliva, and faeces. (Palmer et al. 2012, Santos et al. 2015a). Carnivores, such as badgers and

red foxes, also exhibit respiratory-centric TB pathology (Delahay et al. 2007, Fitzgerald & Kaneene 2013). Lesions are commonly found in the lungs and lymph nodes, with considerable pulmonary infection. While badgers may not always display visible gross lesions, they can excrete mycobacteria from multiple routes, including the respiratory, digestive, and urinary tracts (Corner et al. 2011, Palmer et al. 2012). Infected red foxes show lower evidence of generalised infection, with limited gross lesions. However, they may also excrete *M. bovis* via faeces (Delahay et al. 2007, Richomme et al. 2020).

1.4 Transmission pathways of *M. bovis* at the wildlife–cattle interface

Transmission is a fundamental step in the life cycle of pathogens, being also one of the most challenging processes to uncover and quantify (McCallum et al. 2017). *M. bovis* transmission among hosts can occur through direct (host-to-host) and indirect routes (host-to-environment) (Pereira et al. 2020). Historically, *M. bovis* transmission was believed to occur primarily through aerosols during direct interactions that require close proximity or physical contact between an infected and a susceptible host (Morris et al. 1994, Palmer et al. 2012). Direct transmission thus occurs primarily via the respiratory route and is likely to have a significant importance in intra-specific transmission (Kukielka et al. 2013, Cowie et al. 2016, Triguero-Ocaña et al. 2020a). This is because direct interactions between individuals of the same species tend to be frequent, particularly in social and gregarious species that live in social groups, as the case of the wild boar, red deer, and badgers (Drewe et al. 2013, Cowie et al. 2016). On the other hand, direct transmission is expected to play a minor role in transmission of *M. bovis* between different species. Direct interactions involving wildlife and cattle, as well as among different wildlife species, have been infrequently documented and are described as rare events by some authors (Kukielka et al. 2013, Woodroffe et al. 2016, Campbell et al. 2019, Varela-Castro et al. 2021b). Nevertheless, further investigation is essential to understand these interactions across diverse ecological contexts and landscapes.

Indirect transmission involves shared space use in different time frames, that is, when animals contact asynchronously through contaminated environments (e.g. food or water sites). Indirect transmission has been suggested to occur in several TB disease systems, as the case of ungulates-cattle in the Iberian Peninsula (Barasona et al. 2016, 2017), badger-

cattle in the UK and Ireland (Woodroffe et al. 2017), and white-tailed deer-cattle in the USA (Walter et al. 2014, Wilber et al. 2019). High rates of indirect interactions involving cattle, ungulate reservoir hosts (red deer and wild boar) and suspected spillover hosts (e.g. red fox) have been reported in Iberian contexts, where farming typically occurs close to wildlife habitats (Cowie et al. 2016, Triguero-Ocaña et al. 2019, 2020a, Varela-Castro et al. 2021b). Interspecies transmission of *M. bovis* may occur when cattle/wildlife come into contact with wildlife-contaminated fomites (e.g. badger latrines). However, the use of farm-related resources (e.g. water sites) seems to be the most frequent and significant scenario in some areas of Iberian Peninsula (Drewe et al. 2013, Carrasco-Garcia et al. 2016, Balseiro et al. 2019). This mode of transmission should be particularly important in TB dynamics within agroforestry systems where wildlife coexists in sympatry with cattle, and has access to farm resources, thus favouring sharing of potentially contaminated resources (Kukielka et al. 2013). Indeed, contamination of environmental substrates (e.g. water and soil) with MTBC DNA has been evidenced in Mediterranean TB areas across the Iberian Peninsula (Santos et al. 2015b, Barasona et al. 2016, Pereira et al. 2023). Given the viability maintenance of MTBC at various environmental sites used by a variety of hosts, animals are thus exposed to increased infection risk (Vicente et al. 2013, Barasona et al. 2016, Triguero-Ocaña et al. 2020b, Pereira et al. 2023). In recent years, indirect transmission of *M. bovis* through shared contaminated environments has been hypothesised as one of the likely main mechanisms of interspecies disease transmission (Allen et al. 2021), though animal-environment transmission has only been confirmed very recently (Pereira et al. 2024b).

1.5 Ecology of cattle and wildlife hosts in the Iberian Peninsula

1.5.1 Cattle

Cattle belongs to the Bovidae family. Domestic cattle have various breeds, each adapted as a function of environmental conditions and management practices across the world (Felius et al. 2011, Zhang et al. 2020). In beef cattle farms of Mediterranean regions of the Iberian Peninsula, cattle (e.g. *Mertolenga* breed, <u>Figure 1-5 a</u>) are a free-ranging ruminant, reaching a mature weight of ~700kg (Reis et al. 2001). Cattle concentrate their activity nearby human settlements and in natural pasture areas mostly managed by humans. Cattle can explore medium to large areas (Home range = 2km^2 to 18 km^2; Barasona et al. 2014, Cowie et al.

2016), preferring open landscapes within agroforestry systems, but also can make use of more forested areas or other types of habitats (Zengeya et al. 2014, Triguero-Ocaña et al. 2019). Agroforestry landscapes provides a mosaic of pastures with shrub areas where cattle can roam (Pinto-Correia et al. 2018). Cattle, however, exhibit non-uniform use of available space in such landscapes, frequently preferring sites that provide access to essential resources, like water, food, and shaded areas (Carrasco-Garcia et al. 2016, Von Müller et al. 2017, Costa et al. 2018). The use of water sites becomes particularly evident during summer periods when water resources are limited – in number and size – (Kukielka et al. 2013), and the temperatures may soar to high values, reaching up to 45° C. In terms of activity, cattle are a diurnal species with two main peaks of activity, one concentrated into early morning (6-8h) and other during the late afternoon/dusk period (17-21h) (Kukielka et al. 2013)

1.5.2 Wild ungulates

The wild boar (Figure 1-5 c) and red deer (Figure 1-5 d) belong to the taxonomic families Suidae and Cervidae, respectively, order Artiodactyla. Over the last decades, wild ungulates have undergone a significant population growth and an expansion of their geographic range across the Iberian Peninsula (Acevedo et al. 2011), linked to changes in farming practices, wildlife management (e.g. hunting purposes) and rewilding policies (Massei et al. 2015, Laguna et al. 2021b). In the Iberian Peninsula, the wild boar and red deer are big-game species, highly exploited in the hunting sector.

The wild boar is a large omnivorous species with marked sexual dimorphism. Females weight range between 30 to 70 kg, while males tend to be larger (60-118 kg) (Fernández-Llario & Mateos-Quesada 1998, López-Martín et al. 2007). Nowadays, the species has a widespread distribution across the Iberian Peninsula (present all over the Portuguese continental territory; Bencatel et al. 2019), and can reach high population densities in certain areas (> 10 individuals/km^2), depending on the habitat and management practices (Giménez-Anaya et al. 2020, Laguna et al. 2021a). The wild boar is a generalist and opportunistic species, being able to explore different habitats and a variety of food resources (e.g. vegetable matter, vertebrates, and invertebrates). They prefer forested areas with dense shrub cover, but species spatial behaviour is notably affected by human disturbance and land use management (Laguna et al. 2021a). The wild boar can display an annual home range of 17.88 km^2 (Barasona et al. 2014), with varying estimates in agroforestry systems during the hunting

season (Laguna et al. 2021a). Females live in social groups made up of one to several other females, while mature males are typically solitary (but younger males may also reside in social groups) (Giménez-Anaya et al. 2020, Laguna et al. 2021a). Wild boars are mainly active during nocturnal and crepuscular periods, with a peak of activity between 23h and 5h (Kukielka et al. 2013).

The red deer <u>(Figure 1-5 d)</u> is a large herbivorous species with marked sexual dimorphism. Adult females weight ranges between 50-100 kg, while males tend to be larger (80-160 kg) (Gebert & Verheyden-Tixier 2001, López-Martín et al. 2007, Rodriguez-Hidalgo et al. 2010). The species is now common and widespread in all the Iberian Peninsula territory. In Portugal, the most representative populations are located near boarder areas with Spain (e.g. Barrancos and Montesinho), and in the Lousã region due to a successful reintroduction programme (López-Martín et al. 2007, Bencatel et al. 2019). Red deer densities can vary from moderate (6.3 individuals/km^2) to high as 46.5 individuals/km^2 in the Iberian Peninsula (Laguna et al. 2021b). The species tend to prefer forested areas and patches with high shrub cover, avoiding human settlements and open areas (Carvalho et al. 2018, Ares-Pereira et al. 2022). They can explore large areas, with home ranges extending up to 7.25 km^2, depending on the landscape context (Cowie et al. 2016, Laguna et al. 2021b). Females tend to live in social family groups year-round. Males are more solitary than females, but can form male-only groups outside the breading season (Jaedrzejeski et al. 2006, Bocci et al. 2012). The species is often described as crepuscular/nocturnal, with two peaks of activity, during the sunrise (5-8h) and sunset (19-22h) (Kukielka et al. 2013).

1.5.3 Carnivores

The red fox <u>(Figure 1-5 e)</u> is a medium-sized mesocarnivore. Adult females weight ranges between 3.1-7.8 kg, while males tend to be slightly larger (4.6-8.6 kg) (López-Martín et al. 2007). Red fox occurs throughout all the territory of the Iberian Peninsula, and it is one of the most widespread and abundant carnivores (Bencatel et al. 2019). As a generalist species, red fox can exploit a variety of habitats, including forest and agricultural areas, and is often found in agroforestry systems. In *Montado* landscapes, the species tends to use more intensively areas with dense shrubby understory, while avoiding semi-disturbed mosaics of sparse shrubs (Curveira-Santos et al. 2017, Alexandre et al. 2020). In terms of diet, most studies indicate that the species is a generalist and opportunistic predator that uses

resources (e.g. fruits, invertebrates, lagomorphs) based on their availability (Díaz-Ruiz et al. 2013). Red fox densities vary across regions according to ecological contexts, but could be as high as 20 individuals/km^2 (López-Martín et al. 2007). In *Montado* landscapes, Palencia et al. (2021) documented red fox density estimates of less than one individual/km^2. The few studies conducted in the Iberian Peninsula revealed red fox mean home ranges around 2.18 km^2 (Travaini et al. 1993). The species is considered as a solitary forager that might form groups in certain circumstances. The species exhibits predominantly nocturnal activity, although it also displays crepuscular behaviour and some daytime activity (Curveira-Santos et al. 2017).

Badger (Figure 1-5 b) is a medium-sized mesocarnivore having a reduced sexual dimorphism. Adult females weight ranges between 5.6 and 7.4 kg, while males are slightly larger (5.6-8.8 kg) (Rosalino et al. 2005c, López-Martín et al. 2007). The species has a generalised distribution in the Iberian Peninsula, with a more pronounced presence in the south of Portugal (Bencatel et al. 2019). Badger uses preferentially forest areas as well as agroforestry landscapes, although they may occur in other habitat types (Hipólito et al. 2018). In *Montado* landscapes, the species favours homogenous areas such as forest areas with minimum shrubby understory and riparian vegetation (Rosalino et al. 2004, Curveira-Santos et al. 2017). The species behaves as a generalist predator, or a season specialist, adapting its diet to the abundance and availability of resources (e.g. fruits and arthropods) throughout the year (Rosalino et al. 2005a, Hipólito et al. 2016). In agroforestry systems in Mediterranean Europe, badger populations can reach densities of 0.36-0.48 individuals per km^2, although higher densities (3.8 individuals/km^2) have been documented in other Iberian contexts (e.g. Atlantic Spain) (Acevedo et al. 2014). Badgers use home range areas averaging 4.46 km^2, frequently occupied by multiple individuals organised into social groups. (Rosalino et al. 2004). The species is predominantly nocturnal, with limited activity during dusk and dawn (Rosalino et al. 2005b, Curveira-Santos et al. 2017).
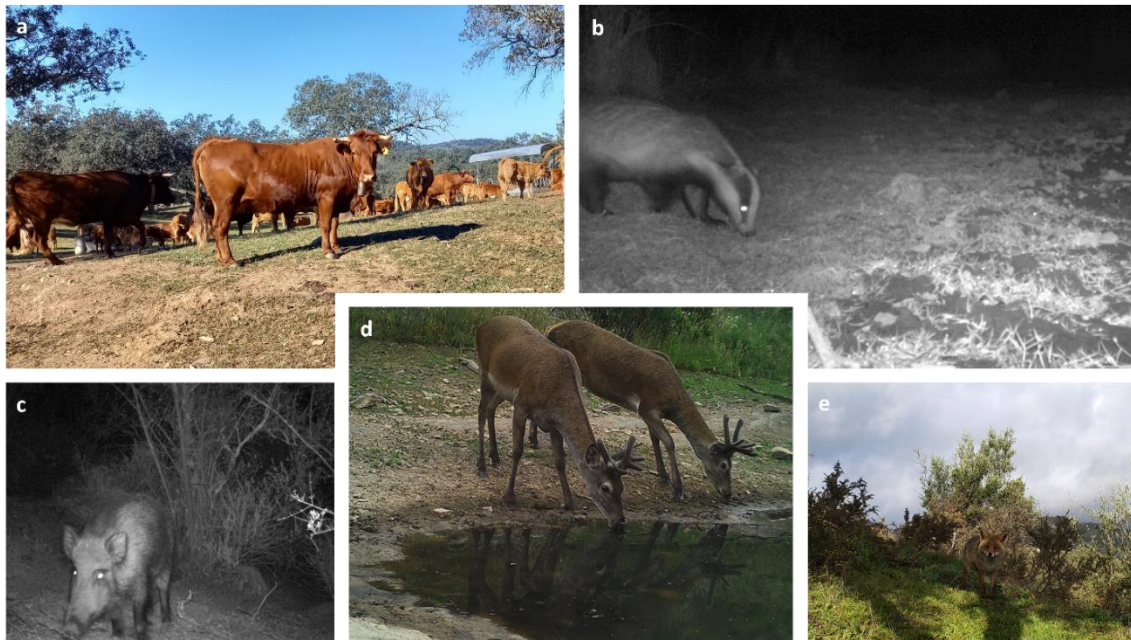
Figure 1-5. Pictures of the species targeted in this study. a) cattle, including adults and juvenile; b) badger; c) wild boar; d) red deer (males drinking in a water site) and e) red fox. Photograph credits: Eduardo M. Ferreira.

1.6 Control of TB at the wildlife–cattle interface

Disease control can take many forms, including preventive actions, host population control, environment/habitat management and vaccination. However, a significant amount of actions applied so far in the context of TB are centred in reducing wildlife density through massive culling (Gortazar et al. 2015, Ham et al. 2019). In principle, culling wildlife species is expected to reduce transmission by decreasing infection prevalence among wildlife populations and limiting disease-relevant interactions between wildlife cand cattle (Wobeser 1994, Gortazar et al. 2015). However, culling actions towards TB control in wildlife populations have shown varying degrees of success, and sometimes counterproductive results as well, depending on the study system. Successful eradication of TB in wildlife through culling has been achieved in Australia, targeting water buffalo populations (considered a reservoir between 1970 and 1990s), and in Minnesota, targeting white-tailed deer populations (not a reservoir in the region) (Radunz 2006, Palmer 2013). In addition, the implementation of culling showed promising results in New Zealand, focusing on the local reservoir, the brushtail possum (Livingstone et al. 2015). However, TB eradication has not been achieved in New Zealand and in other geographic regions where culling has taken place, namely the UK, the Republic of Ireland, and even in certain regions of the USA (Palmer

et al. 2012, Gortazar et al. 2015). Wild boar culling has also been implemented in the Iberian Peninsula (Boadella et al. 2012). Reduction of TB in wild boar has been observed in intervened areas, with positive effects extending to the reduction of TB prevalence in other host species (red deer) (Boadella et al. 2012). Nevertheless, despite its widespread use, culling can lead to mixed results and has rarely proved successful at reducing the threat of TB infection from wildlife (Gortazar et al. 2015). Thus, this strategy is not universally accepted.

Other methods to control TB shared between wildlife and cattle include wildlife vaccination and actions upon the environment in terms of management (Gortázar et al. 2016). Vaccination is considered a promising approach and has garnered significant attention in recent years (Gortázar et al. 2015). Studies have progressed from testing vaccines in experimental settings to evaluating their efficacy in wildlife populations under field conditions (Buddle et al. 2013). Some studies have demonstrated that vaccination can induce protection against *M. bovis* in controlled environments, with lesion severity considerably reduced (e.g. wild boar as a case study in Spain) (Buddle et al. 2013, Gortazar et al. 2014, 2015). Attempts have also been made in wildlife free-raging populations, but these efforts have faced numerous challenges (Díez-Delgado et al. 2018). Vaccination of cattle is prohibited in many countries (including Portugal), while trials are in progress in several countries to evaluate the use of the currently available vaccine Bacillus-Calmette-Guérin (BCG) against *M. bovis* infection (Srinivasan et al. 2021). Regarding environmental and habitat-related control measures, a common rationale is to prevent or reduce disease-relevant interactions between infected and susceptible hosts by minimizing shared space use (Gortázar et al. 2015, 2016). These actions consist of biosecurity measures. For example, studies have evaluated the effectiveness of wildlife and cattle-specific fencing around key aggregation sites (i.e. water sites) in shared interfaces, while others have examined the impact of supplementary feeding for wildlife and related management actions (e.g. prohibition of supplementary feeding) (Gortazar et al. 2011, Barasona et al. 2013, LaHue et al. 2016).

Despite the exploration of various control strategies focusing on wildlife species and the implementation of cattle eradication programmes, these approaches have not been sufficient to effectively control TB, particularly in multi-host areas with complex epidemiology. Consequently, new strategies based on interdisciplinary research are required

to address the growing challenge of managing and control TB at the wildlife–cattle interface (Conteddu et al. 2024, Jori & Fine 2024).

1.7 The need of multidisciplinary research approaches to curb animal tuberculosis

Animal Tuberculosis remains a costly One Health challenge in many TB epidemiological scenarios. From an ecological perspective, different approaches (e.g. camera-trapping, GPS telemetry; proximity collars) have been used to characterise and gain better understanding of host ecology within TB host communities (Kukielka et al. 2013, Cowie et al. 2016). Interaction patterns between *M. bovis* hosts have been serving as a reasonable proxy to discuss potential pathogen transmission risk, since it is very difficult to record real transmission events (e.g. Cowie et al. 2016, Campbell et al. 2019, Triguero-Ocaña et al. 2019). While studies at a local/regional scale are available, there is a lack of evidence at a global scale. Uncovering interaction patterns globally is a necessary first step to understand the transmission pathways encompassing various wildlife–cattle interfaces. Nevertheless, the lack of standardisation in experimental approaches regarding data collection and expression poses constraints on the comparability of research results in this field.

Pathogen transmission occurs where host and pathogen movement paths intersect (Manlove et al. 2022). In this sense, transmission risk depends not only on the spatial ecology of hosts (e.g. interactions, animal abundance), but also on the extent of environmental contamination (Dougherty et al. 2018, Allen et al. 2021). This is particularly relevant in Mediterranean areas where TB transmission pathways likely include a strong environmental component, given the substantial evidence of environmental contamination with *M. bovis* (Santos et al. 2015b, Barasona et al. 2016, Pereira et al. 2023) and the frequent shared space among sympatric hosts (Kukielka et al. 2013, Triguero-Ocaña et al. 2020, Varela-Castro et al. 2021b). Despite the evident need to integrate animal ecology and disease fields, multi-disciplinary analyses are still uncommon, representing an under-explored avenue for investigation of disease systems at the wildlife-livestock interfaces (Dougherty et al. 2018, Manlove et al. 2022). To date, no spatial attempts have been made to integrate ecological host data with environmental exposure to TB. Linking these components is crucial for predicting the spatial likelihood of transmission risk in multi-hosts communities and

identifying specific TB hotspot areas where control strategies should be a priority. In the Iberian context, a significant proportion of high-risk areas regarding TB in wildlife is located along the terrestrial border between Portugal and Spain (Santos et al. 2018). Regardless of the eco-epidemiological similarities between Portugal and Spain (e.g. in terms of hosts, land use, environmental conditions), Portugal may harbour specific conditions related to *M. bovis* hosts, and, consequently, TB dynamics. The composition, spatial ecology, and interaction dynamics of TB communities within wildlife–cattle high-risk areas remain unknown in Portugal, and so several ecological and epidemiological questions cannot be solved, and transmission risk remains a key challenge.

1.8   Thesis focus and main goals

This thesis focuses on modelling the transmission risk of animal tuberculosis (TB) in a Mediterranean multi-host system involving wildlife species (ungulates and carnivores) and cattle, in the national context, Portugal, with incorporation of host ecology and environmental contamination. More specifically, this study intends to disclose and quantify the importance of indirect transmission in multi-host TB epidemiology by considering space use and interactions of different hosts in shared environments at the wildlife–cattle interface within the *Montado* ecosystem. Accordingly, the following specific research goals were defined:

1   Review the main criteria used to define wildlife–cattle interactions relevant to *M. bovis*/MTBC transmission considering the main methodological approaches reported in the scientific literature **(Chapter 3)**;

2   Estimate direct and indirect interaction rates between wildlife and cattle TB hosts on a global scale, and identify key factors likely responsible for variation in interaction patterns **(Chapter 3)**;

3   Assess the spatial-temporal interaction patterns among a multi-host mammal community through camera-trapping in a TB endemic area within a Mediterranean agroforestry system (*Montado*) **(Chapter 4)**;

4   Identify main ecological and environmental factors regulating wildlife–cattle and wildlife-wildlife interactions across spatial-temporal scales **(Chapter 4)**;

5   Evaluate the extent of environmental contamination with MTBC (a proxy for *M. bovis*) within a Mediterranean agroforestry system **(Chapter 5)**;

6   Identify main ecological and environmental factors influencing patterns of spatial contamination with MTBC **(Chapter 5)**;

7   Predict potential high risk-areas for MTBC transmission at the wildlife–cattle interface **(Chapter 5)**;

8   Identify wildlife hosts that most likely contribute to TB maintenance within the multi-host community under focus in the study area **(Chapters 4 and 5)**;

1.9   Thesis outline

This thesis is structured into six Chapters. **Chapter 1** corresponds to the introduction, while **Chapter 2** focuses on the selected study area. The results section opens with the investigation of host ecological interactions at both global and local scales **(Chapters 3 and 4, respectively)**, followed by the evaluation of environmental contamination by MTBC in the study area and the assessment of transmission risk gradients **(Chapter 5)**. The Chapters presenting results relate to three independent scientific manuscripts: two published **(Chapters 3 and 4)**, and one submitted **(Chapter 5)** to peer reviewed journals. The six chapters are organized as follows:

- **Chapter 1** is the general introduction which includes background information and research goals, corresponding to the present chapter.

- **Chapter 2** includes the biophysical and environmental description of the study area (SA) in the Barrancos region (southeast Portugal), detailing the wildlife–cattle interface in terms of wildlife occurrence and cattle management. Also, it provides a concise overview of temporal trends in TB epidemiology among wildlife and cattle for the region.

- **Chapter 3** presents a systematic review of the criteria used to define and express wildlife–cattle interactions in TB context, considering the main methodological approaches in the field. This chapter also includes a meta-analysis on wildlife–cattle interaction estimates on a global scale, and identifies the underlying ecological factors influencing interaction patterns. Recommendations for future research to

establish harmonised methodological protocols within an eco-epidemiological perspective are provided.

> **Paper #1** – Ferreira EM, Duarte EL, Cunha MV, Mira A, Santos SM (2023) Disentangling wildlife– cattle interactions in multi- host tuberculosis scenarios: systematic review and meta- analysis. *Mammal Review*, 53, 287–2023. https://doi.org/10.1111/mam.12324

- **Chapter 4** examines the spatial-temporal patterns of interactions between wildlife and cattle and between different wildlife species in the national context, Portugal. Conducted in an endemic TB risk area within a Mediterranean agroforestry system (*Montado*) in the Barrancos region, southeast Portugal, this study used a longitudinal experimental design with camera-trapping to determine interspecies interactions within a multi-host mammal community across spatial-temporal scales. Generalized Linear Mixed Models (GLMM) were employed to assess the potential influence of ecological factors on interspecies indirect interaction patterns during wet and dry seasons. Results identified the host species most likely involved in TB dynamics and highlight conditions potentially favouring transmission of *M. bovis* at the wildlife–cattle interface.

> **Paper #2** – Ferreira EM, Cunha MV, Duarte EL, Gonçalves R, Pinto T, Mira A, Santos SM (2024) Host-, environment-, or human-related effects drive inter-species interactions in an animal tuberculosis multi-host community depending on the host and season. *Transboundary and Emerging Diseases* 2024: 9779569. https://doi.org/10.1155/2024/9779569

- **Chapter 5** investigates the extent of environmental contamination with MTBC in the study area and identifies environmental and ecological factors influencing the spatial occurrence of MTBC in environmental matrices. Second, transmission risk maps were generated by overlaying host space use data with predicted areas of MTBC occurrence. Based on the results, implications for disease management are evaluated in this chapter, highlighting potential high risk-areas for MTBC transmission according to different ecological scenarios.

> **Paper #3** – Ferreira EM, Cunha MV, Duarte EL, Mira A, Pinto D, Mendes I, Pereira AC, Pinto T, Santos SM. Mapping high-risk areas for *Mycobacterium*

tuberculosis complex bacteria transmission: linking host space use and environmental contamination. *Science of the Total Environment*, submitted.

- **Chapter 6** corresponds to the General Discussion, summarising the main findings from the previous three chapters integrating and discussing the key achievements in a broader perspective. It concludes by outlining suggestions of future research and offering concluding remarks.

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Chapter 2

Study area

Chapter 2 – Study area

This study was carried out within Moura-Barrancos Natura 2000 site (Council decision 76/2000; directive 92/43/CEE), specifically encompassing the agroforestry areas north of the village of Barrancos, located in the southeast and near the Portuguese-Spanish border (Figure 2-1). The study area (SA) comprised five major adjoining farms with similar management practices, that may be considered a single epidemiological unit, comprising an area of ~ 3048 ha (farm size ranging from 148 ha to 980 ha).
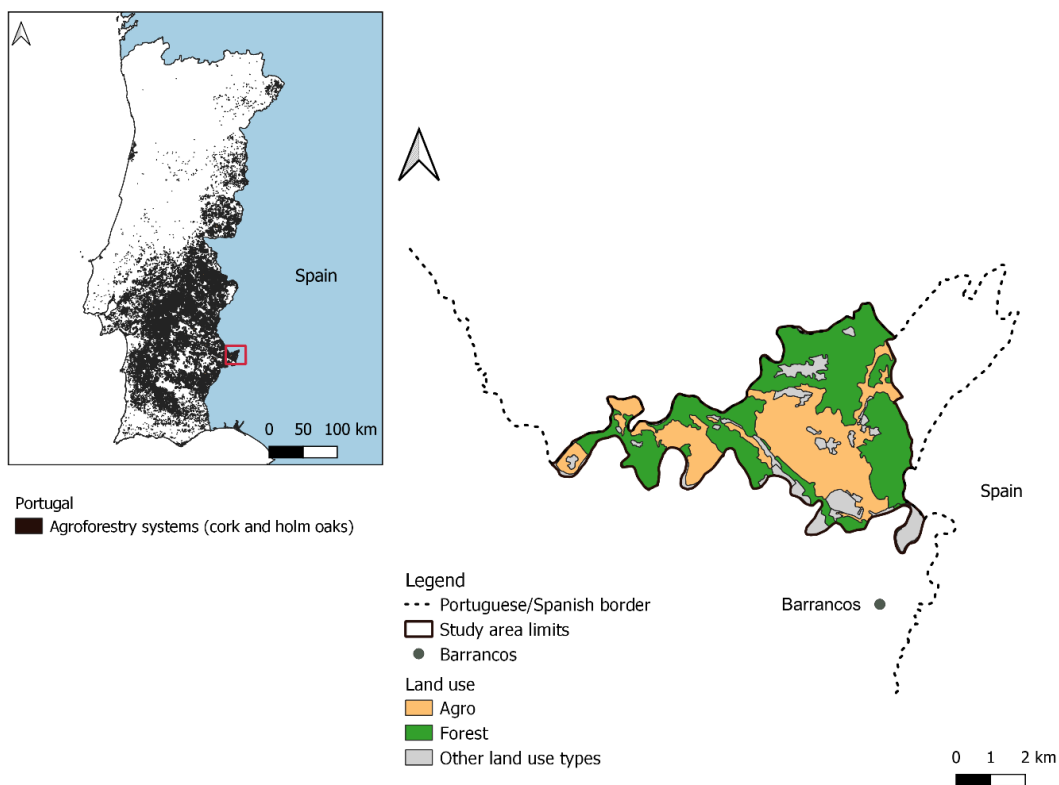
Figure 2-1. Location of the SA in Barrancos region, showing main land uses (right image), plotted against the distribution of agroforestry systems (including cork and holm oaks) in the mainland territory of Portugal (left image). Source: Land use and land cover map of continental Portugal for 2018 – COS2018.

2.1 Climate

The climate in the SA is Mediterranean, characterised by mild and wet winters and hot and dry summers. Mean winter temperatures (January) range from 5 °C to 14 °C, while mean summer temperatures (July) range from 15 °C to 34 °C (IPMA 2023). The mean temperature during the research period (2021-2022; **Chapter 4**) was 8.9°C in January and 25.5°C in July. The average annual precipitation is 555mm, concentrated between October and May (IPMA 2023).

2.2 Landscape and land use types

The dominant landscape is a typical Mediterranean forest area, dominated by holm oak woodlands (*Quercus rotundifolia*), known as *Montado*, a savannah-like open tree forest with varying tree and shrub densities (Figure 2-2 a). The *Montado* is characterised as an agro-silvo-pastoral system, resulting from a gradient of human intervention, which can be expressed by a combination of extensive agriculture, livestock grazing and forest activities (Pinto-Correia et al. 2011). This system is considered a high nature value farming system, with significantly high socio-economic importance (Pinto-Correia et al. 2011, Plieninger et al. 2021). In the SA, the landscape is dominated by holm oak stands or mixed woodland patches with high shrub cover (hereafter referred to as forest, representing 50% of the study area; Figure 2-1 and Figure 2-2 b) and holm oak stands with low or absent shrub cover due to grazing and other pastoral activities (36%; hereafter referred to as agro land use; Figure 2-1 and Figure 2-2 c). Other less representative land use types in the SA (14%) include olive trees, pastures, and scattered patches of agricultural and shrub areas. The SA is delimited, for most of its length, by the Múrtega stream to the south and the Ardila river to the north, with the latter forming the border with Spain. Several artificial and natural water points (Figure 2-2 c) are scattered throughout the SA, and a few temporary natural water ponds are also available during the wet season. Water availability is low during the dry season, when the majority of water sites and streams dry out, and thus water is limited to the river and a few artificial water sites.
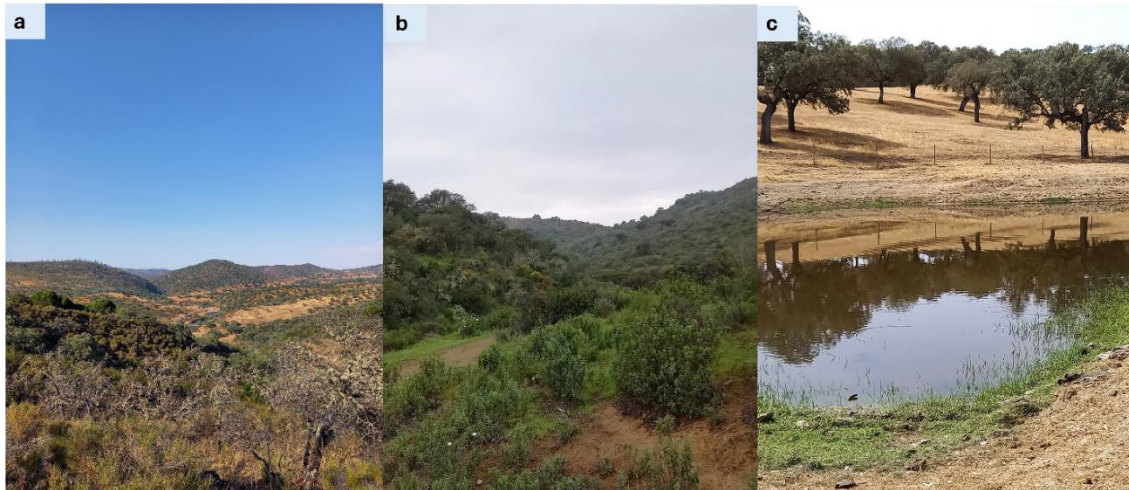
Figure 2-2. Example of the landscape (*Montado*) in the study area: (a) general landscape view, b) forest land use (b) and (c) agro land use, which includes an example of an artificial water site.

2.3   Wildlife – TB hosts

Montado systems support high levels of biodiversity (Curveira-Santos et al. 2017, Salgueiro et al. 2018, Aubard et al. 2019, Medinas et al. 2019). Ungulates are abundant in the region (wild boar density = 3-4 individuals/km^2; red deer density = 4-8 individuals/km^2) (Santos et al. 2022). Both species are widespread in the SA, with higher abundance estimates compared to other TB settings farther from the Portuguese-Spanish border, as evidenced by Santos et al. (2022). In addition to the red fox and badger, which are present in the study area and tend to be widespread in agroforestry systems (Grilo et al. 2016, Curveira-Santos et al. 2017), other carnivore species such as the Egyptian mongoose (*Herpestes ichneumon*), genet (*Genetta genetta*), and stone marten (*Martes foina*) are documented in the region (Bencatel et al. 2019). The four target species addressed in this study are classified as Least Concern according to the recent national assessment '*Livro Vermelho dos Mamíferos de Portugal Continental*' (Mathias et al. 2023).

2.4   Human activities

Within the study area, no villages are present, and human settlements manifest as isolated traditional farms. One of these farms encompasses a touristic facility (Parque de Natureza de Noudar), with seasonal visitors. Extensive cattle production coexists with large game hunting in all five farms. Cattle can room freely across most of the study area. However, on all farms, in certain periods, cattle are confined to parcels (fenced sub-units/grazing plots)

that restrict their movement. This confinement is due to management actions that are controlled throughout the year. Given that, cattle have access to multiple water sites across the SA – which are accessible to wildlife – depending on the specific parcels where they are located. There are few artificial feeding sites where supplementary food is provided for cattle (these sites are not selective, meaning wildlife can theoretically access them as well). During the study period, an average of ~136 adult cows per farm was recorded (ranging from 50 to 185), mainly comprising the *Mertolenga* breed. One farm also hosted animals belonging to the *Garvonesa* breed. In terms of hunting activity, the regular period during which authorized hunters can engage in driven hunt campaigns for wild boar and red deer spans from October to February (Law-Decree n. º 202/2004). In addition, wild boar can be hunted by night waiting during the full moon, which could be year-round (ICNF 2023). Other human activities such as hiking, biking and offroad 4WD occasionally occur in some parts of the study area, mainly associated with local public unpaved roads. Indeed, the road network in the SA consists solely of unpaved roads (i.e. dirt roads).

2.5   Epidemic situation in Barrancos region

Rates of TB herd prevalence in the Alentejo region have remained consistently low since 2012, dropping from over 4% in 2010 to below 1.5% since 2012, with trends similar to other regions of mainland Portugal. However, there has been a slight increase in recent years (1.09% in 2017; 1.38% in 2021) (DGAV 2017, 2018, 2022). Currently, the Alentejo region exhibits the highest rates of herd prevalence (1.83% in 2022;DGAV 2023), with Barrancos identified as a central component in TB epidemiology over the last decades (Santos et al. 2018, Reis et al. 2020). During the field work (2021 to 2022), cases of TB were confirmed in cattle farms in the area (unpublished data), thereby confirming the circulation of *M. bovis* in the region. Santos and co-authors demonstrated a strong spatial structure of TB in wildlife in Portugal, with a main cluster identified in southern Portugal encompassing Barrancos region (Santos et al. 2018). The few studies available – master's thesis – focusing on wildlife epidemiology in the region suggest lower rates of TB prevalence for both wild boar (3.1%) and red deer (1.8%) (assessed via screening of hunting bags) (Costa 2015). Furthermore, the estimated distribution of true prevalences for both species, as documented by Santos et al. (2022), revealed a median prevalence of 25% for wild boar and 7% for red deer, with the wild boar described as the maintenance host in the SA. Although TB has been confirmed in

badgers and red foxes in other multi-host systems across the Iberian Peninsula (e.g. Idanha-a-Nova; Matos et al. 2014), we did not find any prevalence estimates for the study area or surrounding areas. Thus, the potential role of carnivores in TB epidemiology in this area remains undefined (Gortázar et al. 2012, Santos et al. 2022).

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Chapter 3

Disentangling wildlife–cattle interactions in multi-host tuberculosis scenarios: systematic review and meta-analysis

Chapter 3 – Disentangling wildlife–cattle interactions in multi-host tuberculosis scenarios: systematic review and meta-analysis

Abstract

1. Ecological interactions involving wildlife (wild mammals) and cattle *Bos taurus* are considered fundamental drivers of animal tuberculosis (TB) caused by *Mycobacterium bovis* at the wildlife–livestock interface. Despite recent insights about the role of direct and indirect interactions on TB dynamics, a mechanistic evaluation of studies addressing patterns of wildlife–cattle interaction at the global level is lacking, and the most likely factors explaining interaction rates under different epidemiological scenarios remain poorly understood.

2. We began by reviewing the main criteria used to define a wildlife–cattle interaction relevant to *Mycobacterium bovis* transmission under different methodological approaches (camera-trapping, proximity loggers and Global Positioning System collars). Secondly, we applied a generic framework to estimate and characterise interaction patterns between susceptible wildlife and cattle hosts worldwide, testing the effect of potential ecological and methodological factors on interaction rates.

3. We synthesise two main criteria to define direct interactions and five criteria to define indirect interactions between wildlife and cattle. Using data from 31 studies, our meta-analysis showed that wildlife–cattle direct interaction rates were low (mean = 0.03 interactions/month per species pair, range: 0.00–0.12). In contrast, indirect interaction rates were 154 times higher than the mean of direct interaction rates (mean = 4.63 interactions/month per species pair, range: 0.16–30.00).

4. To prevent TB transmission to cattle, attention should be given to indirect interactions between wildlife and cattle in shared environments. Indirect interactions significantly increase with increasing wildlife density, which, hypothetically, could result in a higher TB transmission risk for cattle. We outline recommendations to achieve harmonised integration and comparison of results in future studies. Consolidation of knowledge in this field will

contribute towards guiding control and biosecurity measures, also applicable to other infectious diseases at the wildlife, domestic species and human interfaces.

Keywords

bovine TB *Mycobacterium bovis* transmission, camera- trapping, Global Positioning System (GPS) collars, multi- host pathogens, proximity loggers, wildlife– livestock interface, wild mammals

3.1 Introduction

The emergence of infectious diseases at the wildlife-livestock-human interfaces has become a significant concern worldwide (Alexander et al. 2018, White & Razgour 2020, Hassell et al. 2021). Animal tuberculosis (TB), caused by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex, is among the most widespread zoonotic diseases (Palmer et al. 2012, Fitzgerald & Kaneene 2013). Interspecific transmission of this pathogen occurs in complex socio-ecological contexts, potentially affecting humans, livestock and a wide range of wildlife (wild mammal) species (Duarte et al. 2008, Palmer et al. 2012, Gortázar et al. 2015), with economic, animal and public health implications (Cunha et al. 2011, Palmer et al. 2012; Hardstaff et al. 2014). Despite considerable efforts and long-standing programmes to control TB in livestock, eradication has not yet been achieved in many countries (Cunha et al. 2012, Pereira et al. 2020, Ramos et al. 2020). Wildlife reservoir hosts are considered to be the main obstacle for eradication worldwide (Palmer 2013, Gortázar et al. 2015), with recent data evidencing a high TB burden in non-bovine hosts (Santos et al. 2020). *Mycobacterium bovis* infection is maintained within intra- and interspecific wildlife populations, contributing to the geographical expansion of animal TB and *Mycobacterium bovis* dissemination to other species, and thus increasing the risk of spill-back to livestock under extensive husbandry (Duarte et al. 2008, Santos et al. 2012, Fitzgerald & Kaneene 2013, Van Tonder et al. 2021). In this context, spatial-temporal interactions between (and within) wildlife species and livestock have been recognised as a central driver of inter-specific transmission (Kukielka et al. 2013, Barasona et al. 2014, Cowie et al. 2016, Wilber et al. 2019).

Mycobacterium bovis transmission between individuals can occur either through direct host-to-host interaction or through indirect interaction via contaminated environments and asynchronous space-use overlap (Humblet et al. 2009, Fitzgerald & Kaneene 2013, Pereira et al. 2020). Historically, *Mycobacterium bovis* transmission was believed to occur primarily through aerosols during direct interactions that require close proximity or physical contact between infected and susceptible hosts (Morris et al. 1994, Palmer et al. 2012). However, recent studies focusing on spatial ecology of wildlife and livestock suggest that this mode of transmission is infrequent, even rare, in different epidemiological scenarios. Direct interactions between wildlife and cattle *Bos taurus* have been infrequently documented in England (e.g. badger *Meles meles* as a case study; Drewe et al. 2013, Woodroffe et al. 2016), in North America (e.g. white-tailed deer *Odocoileus virginianus* as a case study; Lavelle et al. 2016), and even in the Iberian Peninsula (Kukielka et al. 2013, Cowie et al. 2016) where TB is maintained in a multi-host system (Cunha et al. 2011, Santos et al. 2012, Reis et al. 2021). Even if direct interactions are infrequent, they should be considered in disease dynamics, because, when occurring, they increase the risk of disease transmission (Wilber et al. 2019).

High rates of indirect interactions have been reported in Spain, France and the USA, involving ungulate reservoir hosts (e.g. red deer *Cervus elaphus*, wild boar *Sus scrofa*, white-tailed deer) and suspected spillover hosts (e.g. red fox *Vulpes vulpes*), where farming typically occurs close to wildlife habitats (Barasona et al. 2016, Payne et al. 2016, Wilber et al. 2019, Martínez-Guijosa et al. 2021). Indirect transmission can take place via shared space use at different times and common exposure to an infectious off-host environment. Inter-species transmission may occur when livestock or wildlife come into contact with *Mycobacterium bovis*-contaminated fomites, such as badger latrines (e.g. Drewe et al. 2013, Campbell et al. 2019), although the use by wildlife of farm and cattle-related resources, such as food and water sites, seems to be more frequent (Carrasco-Garcia et al. 2016, Payne et al. 2016, Balseiro et al. 2019, Campbell et al. 2019). This mode of transmission might be particularly relevant in agroforestry and pastoral landscapes, where abundant wildlife occurs in sympatry with extensively reared livestock (Payne et al. 2017, Caron et al. 2021), or has access to potentially contaminated farm resources (Tolhurst et al. 2009, Campbell et al. 2019). Mycobacteria are considerably resistant to adverse environmental conditions (Fine et al. 2011, Allen et al. 2021), and widespread bacterial contamination can occur at different sites (e.g. water sites; Santos et al. 2015, Barasona et al. 2016) used by a variety of hosts, therefore, increasing transmission risk in shared environments.

Despite recent insights about the role of direct and indirect interactions on TB dynamics (Gortázar et al. 2015, Wilber et al. 2019), differences in interaction patterns derived from multiple studies hamper inferences on the underlying transmission processes. Interaction type and frequency may vary according to host species (White et al. 2018a, Wilber et al. 2019). Depending on their behavioural and ecological traits, different species, and even individuals of the same species, could contribute differently to epidemiologically meaningful interactions (Craft 2015, Silk et al. 2017, Triguero-Ocaña et al. 2020). In turn, species' traits considered relevant for disease dynamics (e.g. social behaviour; habitat selection) are shaped by environmental factors, such as resource availability and landscape patterns (Morris et al. 2016, Albery et al. 2021, Triguero-Ocaña et al. 2021).

Also, the different methods employed to study host interactions in TB epidemiology raise several issues (Bacigalupo et al. 2020). Technology-based approaches applied on this theme range from non-invasive and cost-effective methods, such as camera-trapping (e.g. Barasona et al. 2013, Kukielka et al. 2013), to proximity loggers that enable researchers to record animals' social interactions (Wilber et al. 2019). Additionally, the use of high-resolution Global Positioning System (GPS) tracking devices capable of co-locating individuals at fine spatial-temporal scales have been reported as being useful (e.g. Triguero-Ocaña et al. 2019), although their use requires animal capture and handling (Barasona et al. 2014). Those methods tend to provide information at distinct spatial and temporal resolutions, wherein standardisation of experimental approaches (within and between methods) is significantly lacking. Furthermore, criteria used to define and evaluate animal interactions vary widely and need harmonisation (Bacigalupo et al. 2020), as different methodologies and study goals may lead to different research outputs. This poses significant constrains for comparisons of research results, thus hampering inferences on global transmission pathways and on wildlife hosts' relative importance in TB dynamics. Despite recent improvements in this direction (see Bacigalupo et al. 2020), generic mechanistic evaluations to characterise animal interactions at the wildlife–cattle interface on a global level remain a key challenge in TB epidemiology. Specifically, to date, a comprehensive comparison between studies addressing the type and frequency of interactions between wildlife hosts of *Mycobacterium bovis* and cattle has not been attempted. Moreover, obtaining estimates of wildlife–cattle interaction rates would allow us to identify the factors that are most likely to be responsible for variation in interaction patterns, an aspect that, until now, has not been assessed.

We systematically review the main criteria used to define wildlife–cattle interactions relevant to *Mycobacterium bovis* transmission and examine the main interaction patterns between *Mycobacterium bovis's* wild mammal hosts and cattle worldwide. Specifically, we aimed to: 1) synthetise the main criteria used to define wildlife–cattle interactions relevant to TB epidemiology; 2) assess the geographical and temporal trends of the studies addressing wildlife–cattle interactions; 3) assess the most frequent host species and the methods used in studies addressing wildlife–cattle interactions; 4) quantify rates of direct and indirect wildlife–cattle interactions; and 5) identify which factors influence interaction rates.

Based on our findings, we propose recommendations for future studies that will enable an improved integration and comparison of interaction results across studies. This knowledge is key for quantifying the overall relative importance of direct and indirect transmission routes and the ecological mechanisms underlying TB epidemiology at the wildlife–cattle interface on a broad scale.

3.2   Material and Methods

3.2.1   Literature search

A literature search was conducted through the ISI's Web of Science focusing on all English language published studies (with no year restrictions) and following the PRISMA (preferred reported items for systematic reviews and meta- analyses; Moher et al. 2009) statement. The query used was as follows: ((*Mycobacterium bovis* OR tuberculosis OR TB) AND (interactions OR contact OR movement OR visit OR spatial ecology) AND (Wildlife)), retrieving 381 studies (Supplementary material, Figure S3-1). Additionally, we examined the reference list of a systematic review on a similar topic (Dougherty et al. 2018), yielding 70 further studies. This initial list was then expanded by adopting a 'snowball' approach (e.g. Prugh & Sivy 2020), where relevant literature cited there was scanned, yielding 28 additional studies (Supplementary material, Figure S3-1). The result was 479 studies in total. Our final search took place on 13 August 2020. Although the terms 'interaction' and 'contact' are used as synonyms in TB related literature, we chose to use 'interaction' throughout the text for consistency.

3.2.2 Inclusion and exclusion criteria

After removing duplicates (n = 31), the titles and abstracts of the remaining 448 studies were screened for study goals, target species, region, methods and TB context to meet our research goals (screening stage). This resulted in the selection of 128 relevant studies that were afterwards fully examined by one person. Hereafter, two distinct frameworks of inclusion/exclusion criteria were conducted, producing two datasets. For the systematic review of criteria defining an interaction (objective 1), we specifically focused on peer-reviewed studies that: 1) assessed interaction pat- terns between *Mycobacterium bovis's* wildlife hosts and cattle, and 2) included a definition of interaction by the authors in the context of TB transmission (Criteria Dataset including 13 studies; Supplementary material A, Figure S3-1).

For the analysis of the interaction patterns (objectives 2– 5), we examined the bulk of studies identified in the screening stage (n = 128). Studies that explicitly investigated interaction patterns between wildlife and cattle were retained. Moreover, we included studies that, despite not addressing ecological interactions or where the authors adopted different terminologies (e.g. visit, spatial occurrence), still provided informative spatial–temporal metrics regarding shared space use at the wildlife–cattle interface. Only studies using technology-based methods such as remote cameras (hereafter termed as camera-trapping), proximity loggers and GPS tracking devices, which focused on *Mycobacterium bovis* wildlife hosts, were included. Exclusion criteria were as follows: 1) studies neither applicable to the animal TB context nor focusing on wildlife hosts; 2) experimental studies (e.g. not free-ranging animals and animal translocations) or studies not based on technological methods (e.g. observational studies and questionnaires); 3) studies not reporting raw counts or means of interactions; and 4) studies based on the same data as other studies already included in the dataset (Patterns Dataset including 31 studies; Supplementary material A, Figure S3-1).

3.2.3 Data extraction

For each study included in the systematic review of interaction definitions (Criteria Dataset), the following information was recorded: first author, year of publication, continent, country where the study took place, year of publication, method, wildlife host(s), criteria defining interactions and type of interaction evaluated. For each study included in the meta-analysis

of interaction patterns (Patterns Dataset), the following data were extracted: first author, year of publication, continent, country where the study took place, wildlife species and study period. Additionally, data regarding 10 predictors that could potentially explain variation in interaction patterns were extracted: species pair (wildlife host species – cattle), host taxonomic family, host type (reservoir vs. susceptible), method (camera-trapping, GPS collars and proximity loggers), dominant habitat, study area range, farm size, number of farms, cattle density and wildlife density (Supplementary material A, Table S3-1).

3.2.4 Calculation of interaction rates

Raw counts and means (e.g. daily/weekly means) of wildlife–cattle interactions and presence/absence of interactions (when exact quantification was unavailable) were extracted. Each observation in our Patterns Dataset corresponded to a particular species pair, that is, to a potential interaction (≥0) between one wildlife host species and cattle. Whenever a study reported results on interaction values for multiple wildlife species or for different landscapes that study contributed with more than one observation in the dataset. Interaction values for each species pair were standardised to allow data comparisons. For each species pair combination, the number of interactions per month (*RatesInt*) was calculated as a function of reported number of interactions (nr of interactions), sampling effort (sampEffort) and study period in months (time) as:

RatesInt=nr of interactions∕sampEffort ∗ time.

RatesInt was calculated separately for direct and indirect interactions. Since the experimental design of the studies in the review varied according to the methods used to assess wildlife–cattle interactions, sampEffort corresponds to different quantifications of observation effort: 1) the number of monitoring points (direct and indirect interactions with camera-trapping); 2) the number of collared individuals, including wildlife and cattle (direct interactions with proximity loggers and indirect interactions with GPS collars); 3) the mean number of collared individuals (including wildlife and cattle) plus the number of monitoring points (indirect interactions with proximity loggers); or 4) the mean number of collared wild individuals plus the number of monitoring points (indirect interactions through environment with GPS collars). Monitoring points are defined as potential fomites sites with high-frequency use by wildlife and/or cattle (badger latrines, water sites and cattle feeding sites),

as defined *a priori* in each study, more frequently in studies using camera-trapping and proximity loggers.

We also included interaction measurements estimated directly from embedded Figures (eight observations from two studies; potential reading error standard deviation ± two interactions) and considered the studies that only provided the number of days with occurrence of interactions as a proxy to the number of interactions (17 indirect observations from 5 studies). To assess the potential effect of pooling these observations on the *RatesInt*, we applied generalised linear models (GLMs) using data source as a predictor (numbers of interactions reported by authors vs. numbers estimated from Figures and proxies). No significant differences between direct and indirect *RatesInt* obtained from authors' values and estimates were recorded (direct GLM: coefficient – coef = 0.015, 95% confidence intervals – CI 95% [−0.186; 0.216]; indirect GLM: coef = −0.363, CI 95% [−0.925; 0.200]). We also performed GLM models to inspect variation on *RatesInt* between methods (direct *RatesInt*: camera-trapping and proximity loggers; indirect *RatesInt*: camera-trapping, GPS collars and proximity loggers). No differences in *RatesInt* between methods were observed, either for direct interactions (coef = 0.094, CI 95% [−0.046; 0.234]) or for indirect interactions (coef = −1.040, CI 95% [−3.361; 1.281]; coef = −1.530, CI 95% [−6.014; 2.955]). Therefore, all observations were pooled in further analyses.

3.2.5 Statistical analyses of factors influencing patterns of interaction rates

A meta-analysis was conducted to identify factors influencing estimated interaction rates. We used general linear mixed models (GLMMs) with study identification number as a random effect to account for dependence among observations (e.g. multiple observations from the same study) and detectability artefacts inherent to each study design. For direct interactions, we used the binomial family, with presence/absence of interactions as the response variable (function glmer, R package 'lme4'; Bates et al. 2015); for indirect interactions, the response variable was *RatesInt* and models were fitted with a Gaussian family (function lme, R package 'nlme'; Pinheiro et al. 2018). However, if a likelihood ratio test between a GLMM and a corresponding reduced model (without random structure) showed no improvement by adding a random effect, we opted for GLMs (Zuur et al. 2009). Host species, taxonomic family (those with high numbers of observations), host type

(reservoir/susceptible host), dominant habitat, study area range, farm size, number of farms, cattle density and wildlife density were tested as predictors (Supplementary material A, Table S3-2). Numeric predictors with skewed distributions were transformed (square root/ logarithmic) to reach normality and reduce the influence of outliers (Zuur et al. 2009).

The number of observations (<25) in the Patterns Dataset was insufficient for building models with multiple predictors and for multi-model inference (Jenkins & Quintana-Ascencio 2020). Thus, univariate models were applied, testing one predictor at a time, ensuring a plausible number of observations to explore data patterns adequately in the context of mixed-regression analysis (Zuur et al. 2009, Jenkins & Quintana- Ascencio 2020). Fitted univariate models were compared with their corresponding null models using AICc, Akaike's Information Criterion adjusted for small sample sizes (Burnham & Anderson 2002). A statistically informative model was considered when: 1) a delta AIC (ΔAICc) > 2 was obtained between the null model and the model testing one predictor; and 2) the 95% confidence intervals (CI 95%) of predictor coefficients (coef) did not include zero (Burnham & Anderson 2002, Zuur et al. 2009). Only models with ΔAICc >2 were considered for discussion in this study.

3.3 Results

3.3.1 Review of criteria used to define wildlife–cattle interactions relevant to TB transmission risk

Considering the 128 eligible studies, only 10% (n = 13; Criteria Dataset) stated the criteria used to define a wildlife–cattle interaction. Among these, seven studies investigated and defined both direct and indirect interactions, whereas five studies were focused exclusively on direct interactions and one study only on indirect interactions (Supplementary material A, Table S3-3). We summarised two criteria used to define direct interactions between wildlife and cattle (using data obtained from camera-trapping and proximity loggers) and five main criteria to define indirect interactions (through camera-trapping, proximity loggers and GPS collars; Table 3-1)

Table 3-1. Synthesis of the main criteria used to define a wildlife–cattle interaction in the context of tuberculosis transmission, according to the interaction type (direct and indirect) and method (camera-trapping, proximity loggers and global positioning system [GPS] collars). Monitoring points are potential fomites sites with high use frequency by wildlife and/or cattle (badger latrines, water sites and cattle feeding sites), as defined a priori in each study.

Method	Direct interaction	Indirect interaction
Camera-trapping	When two individuals of different species (wildlife and cattle) are recorded in the same images or video (in some cases including only individuals within a pre-established close distance or making physical contact).	When two animals of different species (wildlife and cattle) are recorded in the same monitoring point within a critical time window (CTW). When a wildlife species occurs in a monitoring point.
Proximity loggers	When two collared animals of different species establish a contact within a pre-defined proximity (frequently set up to 1.5-2 m) at a given time.	When two collared animals of different species contact a base station deployed at a potential fomites site within a given critical time window (CTW) or spatial-time window (STW). When a collared animal contacts a base station deployed at potential fomites site.
GPS collars		When a pair of spatial locations of wildlife and cattle fall within a pre-defined spatial-temporal window (STW).

3.3.1.1 Direct interactions

To assess direct interactions that could potentially increase *Mycobacterium bovis* transmission risk between wildlife and cattle, researchers reported the simultaneous

presence of two individuals at a given time at a specific location (Table 3-1). A first criterion identified was related to data from proximity loggers (Supplementary material A, Table S3-3). Proximity loggers deployed on animals automatically record contact frequency and duration of contact between devices (Woodroffe et al. 2016). Accordingly, a direct interaction is defined (first criterion) when two collared animals (wildlife and cattle) come within a predefined short distance of one another (up to 20 meters, but most often set up to 1.5–2 meters), triggering a contact (Böhm et al. 2009, Drewe et al. 2013, Triguero-Ocaña et al. 2020). These events are considered direct interactions since short distances detected by loggers are likely to be epidemiologically meaningful due to possible aerosol transmission of *Mycobacterium bovis* (Sauter & Morris 1995). A second criterion to generate disease-relevant interactions involved the use of camera-trapping: infrared motion-triggered cameras are deployed near key resources (e.g. water sites and cattle feeding sites) used by wildlife and/or cattle, where *Mycobacterium bovis* contaminated fomites could endure (Campbell et al. 2019). Cameras are set up to operate 24 h a day, recording multiple and successive images (Kukielka et al. 2013) or short videos (Payne et al. 2016). The simultaneous presence of cattle and wildlife in the same images or video (in some cases including only individuals within a pre- established close distance or making physical contact) is defined as a direct interaction (Tolhurst et al. 2009, 2011, Barasona et al. 2016). Overall, these recorded events are assumed as proxies of closeness between species, possibly leading to an infected and a susceptible host physically interacting with one another within the same time and space frame.

3.3.1.2 Indirect interactions

Proximity loggers combined with base stations (static devices in the field that record connections to the loggers) deployed at key resources (e.g. food sites) have also been used to evaluate indirect wildlife–cattle interactions. An indirect interaction (first criterion) via environment is defined when two collared individuals (wildlife and cattle) contact a base station at different times but within a pre-established critical time window (CTW) or spatial temporal window (STW; Table 3-1; Supplementary material A, Table S3-3). The CTW and STW are chosen according to *Mycobacterium bovis*'s environmental survival time and device specificities (e.g. positional error; Cowie et al. 2016, Lavelle et al. 2016, Wilber et al. 2019, Triguero- Ocaña et al. 2020). A second criterion defining indirect interaction through proximity loggers was implemented by Drewe et al. (2013). They defined an indirect

interaction after a collared animal (wildlife or cattle) contacted a base station specifically deployed at a site that was potentially contaminated with *Mycobacterium bovis* (e.g. a badger latrine), regardless of sequential use by other animals.

For studies using camera-trapping, there are two main criteria to define an indirect wildlife–cattle interaction. One study – focused on the multi-host ungulate community in Spain – defined an indirect interaction as the occurrence of wildlife and cattle at the same monitoring point at different times, but within a CTW that was consistent with *Mycobacterium bovis's* environmental survival (Kukielka et al. 2013). A second criterion, defined as the simple occurrence (often called a 'visit') of a wildlife species at a site used by cattle (farm building and pastures), was implemented by Payne et al. (2016) in a TB-infected area in France.

Despite providing high-resolution spatial–temporal data on animal locations, GPS collars were used exclusively to assess indirect patterns of wildlife–cattle interactions within a TB hotspot area. A single criterion to define an indirect interaction was identified in two studies, both conducted in the same multi-host system in Mediterranean Spain (Triguero-Ocaña et al. 2019, 2020). An indirect interaction was defined when a given pair of GPS spatial locations of wildlife and cattle fell within a pre-defined STW, related to the mean positioning error of the GPS devices and the time lag between successive spatial locations. In contrast to camera-trapping and proximity loggers, indirect interaction events from GPS data are registered independently of potential fomites sites (e.g. water and food sites) under monitoring.

3.3.2 Global patterns of wildlife–cattle interactions

3.3.2.1 General scope and geographic and temporal trends of the studies

A total of 31 studies on wildlife–cattle interactions concerning animal TB epidemiology (Patterns Dataset) were scrutinised (Supplementary material A, Table S3-4). Most studies were conducted in Europe (84%; n = 26), with the highest percentage in England (34%; n = 11) and Spain (25%; n = 8), followed by Northern Ireland (9%; n = 3), France (6%; n = 2), Ireland (6%; n = 2) and Wales (3%; n = 1; Figure 3-1). The few studies from North America (16%; n = 5) were from the USA (9%; n = 3) and Canada (6%; n = 2). All studies are relatively recent ranging between 2002 and 2020 (Figure 3-2). A growing number of studies was recorded since 2008 with a peak in 2016, slightly decreasing thereafter.
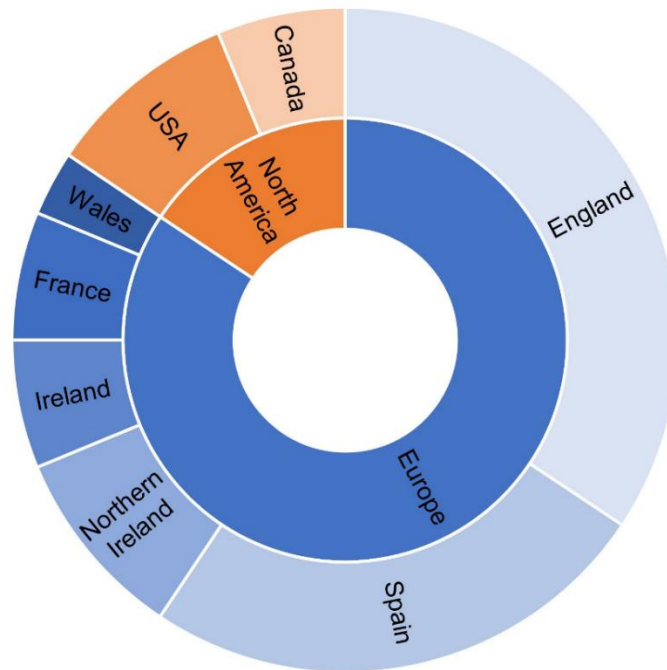
Figure 3-1. Locations of study sites used in research included in the meta-analysis of wildlife–cattle interactions and tuberculosis (n = 31 studies): continent (inner circle) and country (outer circle).
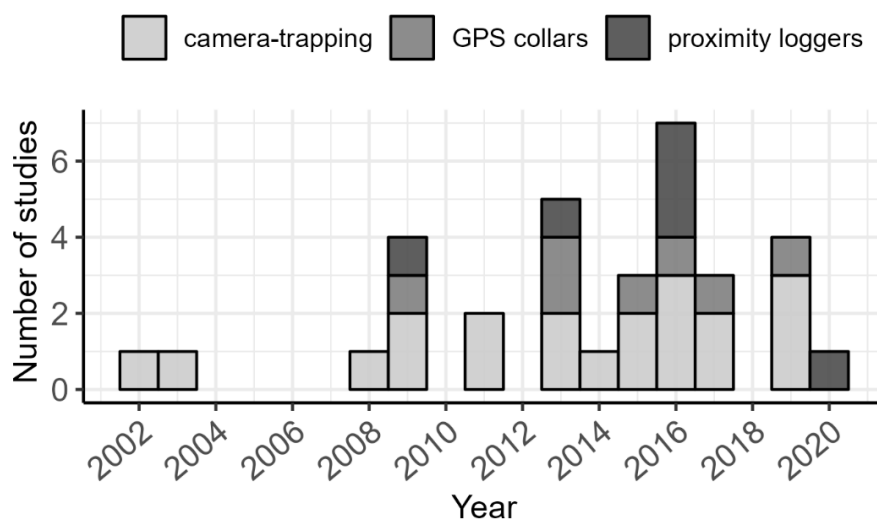
Figure 3-2. Publication year of the studies included in the meta-analysis according to type of method used.

Overall, the studies covered a wide range of *Mycobacterium bovis* wildlife hosts. Four wildlife species were described as reservoirs depending on the ecosystem: European badger, wild boar, red deer and white-tailed deer; while another six, comprising red fox, roe deer *Capreolus capreolus*, raccoon *Procyon lotor*, fallow deer *Dama dama*, opossum *Didelphis virginiana* and wapiti *Cervus elaphus canadensis*, were defined as susceptible spillover hosts (Figure 3-3). A significant percentage of the studies (42%; n = 13) addressed multiple hosts. The European badger (31%) was the most frequently studied host, followed by the wild boar (17%), the red deer (14%) and the red fox (10%). The remaining wildlife species were less commonly represented (<7% of the studies). Camera-trapping was the most frequently used method (61%; n = 19). Proximity loggers and GPS collars came in second, equally represented (16% each, n = 5; Figure 3-2). Additionally, 6% of the studies were multi-method (n = 2: camera-trapping/GPS collars; GPS collars/proximity loggers).
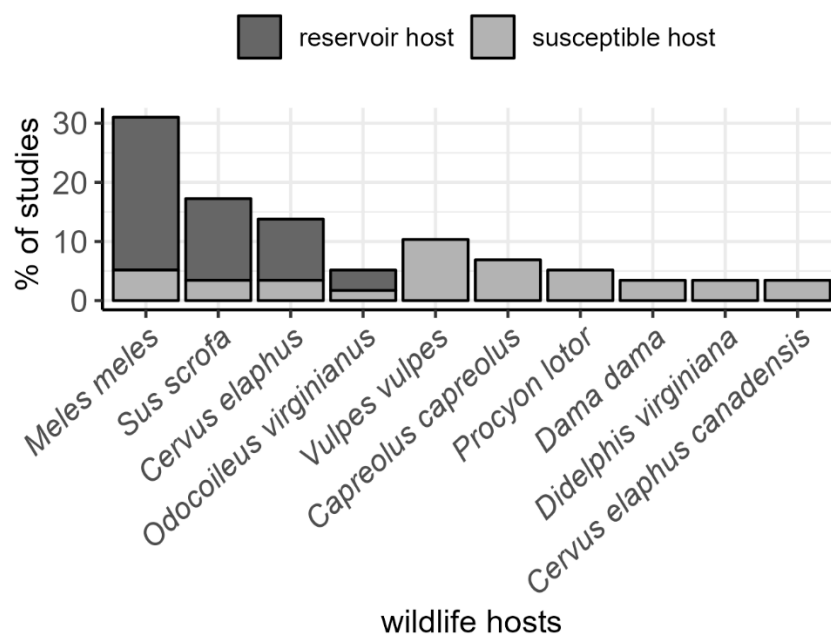
Figure 3-3. Percentage of studies (n = 31) included in the meta-analysis according to wildlife hosts and considering their presumed epidemiological role in the citing study.

3.3.3 Rates of wildlife–cattle interactions

We counted 35 records of direct interactions involving nine species pairs from 18 studies. Additionally, 59 records of indirect interactions comprising nine species pairs from 26 studies were recorded. The overall mean of direct *RatesInt* was 0.03 interactions/month [range:

0.00– 0.12] per species pair, while indirect *RatesInt* was 4.63 interactions/month [range: 0.16– 30.00] per species pair, representing 154 times more than the mean of direct interactions (Figure 3-4).
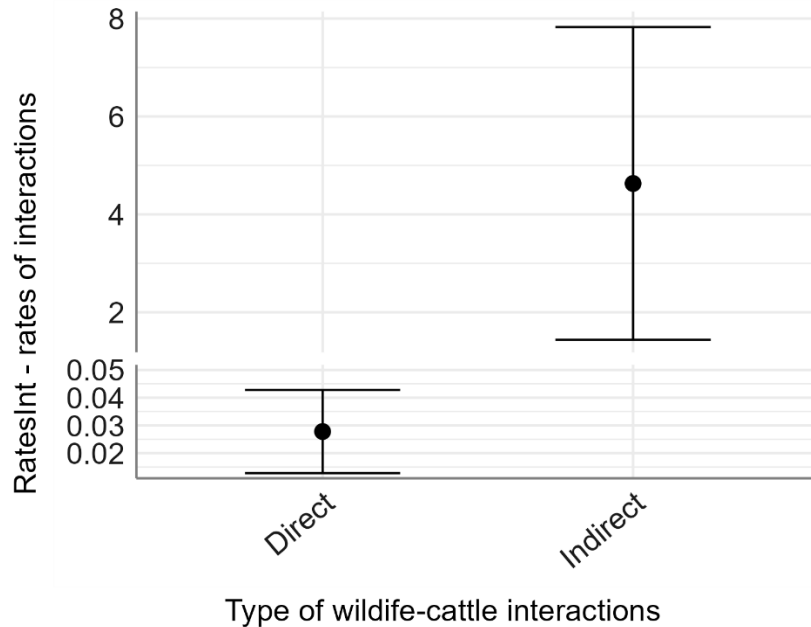
Figure 3-4. Overall rates of wildlife– cattle interactions (*RatesInt* [interactions/ month]). Weighted means (dots) and standard errors (bars) of rates are displayed by interaction type (direct or indirect). Note the broken Y-axis.

Badger–cattle (MM) and fallow deer–cattle (DD) were the species pairs showing the highest rates of direct interactions, ranked as first and second respectively (Table 3-2, Figure 3-5). The remaining species pairs had mean interaction rates lower than the overall mean. Wild boar–cattle (SS) presented the highest rate of indirect interactions. The pairs raccoon–cattle (PL) and red fox–cattle (VV) had moderate rates of indirect interactions, yet lower than the overall mean. The remaining species pairs made a lower contribution to the bulk of indirect interaction rates (less than two interactions/month per species pair).

Table 3-2. Summary of the rates of wildlife–cattle interactions (*RatesInt*) described for species pairs. For each species pair, we provide detailed information on numbers of records (n), means, standard errors (se), and range values (min-max) displayed by type of interactions (* is used to mark means, se and min-max based on < 3 records; NA = not applicable).

Species pair	Scientific name – wildlife host	Species pair acronym	Direct interactions per month				Indirect interactions per month			
			n	Mean	SE	Min–Max	n	Mean	SE	Min– Max
Badger–cattle	*Meles meles*	MM	12	0.12	0.09	[0.00 – 1.08]	26	1.90	0.96	[0.00 – 22.33]
Fallow deer–cattle	*Dama dama*	DD	2	0.09*	0.09*	[0.00 – 0.18]*	NA	NA	NA	NA
Opossum–cattle	*Didelphis virginiana*	DV	1	0.00*	NA	[0.00 – 0.00]*	2	0.30*	0.29*	[0.02 – 0.59]*
Raccoon–cattle	*Procyon lotor*	PL	1	0.01*	NA	[0.01 – 0.01]*	4	4.24	2.76	[0.02 – 12.11]
Red deer–cattle	*Cervus elaphus*	CE	6	0.02	0.01	[0.00 – 0.08]	5	1.29	0.79	[0.03 – 4.33]
Red fox–cattle	*Vulpes vulpes*	VV	2	<0.01*	<0.00*	[0.00 – 0.01]*	6	2.26	1.33	[0.13 – 8.46]
Roe deer–cattle	*Capreolus capreolus*	CC	4	<0.01	0.00	[0.00 – 0.00]	4	0.84	0.72	[0.03 – 3.00]
Wapiti–cattle	*Cervus elaphus canadensis*	CCA	NA	NA	NA	NA	2	0.16*	0.16*	[0.00 – 0.31]*
White-tailed deer–cattle	*Odocoileus virginianus*	OV	1	0.00*	NA	[0.00 – 0.00]*	3	0.72	0.66	[0.00 – 2.03]
Wild boar–cattle	*Sus scrofa*	SS	6	0.00	0.00	[0.00 – 0.03]	7	29.97	29.28	[0.11 – 205.63]
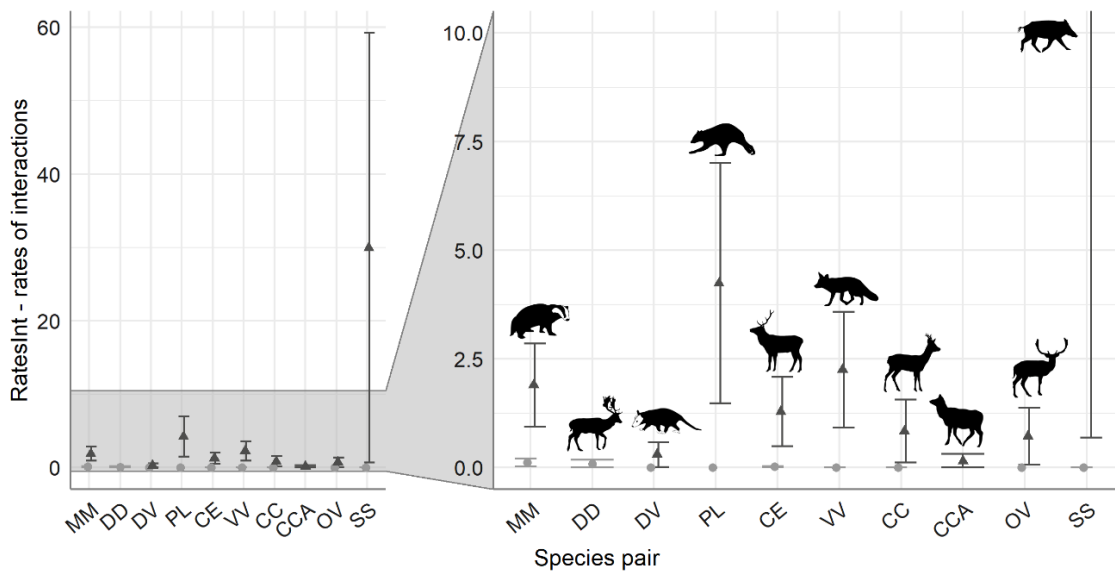
Figure 3-5. Rates of wildlife–cattle interactions (*RatesInt* [interactions/month]) summarised by species pairs. Weighted means and standard errors of rates are displayed by interaction type. Grey dots and dark triangles represent direct and indirect interactions, respectively. Species pair acronyms are (MM) badger–cattle, (DD) fallow deer–cattle, (DV) opossum–cattle, (PL) raccoon– cattle, (CE) red deer–cattle, (VV) fox–cattle, (CC) roe deer–cattle, (CCA) wapiti–cattle, (OV) white-tailed deer–cattle and (SS) wild boar–cattle. Silhouette images of animals are from http://phylo pic.org/ (*Meles meles*, *Dama dama* and *Vulpes vulpes* photos by: Anthony Caravaggi; *Didelphis virginiana* by Gabriela Palomo- Munoz).

3.3.4 Factors influencing interaction rates

Two models regarding the influential factors on the occurrence of direct interactions presented ΔAICc >2 and statistically significant coefficients: farm size (coef = 1.323, CI 95% = [0.501; 2.780]) and cattle_dens (coef = −1.606, CI 95% = [−3.743; −0.537]; Supplementary material A, Table S3-5). According to the models, direct interactions were more likely to occur in larger farms and at low cattle densities (Figure 3-6 a, b). Only one model concerning factors influencing indirect interaction rates had ΔAICc >2 and a significant coefficient: wild_dens (coef = 0.912, CI 95% = [0.350; 1.474]; Supplementary material A, Table S3-5). Rates of indirect interactions significantly increased with higher wildlife density (Figure 3-6 c). Regardless of the type of interactions considered, we found no evidence of significant effects of the remaining predictors we tested.
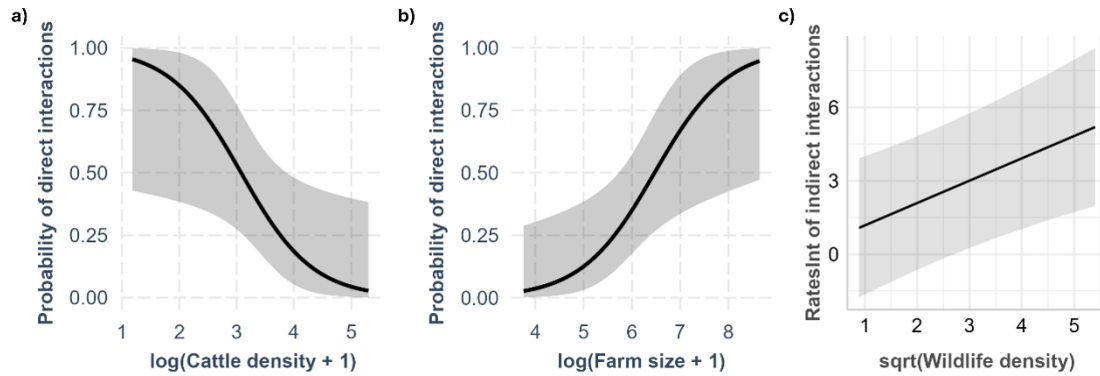
Figure 3-6. Effect of cattle density (a) and farm size (b) on the probability of occurrence of direct interactions (± 95% confidence intervals). Image c represents the effect of wildlife density on indirect interaction rates (± 95% confidence intervals).

3.4 Discussion

Ecological interactions between disease hosts have been widely acknowledged as determinants of pathogen transmission, from domestic species to wildlife and *vice versa*. In this sense, understanding the underlying biological processes that are responsible for the transmission of infectious agents between susceptible animal hosts is becoming an important research area (Gortázar et al. 2016, Dougherty et al. 2018, Bacigalupo et al. 2020, Caron et al. 2021).

Of the studies explored in this work, only a few used specific criteria for the definition of wildlife–cattle interactions. By providing a synthesis of the main criteria used to express wildlife–cattle interactions across different methods, we hope to encourage future researchers to adopt improved integration and harmonisation of interaction data, especially in terms of frequency and interaction type. To the best of our knowledge, this study applies for the first time a framework to estimate wildlife–cattle interaction rates, allowing the comparison of the relative weight of direct and indirect inter-specific interactions on TB transmission at a global level. Regardless of the study context and wildlife host considered, direct rates of interaction between wildlife and cattle are infrequent. In contrast, special attention should be given to indirect interactions that occur frequently through shared environments, wherein wildlife density may play a key role.

3.4.1 When and where do species interact? – Criteria used to define disease-relevant interactions

Despite adopting a variety of criteria, only a small number of studies used explicit standards to define direct and/or indirect interactions pertinent to *Mycobacterium bovis* transmission. This is in agreement with the only study available on cattle diseases (Bacigalupo et al. 2020), in which the authors identified a wide range of definitions used in wildlife–livestock interaction studies, and reported conflicting or overlapping definitions of direct and indirect interactions, highlighting the need for a common generic framework. We found no conflicting criteria between interaction types, although some overlapping criteria for indirect interactions existed. Moreover, Bacigalupo et al. (2020) demonstrated that interaction definitions were highly dependent on the study context, specifically on the species considered and demographic parameters. However, our results suggest that the criteria employed to define interactions vary with the type of interaction and data collection method, rather than with the species or eco- systems studied.

The criteria adopted to define direct interactions included physical contact between animals, as well as close proximity between individuals as a surrogate of that interaction, since spatial proximity may lead to biologically meaningful interaction, considering that aerosol transmission is possible between animal hosts 1–2 m apart (Sauter & Morris 1995, Humblet et al. 2009, Corner et al. 2011). For indirect interactions, a general baseline considered the use of shared environments by individuals at different times, meaning that shared contaminated sites could be high-risk locations by intermediating indirect transmission of *Mycobacterium bovis* between hosts (e.g. Cowie et al. 2016, Lavelle et al. 2016, Payne et al. 2016). We did not find any criteria based on GPS collars for measuring direct interactions. This could be explained by the inherent spatial location error, which can be high (e.g. 26 m, Triguero-Ocaña et al. 2019), resulting in insufficient precision to co-locate individuals at close contact (<2 metres; but see Cooper et al. 2010).

3.4.2 Do wild hosts and study context shape TB interactions?

3.4.2.1 General trends of the studies

Most of studies covered in our meta-analysis were relatively recent and from Europe, with badger and ungulates – particularly wild boar and red deer – as the most represented. These findings are in agreement with the previous work of Reis et al. (2020a) who found that European countries contributed the most to wildlife TB epidemiology knowledge, suggesting that the ecological field is growing alongside the epidemiological area (see also Gortazar et al. 2011, Reis et al. 2020b). In European industrialised countries, TB is a notifiable disease for which eradication programmes and surveillance are mandatory for cattle production and trade (Hardstaff et al. 2014, Gortázar et al. 2015); surveillance programmes also exist in non-European countries. Improved infra-structure and financial resources are available in countries where surveillance and related research are in place. Additionally, on a global scale, some of the best-studied wildlife TB reservoirs occur in Europe, such as in the UK, Ireland, and the Iberian Peninsula (Palmer 2013, Pereira et al. 2020). The absence of interaction-based studies in our review from South Africa and New Zealand is notable given the occurrence of wildlife TB hosts in both countries (see Pereira et al. 2020). This deficit in research could be related to: 1) different socio-economic contexts, particularly the case of South Africa, with the allocation of resources to other research areas (e.g. Di Minin et al. 2021) or to other more affordable studies to assess wildlife–cattle interfaces (e.g. questionnaires, Meunier et al. 2017); or 2) eligibility rules, as the few studies carried out in these countries did not meet our inclusion criteria (e.g. New Zealand; Ramsey et al. 2002, Rouco et al. 2018).

Regarding the methods applied to assess wildlife–cattle interactions, camera-trapping was the most widely used. This was also reported by Bacigalupo et al. (2020), highlighting its considerable versatility to monitor different species, and its applicability across different study types and ecosystems (Caravaggi et al. 2017, Niedballa et al. 2019). Furthermore, novel applications of camera-trapping have proven useful for generating animal density data and for measuring risky behaviour (e.g. Cadenas-Fernández et al. 2019, Palencia et al. 2021). These applications could be highly useful for the further evaluation of disease-relevant interactions across multi-species interfaces.

3.4.2.2 Rates of wildlife–cattle interactions

We developed a novel framework to estimate interaction rates, considering different sampling efforts inherent to data collection methods, enabling multi-studies comparison. Our findings show that direct interactions are rare, suggesting that cattle are seldom approached by wildlife (or the re-verse). In contrast, indirect interactions are significantly more frequent, with a mean estimated frequency 154 times higher than the mean of direct interactions. These findings underpin the idea that a shared environment should be considered an important risk factor for TB transmission at the wildlife–cattle interface (Drewe et al. 2013, Kukielka et al. 2013, Lavelle et al. 2016, Woodroffe et al. 2016, Wilber et al. 2019, Triguero- Ocaña et al. 2020).

Transmission risk depends not only on the extent of disease-relevant interactions among individuals but also on the probability of infection during a particular interaction event (McCallum et al. 2017). In this context, the likelihood of infection involved in direct host-to-host interactions, even if such interactions occur in low numbers, should not be considered negligible in disease transmission (Wilber et al. 2019). Still, obtaining sufficient empirical evidence to quantify the relative importance of different transmission modes constitutes, to date, a real challenge (Craft 2015, Fenton et al. 2015, Webster et al. 2017), which explains the use of interaction patterns as a proxy to evaluate the risk of pathogen transmission (Triguero-Ocaña et al. 2020, Yang et al. 2021). This risk can be estimated by quantifying and modelling fine-scale movement patterns undertaken by host individuals, to elucidate how pathogens can permeate among individuals of different species, and to improve estimates of transmission risk in multi-host systems (Wilber et al. 2022).

Overall, only three predictors significantly influenced the occurrence and abundance of interaction rates. A first model showed that direct interactions are more probable in larger farms. Those areas often hold a higher number of cattle-related resources (e.g. feeding and water sites) that are attractive for wildlife species, enhancing the opportunities for wildlife and cattle to engage in the same spatial site at the same time (Herrera & Nunn 2019). On the other hand, the negative relationship between the occurrence of direct interactions and cattle density may be a behavioural effect, reflecting the local avoidance by wildlife of areas that are often occupied by cattle (see Schieltz & Rubenstein 2016). Mullen et al. (2013) and Woodroffe et al. (2016) demonstrated that badgers actively avoid cattle while foraging in Ireland and England. A similar pattern was found in Portugal by Curveira-Santos et al. (2017),

where cattle presence had a negative influence on space use by the red fox. Conversely, in Spain, Carrasco-Garcia et al. (2016) found that cattle presence was positively associated with wild boar presence and, to a lesser extent, with that of red deer. Moreover, on farms in Northern Ireland, the presence of badgers was positively associated with cattle herd size, yet negatively associated with cattle presence (Campbell et al. 2019). None of these studies specifically explored the role of cattle density on direct inter-species interactions (but see Yang et al. 2021), but they did demonstrate that host behaviour is highly relevant for the co-occurrence of species, and might determine potential close interactions between hosts (Craft 2015, Herrera & Nunn 2019) and thus contribute to transmission risk.

The positive relationship between wildlife density and rates of indirect interaction in this study is consistent with previous research (Carrasco-Garcia et al. 2016, Campbell et al. 2019, Robertson et al. 2019). This pattern is compatible with a density-dependent mechanism, usually applied to exploring relations between social contact rates and animal densities, and relationships with parasite transmission (Hu et al. 2013, Hopkins et al. 2020). Specifically, this mechanism involves an increase in contact rates with higher animal density. However, to date, the limited knowledge on this theme hinders the identification of general principles that explicitly point out the density-dependent wildlife–cattle relationship and its extent. Nevertheless, patterns of interactions resulting from different density contexts might significantly affect pathogen spread, as previously acknowledged (White et al. 2018a, b, Hopkins et al. 2020, Manlove et al. 2022). Thus, regardless of the type of mechanism underlying inter-specific animal interactions, the role of wildlife density in TB epidemiology at the wildlife–cattle interface must be seriously considered (Fofana & Hurford 2017). Furthermore, animal density must be integrated with the factors driving indirect TB transmission, such as environmental persistence of pathogens (Fine et al. 2011, Santos et al. 2015, Dougherty et al. 2018, Manlove et al. 2022). For instance, high-quality habitats tend to attract a higher variety of wildlife species and support larger host densities, increasing contamination levels of indirectly transmitted pathogens (Leach et al. 2016). In those areas, high concentration of hosts enables pathogens to accumulate in spatial reservoirs, which can be viewed as ecological traps. Accordingly, by selecting such areas, individuals can be exposed to an increased infection risk and are more likely to form consistently infected populations (Leach et al. 2016)

3.4.3 Study limitations

Limitations of systematic reviews and meta-analyses affect researchers' ability to broach specific questions or topics. Our results in the review of interaction criteria mirror what we found in the meta-analysis process (estimates of interactions). Variation in experimental design (e.g. criteria adopted, device settings, camera operational days and frequency of fixes with GPS collars) and reporting inconsistency pose significant difficulties: first, summary statistics of interactions (raw counts and means) were often difficult to extract or were not reported at all; second, information regarding potential predictors were also often poorly re-ported and inconsistently detailed across studies, leading to the use of simplified models. This detailed information should be provided in fine-scale studies since it might be relevant for interpreting interaction rates and for comparing studies.

The conclusions on factors influencing interaction rates should be viewed as preliminary since our sample size was limited. However, despite these limitations, our findings offer major insights into understanding animal TB transmission risk through an ecological perspective, even if our framework and modelling approach are a simplification of a complex system. The results highlight the complex ecological links that help us to understand how animals interact at a global scale and thus are a key addition to the present body of knowledge.

3.4.4 What´s next? Guidelines for future studies

Within an ecological–epidemiological perspective of animal TB, a key focus should be wildlife surveillance through harmonised approaches. We propose that several key points should be considered in future studies targeting wildlife–cattle interactions. We encourage researchers to:

1. Adopt the main criteria summarised here, to standardise approaches, allowing comparison of results with previously published studies and easing the integration of new ones.

2. Adopt the terms interaction or contact in studies instead of other nomenclature (e.g. spatial visit and occurrence).

3. Standardise rates of interaction as a function of the sampling effort (e.g. number of camera stations, monitoring sites or collared animals) and study duration, enabling

comparison of studies differing in duration and survey effort; rigorous study duration estimates must take into account the number of active camera days for camera-trapping studies; in the case of GPS collars and proximity loggers, accurate tracking periods of the collared animals are essential (discriminated by species, i.e. the number of tracking days considering the total number of collared individuals for a given species).

4. Guarantee that important predictors (e.g. seasons and sites) are properly surveyed, accommodating balanced spatial and temporal variabilities, because it is important to know when and where species are interacting, and thus when and where actions should be prioritised.

5. Consider CTWs on estimated rates of indirect interactions. Since transmission depends on the pathogen's survival time in the environment, the use of CTW is important for generating reliable estimates in the case of *Mycobacterium bovis* transmission.

6. Information on the study area (proportion of land uses and geographical coordinates), cattle herd size, TB prevalence, wildlife abundance (even if only proxies are available), raw counts of interactions and rates of interaction should be made available.

7. Evaluate both intra- and inter-wildlife species' interactions in order to improve our understanding of TB transmission dynamics in multi-host communities.

3.5 Conclusions

Quantifying interaction patterns between disease hosts through harmonised approaches is critical for inferring key drivers of differentiation across species and ecological features responsible for interaction rate variations. Our synthesis applied to animal TB highlights that only a few studies used specific criteria to define wildlife–cattle interactions, although different criteria were available. Regardless of the study context and host species considered, direct rates of interactions between wildlife and cattle were proven to be very infrequent. In contrast, the focus is on the indirect interactions that occur frequently in shared environments, wherein wildlife density may play a key role. In this sense, the classical direct aerosol transmission might be of minor importance in inter-species disease transmission, underlining the importance of assessing whether or not indirect interactions are the main driver of wildlife–cattle TB transmission. By dissecting, for the first time, patterns of wildlife–cattle interaction at a global scale and their potential connections with

ecological and methodological factors, we draw a path for future studies regarding shared interfaces and ecology of animal diseases. Ultimately, this could be the cornerstone to support initiatives for a successful reduction of disease-relevant interactions that have been proven to be relevant for disease epidemiology.

3.6 Acknowledgements and funding

3.7 References

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3.8   Supplementary material A

3.8.1   Supplementary Figures and Tables
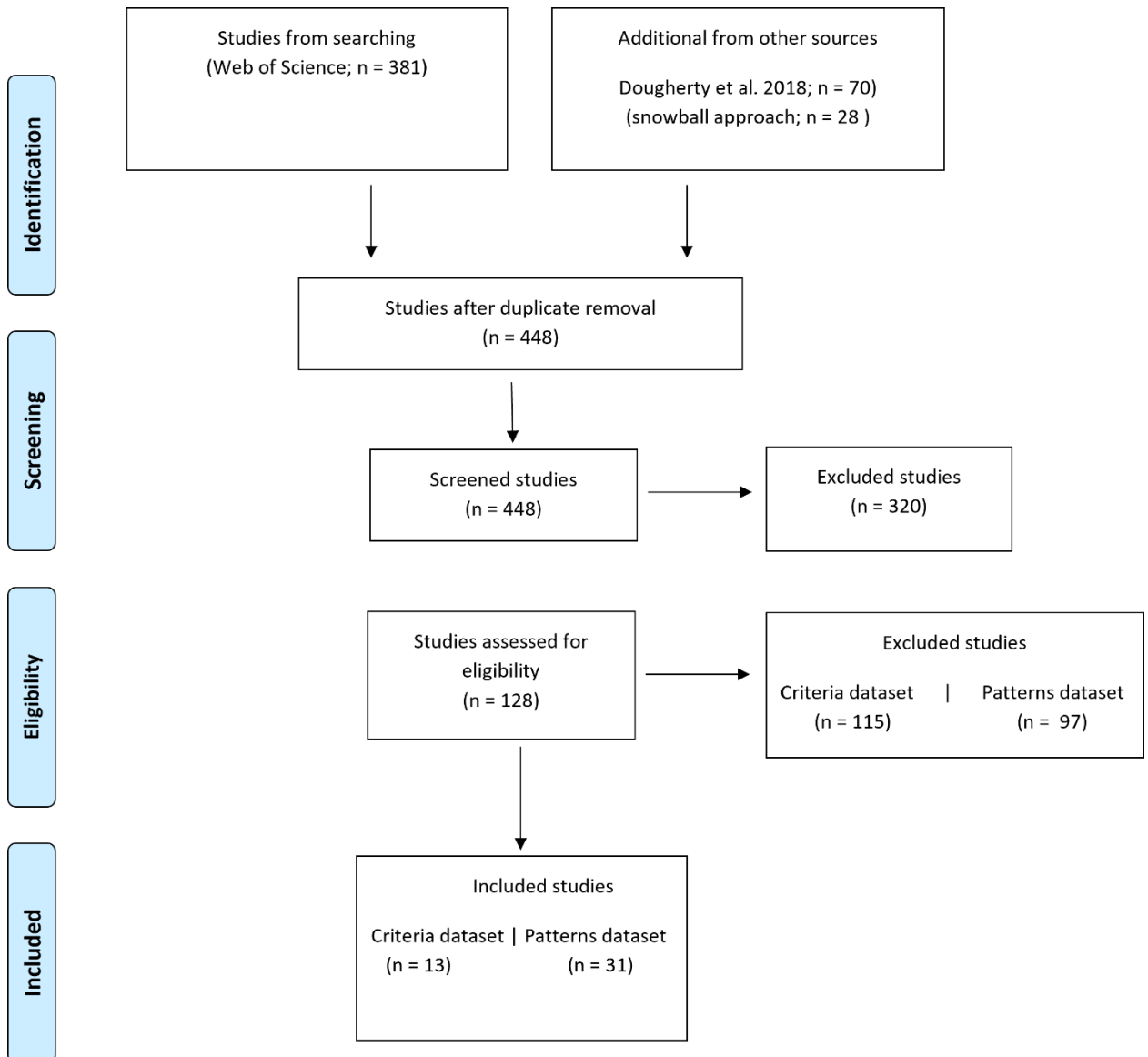
Figure S3-1. Flow diagram of literature search and selection which followed the PRISMA protocol (Preferred Reporting Items for Systematic reviews and Meta-Analyses).

Given its size, supplementary tables can be found online: https://doi.org/10.1111/mam.12324

Table S3-1. Source of the predictors and additional information used in the modelling process for the Patterns Dataset.

Table S3-2. Details of the predictors used in the model- ling of the Patterns Dataset.

Table S3-3. Summary of the studies identified in the literature wherein researchers adopted criteria to define a wildlife– cattle interaction in animal tuberculosis (TB) context.

Table S3-4. Estimates of wildlife–cattle interaction rates used in the modelling of Patterns Dataset, and predictor values extracted of each species pair.

Table S3-5. Summary of generalised linear models (GLMs), liner mixed models (LMMs) and generalised linear mixed models (GLMMs), performed on direct and indirect interactions.

Chapter 4

Host-, environment-, or human-related effects drive interspecies interactions in an animal tuberculosis multi-host community depending on the host and season

Chapter 4 – Host-, environment-, or human-related effects drive interspecies interactions in an animal tuberculosis multi-host community depending on the host and season

Abstract

In many Mediterranean ecosystems, animal tuberculosis (TB), caused by *Mycobacterium bovis*, is maintained by multi-host communities in which cattle and different wildlife species establish interaction networks contributing to *M. bovis* transmission and persistence. Most studies have addressed wildlife–cattle disease-relevant interactions, focusing on reservoir hosts, while disregarding the potential contribution of the so-called accidental hosts and/or neglecting wildlife–wildlife interactions. In this work, we aimed to characterise interspecies interactions in an endemic TB risk area and identify the ecological drivers of interaction patterns regardless of the pre-attributed role of host species on TB epidemiology. For that purpose, spatial–temporal indirect interactions between wildlife mammals and cattle, and between different wildlife species, were investigated through camera trapping. Second, five ecological hypotheses potentially driving species pair interactions in the wet and dry seasons were tested covering water and control sites: human presence (H1), landscape composition (H2), topography (H3), weather (H4), and natural food and water resources (H5). Wild boar (Sus scrofa), red deer (*Cervus elaphus*), and red fox (*Vulpes vulpes*) were the wildlife species mostly involved in indirect interactions. We found that indirect wildlife–cattle interactions were more frequent than wildlife interactions and, for certain species pairs, interaction rates were higher in the wet season in both wildlife–cattle and wildlife groups. Natural food and water resources (H5) was the most supported hypothesis that influenced the abundance of wildlife–cattle interactions, with positive effects during the dry season and negative effects during the wet season. In contrast, the abundance of indirect interactions between wildlife species was mainly supported by the human disturbance hypothesis (H1), with negative effects exerted on the dry season and variable effects on the wet season. Other tested hypotheses also influenced wildlife–cattle and wildlife–wildlife interactions, depending on the season and host species. These results highlight that indirect interactions, and thus conditions potentially favouring the transmission of *M. bovi*s in shared environments, are determined by different ecological backgrounds.

4.1 Introduction

Wildlife–livestock interfaces are physical spaces where wildlife and domestic species can overlap in space and time, along with humans, and where they can potentially interact (Hassell et al. 2017, Caron et al. 2021). Human activities (e.g., agricultural, husbandry practices, deforestation, industry) have been causing marked transformations on habitats (e.g. encroachment into natural areas, habitat fragmentation), shaping these interfaces (Venter et al. 2016, White & Razgour 2020, Meurens et al. 2021). With the loss of natural habitats due to anthropogenic land-use changes, many wildlife species are forced to live in close proximity to those interfaces. In addition, hunting activities have been leading to a notable overlap of large game hunting areas with cattle extensive farming in several regions (Carrasco-Garcia et al. 2016, Carpio et al. 2021). Such changes have profound effects on species interactions and thereby increase the risk of pathogen transmission and the (re) emergence of multi-host diseases (Jones et al. 2013, Alexander et al. 2018, White & Razgour 2020).

Pathogens shared by wildlife and cattle that are of economic and public health concern are considered an increasing problem worldwide (Gortazar et al. 2014, 2015, Webster et al. 2017, Hassell et al. 2021). In the last decades, various studies have been addressing wildlife–cattle interactions in the context of multi-host diseases, including animal tuberculosis (TB), covering different eco-epidemiological scenarios (Ferreira et al. 2023). Animal TB is mainly caused by *Mycobacterium bovis* and is a globally distributed zoonosis, affecting cattle and a wide range of wild mammals (Fitzgerald & Kaneene 2013, Gortázar et al. 2015, Pereira et al. 2020, Ramos et al. 2020). The negative economic impacts of TB on cattle are related to premature culling of animals, animal trade restrictions, rejections at slaughterhouses, and costly eradication plans when mandatory (Zinsstag et al. 2008, Caminiti et al. 2016). Wildlife maintenance hosts, which vary across ecosystems, hamper eradication efforts via pathogen spilling-back to cattle (Corner 2006, Palmer 2013, Pereira et al. 2020). Transmission may occur when a susceptible host comes into close contact with an infected host (direct interaction: same location and time), but also when animals contact asynchronously through contaminated environments (indirect interaction: shared space use in different time frames) (Fitzgerald & Kaneene 2013, Gortázar et al. 2015, Allen et al. 2021). In this sense, defining these spatial-temporal interactions between mammal hosts is of major importance for

understanding TB transmission (Kukielka et al. 2013, Wilber et al. 2019, Albery et al. 2020). This has been recognized as a critical step towards knowing where and when control actions should be prioritized (Triguero-Ocaña et al. 2019, Hayes et al. 2023, Yang et al. 2023).

Local and global studies have previously shown that direct interactions between wildlife hosts and cattle are scarce; in contrast, indirect interactions involving shared environments occur more frequently (Böhm et al. 2009, Lavelle et al. 2016, Campbell et al. 2019, Ferreira et al. 2023). Although explored in fewer studies, similar trends have been observed between different wildlife species, with indirect interactions being more frequent (Cowie et al. 2016, Payne et al. 2017). Opportunities for indirect interactions among wildlife at the wildlife–cattle interface are of particular concern in systems where *M. bovis* circulates in multi-host communities along ecosystem boundaries, potentially favouring pathogen transmission (Borremans et al. 2019, Gortázar et al. 2023). This is the case in Mediterranean ecosystems (Iberian Peninsula), where *M. bovis* is able to infect multiple domestic (cattle, pigs, and goats) and wildlife hosts (ungulates and carnivores) that occur in sympatry (Duarte et al. 2008, Santos et al. 2012, Vicente et al. 2013, Reis et al. 2021).

In Mediterranean ecosystems, the availability and distribution of water and food resources are deemed important for animal aggregation and subsequent interspecies interactions (Barasona et al. 2014, Carrasco-Garcia et al. 2016, Abrantes et al. 2019), with summer-autumn periods promoting increased disease-relevant interactions (Barasona et al. 2014, Triguero-Ocaña et al. 2019). Some studies have examined the effect of host attributes (e.g. animal density; Carrasco-Garcia et al. 2016), as well as of the environment and landscape contexts (e.g. land cover; Triguero-Ocaña et al. 2021) on patterns of interactions between TB hosts at the wildlife–cattle interface. However, the relative importance of different ecological factors, and how they contribute to regulate interspecies interaction patterns in multi-host communities, remains poorly understood (Allen et al. 2018). Moreover, multifaceted studies that also focus on non-reservoir hosts in the host-space-time axes and/or beyond the classic wildlife–cattle binomen are lacking. Considering accidental hosts and their interactions could help reconstruct missing links in *M. bovis* transmission chains, either among wildlife populations or from wildlife to cattle. Therefore, a community-based perspective when targeting complex multi-host TB systems is crucial (Wilber et al. 2019, Gortázar et al. 2023) to identify potential host species and to typify the group of animal

interactions that most likely contribute to TB maintenance within the community (Barroso et al. 2023).

In Portugal, red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) have been recognized as the most TB-relevant wildlife hosts, with reports of environmental contamination of natural substrates (soil and water bodies) in areas where wildlife TB is highly prevalent (Vieira-Pinto et al. 2011, Santos et al. 2015, 2018, Aranha et al. 2021, Pereira et al. 2023). In this work, we aimed to increase global understanding of spatial-temporal indirect interaction patterns within a multi-host mammal community (cattle and wildlife: red deer, wild boar, red fox [*Vulpes vulpes*], and badger [*Meles meles*]), focusing on a high prevalence TB area within a Mediterranean agroforestry system of southern Portugal. Specifically, we aimed to: i) typify the interaction patterns between cattle and wildlife, and between wildlife species, and discuss these patterns in relation to pathogen transmission risk; ii) compare the interaction rates between wildlife–cattle and between wildlife groups in the dry and wet seasons; and iii) evaluate the potential effect of a set of 18 ecological factors related to human disturbance, landscape composition, topography, weather and natural resources on both wildlife–cattle and wildlife-wildlife interactions in the dry and wet seasons.

4.2   Material and Methods

4.2.1 Study area

This study was conducted in Barrancos, located in southeast of Portugal (Alentejo region), close to the Spanish border (38°08' N; 6°59' W) (Figure 4-1). This area is considered a hotspot for TB in cattle and is included in the official epidemiological TB risk area where special measures (a mandatory veterinary examination of carcasses to search for TB-compatible lesions) apply to hunted big game species (red deer and wild boar) (Cunha et al. 2011, DGAV 2011, Santos et al. 2018). Ungulates are abundant in the region (wild boar density = 3-4 individuals/km^2; red deer density = 4-8 individuals/km^2) (Santos et al. 2022). Barrancos is an important *Montado* region (i.e. woodland, a savannah-like open tree forest) with extensively cattle breeding in sympatry with wildlife (e.g. big game). Herd TB prevalence was estimated at 1.83% for the Alentejo region in 2022 (DGAV 2023). A local study specifically conducted in Barrancos in 2014-2015 points towards a TB prevalence of 3.1% and 1.8% for red deer and wild boar, respectively (Costa 2015). While official numbers are remarkably lower, a meta-

regression and systematic review analyses estimated the pooled TB prevalence at a national scale as 27.5% and 13.3% for the red deer and wild boar, respectively (Reis et al. 2020).

The study area (SA) has a Mediterranean climate, with mild and wet winters and hot and dry summers. Mean annual temperature ranges from 5 °C to 14 °C during the winter (January), and from 15 °C to 34 °C during the summer (July) (Beja; 1981–2010; IPMA 2023). During this study period, the mean temperature in January was 8.9°C and in July was 25.5°C. The average annual precipitation is 555mm, concentrated between October and May. The topography is characterised by gentle to moderate undulating relief, with altitude ranging between 160 and 350m a.s.l. The landscape is dominated by holm oak (*Quercus rotundifolia*) *Montado*, with varying tree and shrub density (Agro: holm oak stands with low or absent shrub cover due to grazing and other pastoral activities; Forest: holm oak stands or mixed woodland patches with high shrub cover) (Figure 4-1). Other less representative land cover types include olive groves and few shrub and agricultural area mosaics.
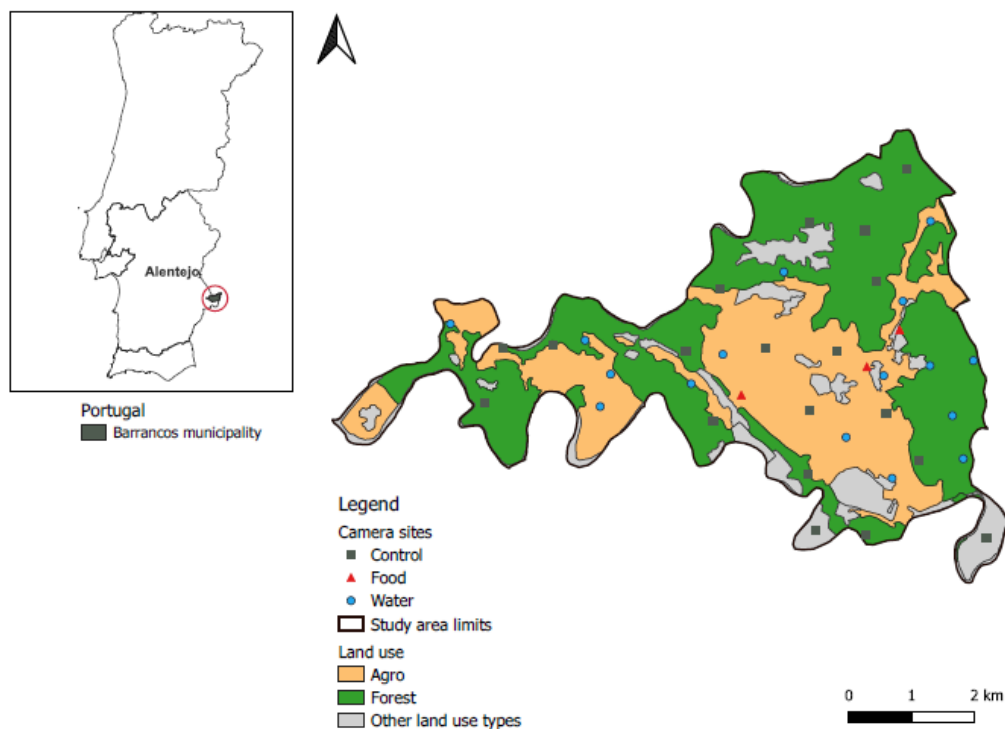
Figure 4-1. Study area location in Barrancos region, Portugal, showing camera sites and main land uses.

4.2.2 Study design

We used camera-trapping to assess spatial-temporal patterns of interactions involving wildlife–cattle and wildlife-wildlife species over a year (from April 2021 to April 2022). Besides cattle, we used as target species the TB reservoir hosts described for Portugal (red deer and wild boar ; Pereira et al. 2020, Santos et al. 2022), and two other susceptible species that occur in the region: the red fox and the badger (Santos et al. 2012, Matos et al. 2014).

We selected five free-ranging adjoining farms with similar management practices, comprising an area of ~3048 ha (farm size ranging from 148 ha to 980ha), with an average of 136 adult cows per farm. A 1 km grid was overlaid on the (SA) (Curveira-Santos et al. 2017, Zanni et al. 2021). One camera was installed on each 1 km^2 cell, to assure spatial independence of sampling sites and land cover representativeness. From this grid, we first selected key sites (water and supplementary food sites; Kukielka et al. 2013) – known as important aggregation points between species – prioritizing sites located in different grid cells (Triguero-Ocaña et al. 2020b), and an even distribution across farms. The remaining empty cells were defined as control sampling sites, and camera-traps were placed on their centroids. A total of 38 sampling sites (hereafter called camera sites; <u>Figure 4-1</u>) were defined: three food sites for cattle (hay feeders); 16 water sites (natural water sources and water trough) and 19 control sites (without any water sources or supplementary food, e.g. forest animal path). Minimum distance between camera sites averaged 686 m (range: 350 m to 1300 m).

Each camera site consisted of a single camera-trap (Busnhell Trophy Cam HD Aggressor or Reconyx Hyperfire) placed 30-50 cm above the ground, attached to trees or artificial stakes. At water and food sites, the cameras were facing towards areas highly used by cattle and wildlife to maximise the detection of interaction between different species. At control sites, we prioritised animal trails or other areas (e.g. resting sites) potentially used by cattle and wildlife in suitable habitats. No bait of any kind was used. We programmed cameras to operate 24 hours a day, taking three sequential pictures per trigger with a 30-second delay between consecutive triggers (Kukielka et al. 2013, Triguero-Ocaña et al. 2020b). On average, every 10-15 days, we visited camera sites for battery and memory card replacement.

4.2.3 Data coding and processing

Pictures recorded by each camera were individually classified by visual observation. The following information was recorded in an Excel database: camera coordinates, camera site type (water, food, or control), target species (cattle, red deer, wild boar, red fox, and badger), and number of individuals (minimum number of individuals recorded in each picture). In addition, date-time of picture capture were retrieved using the open-source software ExifTool (Harvey 2022). An independent observation of the same species (hereafter called 'detections') was considered at a given camera site when pictures were taken at least 15 min apart (Kukielka et al. 2013, Carrasco-Garcia et al. 2016, Martínez-Guijosa et al. 2021).

If cattle were unable to reach a given camera site in a certain period (due to cattle grazing rotation and management), that period from that camera was excluded from analyses. We assumed that the fences were permeable to wildlife (Carrasco-Garcia et al. 2016), as confirmed in the field and several times in the camera pictures. The three camera sites initially classified as food sites had no food for long periods of time, and thus were excluded from further analyses.

4.2.4 Definition and estimation of interactions

An indirect interaction was defined as the detection of one species at a given camera site, following the detection of another species within a pre-established critical time window, CTW, related with estimated *M. bovis's* environmental survival time. A CTW of three days for the dry season (June to September) and of 12 days for the wet season (October to May) was assumed, following the procedures of Kukielka and colleagues (Kukielka et al. 2013) and Cowie and colleagues (Cowie et al. 2016), applied in a similar eco-environmental context (see Supplementary Material B, Figure S4-1 and Figure S4-2). A direct interaction was defined whenever individuals of different species were captured in the same picture (Ferreira et al. 2023), although it was not analysed in this study due to the much lower number of observations recorded.

The number of indirect interactions was calculated for each camera site and month, discriminated by species pairs. The species pairs considered in this study are composed of the combinations of the five target species and are divided into two groups: the wildlife–cattle group includes four species pairs: BT_CE (cattle – red deer), BT_SS (cattle – wild boar),

BT_MM (cattle – badger), and BT_VV (cattle – red fox); and the wildlife group includes six species pairs: CE_MM (red deer – badger), CE_SS (red deer – wild boar), CE_VV (red deer – red fox), SS_MM (wild boar – badger), VV_MM (red fox – badger) and VV_SS (red fox– wild boar). For each species pair and camera site, we calculated monthly rates of indirect interactions (*RatesInt*) as a function of the number of interactions (nr of interactions) per time (*RatesInt* = nr of interactions/time), adapted from (Ferreira et al. 2023). Time was expressed as a proportion, corresponding to active camera days (days when cameras were operational and recording without any interference) divided by the number of days in a given month. We summarised *RatesInt* by species pairs and seasons (indirect interactions/month/camera), computing *RatesInt* means along with the corresponding standard errors. Generalised linear models (GLM) were used to inspect potential differences on *RatesInt* between wildlife–cattle and wildlife groups, across seasons.

4.2.5 Human, landscape, and environment predictors

To address the third objective, we defined a total of 18 eco-environmental predictors that influence the abundance of the target species and thus may influence species interaction patterns. These predictors were arranged according to five ecological hypotheses that might regulate species interactions. H1) human disturbance (n = 4 predictor variables); H2) landscape composition (n = 5); H3) topography (n = 3); H4) weather (n = 3); and H5) natural food and water resources (n = 3) (Table 4-1).

We estimated human disturbance (H1) for each camera site through the total number of days with human records (visually extracted from pictures); and through the Euclidean distance of camera sites to the nearest houses, to hunting sites (stand sites for hunting, where baiting is placed nearby for attracting wildlife) and road density metrics of unpaved roads (length of roads/total area within a given neighbourhood) in the SA (Quantum GIS v. 3.0.3; QGIS 2022). For landscape composition-related predictors (H2), we computed the proportion of land cover, considering the main land uses (agro and forest) occurring in the SA; the Shannon landscape diversity index, and the Euclidean distance of camera sites to forest edges. Those metrics were obtained from the Corine Land Cover (2018) dataset (European Union, Copernicus Land Monitoring Service, European Environment Agency) and were retrieved from the 'landscapemetrics' R package (Hesselbarth et al. 2019). In addition,

tree cover density was derived from the Tree Cover Density (2018) dataset (Copernicus Land Monitoring Service, European Environment Agency) (Table 4-1).

Table 4-1. Study hypotheses and description of the eco-environmental predictors used for modelling interspecies interactions.

Hypothesis	Inclusion rationale	Prediction	Predictor acronym	description
H1. *Human disturbance*	Wildlife species tend to show a spatial-temporal avoidance of humans and to humans related activities, which in turn may influence patterns of interspecies interactions (Alexandre et al. 2020, Laguna et al. 2021, Rosalino et al. 2022).	We expect a negative association between human disturbance and abundance of interspecies interactions. We also expect that a greater human presence may also imply a greater presence of domestic species which increases the likelihood of wildlife–cattle interactions.	Dist_houses	Distance of camera sites to the nearest artificial houses/facilities (m).
			Dist_hunt	Distance of camera sites to the nearest hunting site (m).
			DensRoad	Density of unpaved roads within 100, 250 and 500 m spatial scales around camera sites.
			Human_days	Number of days with occurrence of humans (a proxy for human presence).
H2. *Landscape composition*	The occurrence and distribution of species depend on their habitat requirements, and thus landscape context may be a key driver for interspecies interactions (Carrasco-Garcia et	We predict that landscape composition is the most important mechanism driving interspecies interactions. We expect a positive relationship between forest and heterogeneous areas and wildlife	Agro	Percentage of agroforest land (holm oak stands with low or absent shrub cover due to grazing and other pastoral activities) within 100, 250 and 500 m spatial scales around camera sites (%).
			Forest	Percentage of forest (holm oak stands or mixed woodland patches with high shrub cover) within 100, 250 and 500 m spatial scales around camera sites (%).

Hypothesis	Inclusion rationale	Prediction	Predictor acronym	description
	al. 2016, Curveira-Santos et al. 2017, Laguna et al. 2021).	interactions; and a positive relationship between agro-dominated areas and wildlife–cattle interactions.	TreeD	Proportion of tree cover density within 100, 250 and 500 m spatial scales around camera sites (%).
			Dist_edgeF	Distance of camera sites to the nearest edge of forest patches (m).
			Shidi	Shannon's landscape diversity index within 100, 250 and 500 m spatial scales around camera sites.
H3. *Topography*	Terrain features are important drivers that regulate species co-occurrence and thus influence shared space among host species (Alexandre et al. 2020, Linck et al. 2023).	We expect a negative relationship between topography-based predictors and species interactions.	Altitude	Terrain altitude within 100, 250 and 500 m spatial scales around camera sites.
			Rugg	Terrain ruggedness index within 100, 250 and 500 m spatial scales around camera sites.
			Slope	Topographic slope within 100, 250 and 500 m spatial scales around camera sites.
H4. *Weather*	Weather conditions shape species activity and, in turn, can drive interactions among hosts across space and time gradients (Rivrud et al. 2010, Kukielka et al. 2013, Zanni et al. 2021).	We predict that weather conditions exert positive or negative effects on interspecies interactions, being species-specific and season dependent.	Temp	Minimum monthly temperature (ºC), used in the wet season.
			Temp	Maximum monthly temperature (ºC), used in the dry season.
			Rain	Total monthly accumulated precipitation (mm).
H5. Natural *food and water resources*	Food and water resources can facilitate species aggregation, thus being an important factor shaping spatial and temporal patterns of interactions between mammal host species (Kukielka et al. 2013, Triguero-Ocaña et al. 2019).	We predict that food-rich areas, along with water abundance, have a positive influence on interspecies interactions, particularly during the dry season.	Water_cont	Water content at each camera site (mean monthly water area size; m2), calculated by visual estimation in the field.
			Station_site	Typology of the camera sites: control sites and water sites.
			NDVI	Normalized Difference Vegetation Index within 100, 250 and 500 m spatial scales around camera sites.

Regarding topographic predictors (H3), we estimated elevation from a 30-m Digital Elevation Model (DEM), and derived terrain ruggedness index and slope from the DEM using Quantum GIS v. 3.0.3. Weather-based predictors (H4; i.e. Rain and Temp) were obtained from data collected at a local weather station. Lastly, for H5, the water content (Water_cont) was visually estimated based on the area covered by standing water (using some marks in situ to retrieve estimates) during field work visits throughout the sampling period. The typology of each camera site – Station_site (control or water) – was used as a categorical variable. The normalized difference vegetation index (NDVI) was derived from the LANDSAT 8 image collection (level 2, Tier 1), with a 30 m spatial resolution, and processed in Google Earth Engine (Gorelick et al. 2017). The NDVI has shown high correlation with vegetation biomass and dynamics in various ecosystems worldwide. Several authors have used NDVI to assess vegetation productivity – representing resource quantity and quality – and the dynamics of habitat use by wild mammals, including ungulates and carnivores (Kerr & Ostrovsky 2003, Pettorelli et al. 2006, Santos et al. 2016). For this reason, we used NDVI as a proxy for natural food availability. We only retained high-quality images with ≤ 5% of cloud cover considering the whole SA (more details are available in Pinto et al. 2023). For the missing data in our time series (a three-month gap, non-consecutive months), we used images from the month before and after (time interpolation; Li et al. 2021) to estimate the NDVI values (Oeser et al. 2021).

A multi-scale approach was carried out to cover a wide range of scales and thus maximise potential responses with the target species (Arroyo-Rodríguez et al. 2023). Continuous predictors not based on distances (Dens_roads, TreeD, Altitude, Rugg, Slope and NDVI) were stacked in a 30 m spatial resolution multi-raster layer. We then applied the following spatial scales of analysis: 90, 240 and 510 m focal-radius moving window as a proxy for 100, 250 and 500 m neighbourhood scales of analysis around camera sites. Mean was used to summarize the raster values within each spatial scale. A similar procedure (in terms of scales) was applied to Agro, Forest and Shidi using a spatial resolution basis of 10 m, and thus a focal-radius moving window of 100, 250 and 500 meters (Table 4-1).

We also estimated the relative abundance index of each target species (e.g. RAI), discriminated by camera site and season, to be used as a proxy of animal density in the modelling process. Animal abundance was calculated as the number of detections of each species in a month/(number of active camera days/number of days of a given month).

4.2.6 Modelling: hypothesis explaining interspecies indirect interactions

Interaction analyses were conducted separately for each species pair, and for the dry and wet seasons, allowing the identification of potential differences in the effects of predictors driving interactions between seasons. As pre-modelling procedures, we checked for outliers and inspected collinearity among variable predictors. Pairwise Spearman correlations were calculated among all predictors to check for multicollinearity. Numeric predictors with skewed distributions were transformed (square-root, logarithmic, or arcsine) to approach normality and to reduce the influence of extreme values (Zuur et al. 2009). In addition, all continuous predictors were standardized, allowing comparisons of their strength in the modelling process.

We fitted the response variable – number of species interactions – to generalized linear mixed models (GLMM) with a Poisson or Negative Binomial family distribution and log link (package 'glmmTMB', Brooks et al. 2017), using camera site as a random factor because each camera site was sampled repeatedly through time. The log of the number of active camera-days was used as offset in the models to integrate sampling effort between camera sites over time (Kukielka et al. 2013). This procedure avoided transforming count data (log-transformed data or *RatesInt*), as recommended by Zuur and colleagues (Zuur et al. 2009) and O'Hara & Kotze (2010).

The five ecological hypotheses (H1 to H5) were independently evaluated (Vallejo-Vargas et al. 2022), first through simple models, testing one predictor at a time. These simple models always included the abundances of each species (RAI) involved in a given species pair interaction as fixed predictors, since higher host abundance increases interaction levels (Ferreira et al. 2023). Then, if more than one predictor was informative within a hypothesis, a multivariate model was built for each hypothesis with all informative predictors.

Model example: species pair AB | season

Number of interactions ~ *animal abundance (A)* + *animal abundance (B)* + predictor X + random (1|camera site), *offset* (log(camera days)), *family* (poisson/negative binomial).

A predictor variable was considered informative when: 1) the 95% confidence intervals (CI 95%) of the variable coefficient being tested did not include zero; and 2) a delta AICc > 2 (ΔAICc; Akaike's Information Criterion adjusted for small sample sizes) was obtained when

comparing the tested model with the reference model (without the specific predictor; Burnham & Anderson 2002, Stephens et al. 2005, Zuur et al. 2009). If highly correlated informative predictors (r > 0.7) were identified, we only retained the one producing a lower AICc to be included in the multivariate model. This procedure also involved comparing multiple scales for a given predictor. Multivariate models were built with all possible combinations of the informative predictors of each hypothesis, always keeping animal abundance (RAI) in all competing models, and limiting each model to a maximum of four predictors to avoid model instability. We selected the best multivariate model for each hypothesis using AICc. Models having a ΔAICc < 2 are considered equally supported. When several models had ΔAICc < 2: 1) all associated predictors were included in a single best multi-model (Humphrey et al. 2023) if ≤ four predictors were selected; 2) all models within ΔAICc < 2 of the top-ranked models were retained for interpretation, otherwise.

The dredge function (R package 'MuMIn', Bartoń 2022) was used for model selection. Once we identified all the best models for the hypotheses tested (H1 – H5), we again ran the models with a restricted maximum likelihood (REML). Since it is important to assess model adequacy (Mac Nally et al. 2018, Tredennick et al. 2021), models were evaluated and validated using diagnostic tools (normality, outliers, and zero inflation) available in the 'DHARMa' package (Hartig 2022).

4.3 Results

We obtained a total of 15537 detections of cattle and target wild mammal species over 6170 effective trap days across the 35 camera sites (mean = 176 ± 61 sd trap days per camera site) during the study period. Cattle was the most frequently detected species (66.8%; n = 10379). Red fox (10.5%; n = 1631), red deer (8.6%; n = 1335) and wild boar (7.3%; n = 1141) were detected in similar numbers and were widespread in the SA (detection in > 85% of camera sites). The badger occurred at lower rates (2.5%; n = 382), although it was also widespread in the SA (detection in > 75% of camera sites).

4.3.1 Wildlife–cattle and wildlife species interactions

Wildlife–cattle indirect interactions represented 52.7% (n = 3619) of the interaction data (only 0.1% [n = 7] were direct interactions involving cattle). The wildlife species that were

most frequently involved in these interactions were the red fox (BT_VV; mean *RatesInt*: wet season = 6.1 and mean *RatesInt*: dry season = 4.5), followed by the wild boar (BT_SS; mean *RatesInt*: wet season = 4.8 and mean *RatesInt*: dry season =2.8) and red deer (BT_CE; mean *RatesInt*: wet season = 4.5 and mean *RatesInt*: dry season = 2.5). The badger (BT_MM; mean *RatesInt*: wet season = 1.6 and mean *RatesInt*: dry season = 1.5) interacted less frequently with cattle (Figure 4-2 a). Interactions with cattle involving the three most detected species (red fox, wild boar and red deer) occurred in all farms, at more than 80% of camera sites during the wet season, and at 30% to 60% of camera sites in the dry season. Interaction rates were significantly higher in the wet season for the pairs BT_VV (GLM; coef: wet season = 0.361, CI 95% [0.050; 0.672]), BT_SS (GLM; coef: wet season = 0.304, CI 95% [0.024; 0.585]) and BT_CE (GLM; coef: wet season = 0.441, CI 95% [0.167; 0.714]).
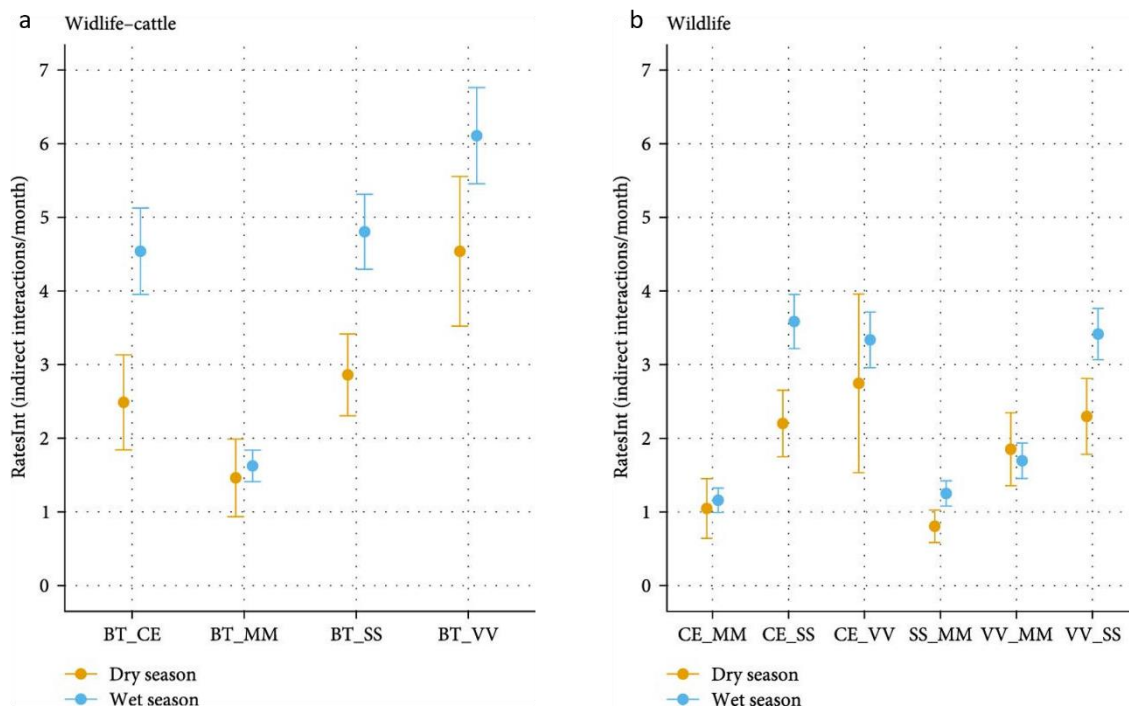
Figure 4-2. Weighted means and standard errors of *RatesInt* (indirect interactions/month) summarized by species pairs and seasons and displayed by animal group (a = wildlife–cattle; b = wildlife). Species pair acronyms are: (BT_CE) cattle – red deer; (BT_MM) cattle – badger; (BT_SS) cattle – wild boar; (BT_VV) cattle – red fox; (CE_MM) red deer – badger; (CE_SS) red deer – wild boar; (CE_VV) red deer – red fox; (SS_MM) wild boar – badger; (VV_MM) red fox – badger; (VV_SS) red fox – wild boar.

Indirect interactions between wildlife represented 46.8% (n = 3210) of the interaction data (only 0.4% [n = 25] were direct interactions). The wildlife species pairs most frequently interacting were CE_SS (mean *RatesInt*: wet season = 3.6 and mean *RatesInt*: dry season = 2.2), CE_VV (mean *RatesInt*: wet season = 3.3 and mean *RatesInt*: dry season = 2.7) and VV_SS (mean *RatesInt*: wet season = 3.4 and mean *RatesInt*: dry season = 2.3) (Figure 4-2 b). Indirect interactions between the three main species (red fox, wild boar and red deer) occurred at more than 80% of camera sites during the wet season, and at 40% to 60% of camera sites during the dry season. Interaction rates were significantly higher in the wet season for the pairs CE_SS (GLM; coef: wet season = 0.283, CI 95% [0.031; 0.535]), CE_VV (GLM; coef: wet season = 0.302, CI 95% [0.045; 0.559]), and VV_SS (GLM; coef: wet season = 0.297, CI 95% [0.038; 0.556]).

4.3.2 *RatesInt* between wildlife–cattle and wildlife groups

Globally, interaction rates (*RatesInt*) were higher in the wet season for both wildlife–cattle and wildlife groups when compared to the dry season. The mean interaction rates of the wildlife–cattle group were 1.8 and 1.6 times significantly higher than the wildlife rates for the dry and wet seasons, respectively (GLM dry season; coef wildlife: -0.156, CI 95% [-0.285; -0.0269]; GLM wet season; coef wildlife: -0.269, CI 95% [-0.354; -0.184]).

4.3.3 Ecological hypotheses driving species interactions

All models were fitted with a Poisson family distribution. The predictors Slope, Rugg, Agro and Forest were not used simultaneously in the same model due to multicollinearity problems. Locations with high terrain ruggedness had also higher slope (rs = 0.99) and low percentage of Agro (rs = - 0.73). On the other hand, locations with high percentage of Agro had low percentage of Forest (rs = - 0.74). Model residual patterns revealed a good to adequate fit of most of the models to the data (Supplementary Material B, Figure S4-3 to Figure S4-6: DHARMa diagnostic plots showing residual, dispersion and zero-inflation fits of the tested models). Four of the five ecological hypotheses tested were significantly associated with abundance of wildlife–cattle interactions, covering one to three species pairs, depending on the hypothesis (Table 4-2, Figure 4-3 a). Three of the five ecological hypotheses tested were significantly associated with the abundance of wildlife interactions, covering from one to four species pairs, depending on the hypothesis (Table 4-3; Figure 4-3

c). Wildlife–cattle interactions were most related to natural food and water resources hypothesis (H5) (Figure 4-3 b), while wildlife interactions were often associated with human disturbance hypothesis (H1) (Figure 4-3 d).
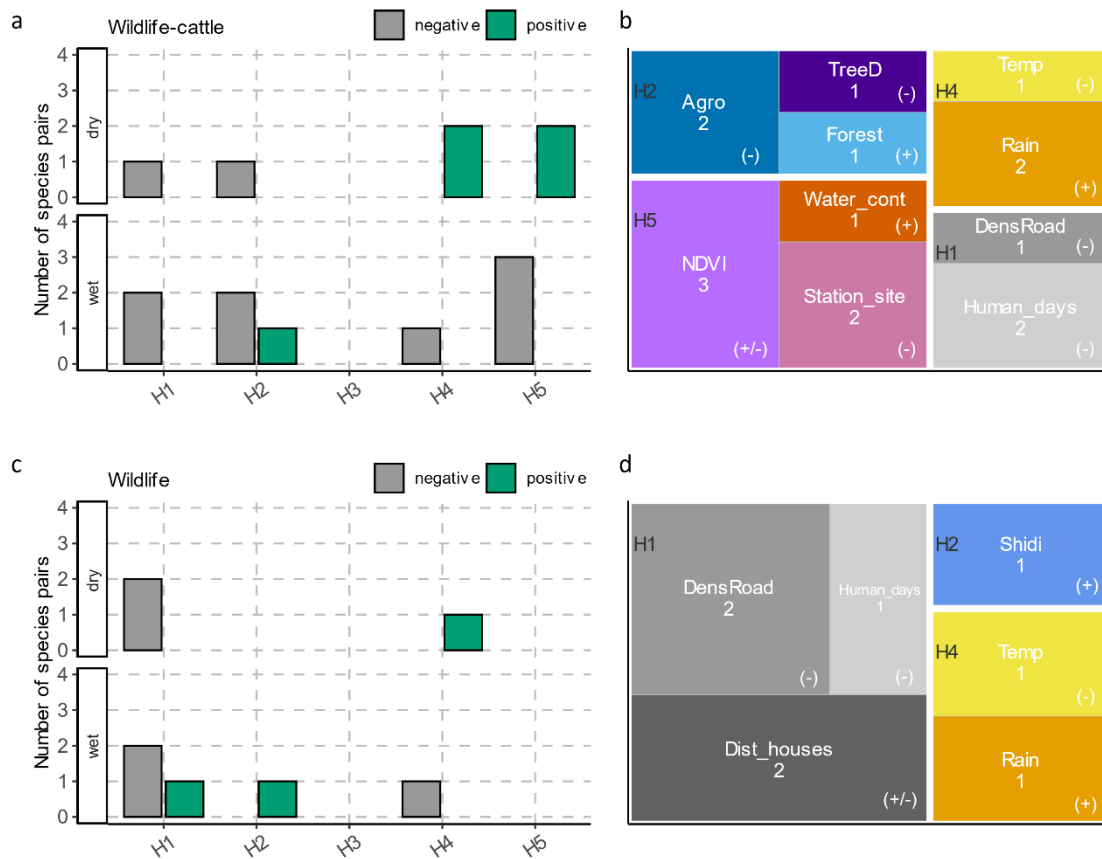
Figure 4-3. Number of species pairs influenced by ecological hypotheses regarding indirect interactions, displayed by wildlife–cattle (a) and wildlife (c) groups and considering sampled seasons. For each hypothesis, the sign of the coefficient effect is shown (positive, negative, or null). Treemaps show the number of times the tested predictors, underlying ecological hypotheses, were associated with species pair interactions, displayed by wildlife–cattle (b) and wildlife (d) groups.

Table 4-2. Summary of the hypotheses (H) tested and predictors (highlighted in bold) significantly related with wildlife–cattle species pairs interactions. For each species pair and season, we provided the best model according to the model´s AICc (Akaike's Information Criterion adjusted for small sample sizes). The AICc of the reference model and the null model is also provided. Delta AICc (ΔAICc) was obtained between the reference model and each best model for a given hypothesis. The coefficients (Coeff.) and corresponding 95% confidence intervals (CI 95%) for each tested predictor are presented. Incidence rate ratios (IRR) are reported as exponentiated results. * (asterisk) was used to mark species pairs and seasons for which we did not find a significant association with the tested hypotheses.

Species pair	Season	H	Model id	Null model AICc	Model ref AICc	Model AICc	deltaAIC	Predictors	Coeff.	CI 95%	IRR
BT_CE	wet	H2	BTCE_wH2	1110.9	601.6	598.1	3.5	**BT abundance**	0.945	[0.792; 1.098]	2.573
								CE abundance	1.021	[0.901; 1.141]	2.776
								Forest_100	0.175	[0.023; 0.326]	1.191
BT_CE	wet	H4	BTCE_wH4	1110.9	601.6	597.0	4.6	**BT abundance**	0.871	[0.726; 1.015]	2.388
								CE abundance	1.065	[0.944; 1.186]	2.902
								Temp	-0.088	[-0.155; -0.020]	0.916
BT_CE	wet	H5	BTCE_wH5	1110.9	601.6	594.7	6.9	**BT abundance**	0.937	[0.794; 1.080]	2.552
								CE abundance	1.090	[0.973; 1.207]	2.974
								Station_site: water	-0.361	[-0.595; -0.127]	0.697
BT_CE	dry	H2	BTCE_dH2	248.7	165.5	161.6	3.9	**BT abundance**	0.626	[0.246; 1.005]	1.870
								CE abundance	1.363	[0.949; 1.778]	3.908
								TreeD_100	-0.868	[-1.659; -0.077]	0.420
BT_CE	dry	H4	BTCE_dH4	248.7	165.5	160.9	4.6	**BT abundance**	1.071	[0.561; 1.580]	2.917

Species pair	Season	H	Model id	Null model AICc	Model ref AICc	Model AICc	deltaAIC	Predictors	Coeff.	CI 95%	IRR
								CE abundance	1.362	[0.936; 1.788]	3.902
								Rain	0.314	[0.061; 0.568]	1.369
BT_CE	dry	H5	BTCE_dH5	248.7	165.5	151.3	14.2	**BT abundance**	0.855	[0.402; 1.309]	2.352
								CE abundance	1.248	[0.852; 1.643]	3.483
								NDVI_500	0.198	[-0.074; 0.471]	1.219
								Water_cont	0.878	[0.367; 1.388]	2.405
BT_SS	wet	H1	BTSS_wH1	1042.8	629.2	624.6	4.6	**BT abundance**	0.743	[0.611; 0.875]	2.103
								SS abundance	1.013	[0.904; 1.123]	2.754
								Human_days	-0.180	[-0.321; -0.039]	0.836
BT_SS	wet	H2	BTSS_wH2	1042.8	629.2	624.7	4.5	**BT abundance**	0.796	[0.662; 0.929]	2.216
								SS abundance	0.998	[0.896; 1.100]	2.713
								Agro_100	-0.141	[-0.247; -0.035]	0.868
BT_SS	wet	H5	BTSS_wH5	1042.8	629.2	625.7	3.5	**BT abundance**	0.707	[0.580; 0.833]	2.027
								SS abundance	1.052	[0.945; 1.159]	2.863
								NDVI_100	-0.088	[-0.162; -0.014]	0.915
BT_SS*	dry	-	-	-	-	-	-	-	-	-	-
BT_VV	wet	H1	BTVV_wH1	1386.5	688.3	683.4	4.9	**BT abundance**	0.985	[0.841; 1.129]	2.678
								VV abundance	1.013	[0.908; 1.119]	2.755
								Human_days	-0.110	[-0.193; -0.026]	0.896
BT_VV	wet	H5	BTVV_wH5	1386.5	688.3	681.9	6.4	**BT abundance**	0.983	[0.841; 1.125]	2.671
								VV abundance	0.999	[0.897; 1.102]	2.717
								NDVI_500	-0.073	[-0.137; -0.008]	0.930
								Station_site: water	-0.308	[-0.571; -0.045]	0.735
BT_VV	dry	H1	BTVV_dH1	287.8	180.3	175.9	4.4	**BT abundance**	0.752	[0.490; 1.013]	2.120
								VV abundance	1.551	[1.215; 1.887]	4.715

Species pair	Season	H	Model id	Null model AICc	Model ref AICc	Model AICc	deltaAIC	Predictors	Coeff.	CI 95%	IRR
								DensRoad_250	-0.384	[-0.682; -0.086]	0.681
BT_VV	dry	H4	BTVV_dH4	287.8	180.3	171.8	8.5	**BT abundance**	1.026	[0.672; 1.380]	2.789
								VV abundance	1.550	[1.179; 1.921]	4.711
								Rain	0.296	[0.111; 0.481]	1.344
BT_VV	dry	H5	BTVV_dH5	287.8	180.3	173.1	7.2	**BT abundance**	0.966	[0.643; 1.289]	2.627
								VV abundance	1.651	[1.262; 2.040]	5.211
								NDVI_100	0.360	[0.124; 0.596]	1.433
BT_MM	wet	H2	BTMM_wH2	651.1	371.3	368.5	2.8	**BT abundance**	0.920	[0.703; 1.137]	2.508
								MM abundance	1.174	[1.037; 1.312]	3.236
								Agro_100	-0.206	[-0.391; -0.020]	0.814
BT_MM*	dry	-	-	-	-	-	-	-	-	-	-

Table 4-3. Summary of the hypotheses (H) tested and predictors (highlighted in bold) significantly related with wildlife species pair interactions. For each species pair and season, we provided the best model according to the model´s AICc (Akaike's Information Criterion adjusted for small sample sizes). The AICc of the reference model and the null model is also provided. Delta AICc (ΔAICc) was obtained between the reference model and each best model for a given hypothesis. The coefficients (Coeff.) and corresponding 95% confidence intervals (CI 95%) for each tested predictor are presented. Incidence rate ratios (IRR) are reported as exponentiated results. * (asterisk) was used to mark species pairs and seasons for which we did not find a significant association with the tested hypotheses.

Species pair	Season	H	Model id	Null model AICc	Model ref AICc	Model AICc	deltaAIC	Predictors	Coeff.	CI 95%	IRR
CE_SS	wet	H1	CESS_wH1	928	539.7	537.8	2.0	**CE abundance**	0.740	[0.632; 0.847]	2.095
								SS abundance	0.788	[0.672; 0.903]	2.198
								Dist_houses	0.083	[0.001; 0.164]	1.086
CE_SS	wet	H4	CESS_wH4	928	539.7	537.1	2.6	**CE abundance**	0.747	[0.638; 0.856]	2.111
								SS abundance	0.752	[0.635; 0.870]	2.122
								Temp	-0.082	[-0.157; -0.007]	0.921
CE_SS*	dry	-	-	-	-	-	-	-	-	-	
CE_VV	wet	H1	CEVV_wH1	906.1	529.9	527.4	2.5	**CE abundance**	0.933	[0.832; 1.033]	2.542
								VV abundance	0.892	[0.784; 1.000]	2.440
								DensRoad_100	-0.116	[-0.224; -0.008]	0.891
CE_VV*	dry	-	-	-	-	-	-	-	-	-	
CE_MM*	wet	-	-	-	-	-	-	-	-	-	
CE_MM	dry	H4	CEMM_dH4	205.4	90.4	83.7	6.7	**CE abundance**	0.757	[0.295; 1.218]	2.131
								MM abundance	1.043	[0.736; 1.350]	2.838
								Rain	0.455	[0.118; 0.791]	1.575
VV_SS	wet	H1	VVSS_wH1	859.4	546.1	543.8	2.3	**VV abundance**	0.837	[0.708; 0.966]	2.310

Species pair	Season	H	Model id	Null model AICc	Model ref AICc	Model AICc	deltaAIC	Predictors	Coeff.	CI 95%	IRR
								SS abundance	0.920	[0.788; 1.051]	2.509
								Human_days	-0.161	[-0.318; -0.004]	0.851
VV_SS	wet	H2	VVSS_wH2	859.4	546.1	538.7	7.4	**VV abundance**	0.855	[0.737; 0.972]	2.350
								SS abundance	0.929	[0.807; 1.051]	2.532
								Agro_100	-0.091	[-0.191; 0.008]	0.913
								Shidi_100	0.108	[0.024; 0.192]	1.114
VV_SS	dry	H1	VVSS_dH1	251.2	136.3	132.3	4.0	**VV abundance**	1.220	[0.838; 1.602]	3.387
								SS abundance	1.054	[0.762; 1.345]	2.869
								Dist_houses	-0.376	[-0.708; -0.043]	0.687
SS_MM*	wet	-	-	-	-	-	-	-	-	-	
SS_MM	dry	H1	SSMM_dH1	173.4	94.3	89.2	5.1	**SS abundance**	1.111	[0.676; 1.545]	3.036
								MM abundance	1.065	[0.811; 1.319]	2.900
								DensRoad_100	-0.635	[-1.122; -0.147]	0.530
VV_MM*	wet	-	-	-	-	-	-	-	-	-	-
VV_MM*	dry	-	-	-	-	-	-	-	-	-	-

4.3.3.1 Modelling: wildlife–cattle interactions

The number of wildlife–cattle interactions, involving the red fox and wild boar, increased in areas with a lower human presence during the wet season (H1, models: BTVV_wH1 and BTSS_wH1; Table 2). Additionally, in this season, interactions encompassing the red deer, wild boar and badger increased in more forested areas (e.g. areas with low Agro cover; H2, models: BTCE_wH2, BTSS_wH2, and BTMM_wH2). More interactions between cattle and red deer were associated with low-temperature periods (H4, model: BTCE_wH4). The higher abundance of interactions, covering red deer, red fox and wild boar, occurred in areas where natural resources are less abundant (i.e. control sites and less productive areas [NDVI]) (H5, models: BTCE_wH5, BTVV_wH5 and BTSS_wH5). During the dry season, wildlife–cattle interactions increased in areas with lower road densities, as evidenced for the red fox (H1, model: BTVV_dH1), and in areas with lower tree cover, in the case of the red deer (H4, model: BTCE_dH2). Rain had a positive influence on the abundance of wildlife–cattle interactions (H4, models: BTCE_dH4 and BTVV_dH4), and interactions were more frequent in sites with higher water content and in more productive areas, for carnivores and ungulates, such as the red fox and the red deer, respectively (H5, models: BTVV_dH5 and BTCE_dH5). Overall, animal abundance had a strong effect size in all models: with one-point increase in animal abundance (wildlife or cattle), number of interactions would be expected to increase by an average IRR of 2.93 (sd = 0.58), holding all variables constant. Ecological predictors, linked to the study hypotheses, had a lesser pronounced effect (positive predictors: average IRR = 1.49, sd = 0.46; negative predictors: average IRR = 0.79, sd = 0.15).

4.3.3.2 Modelling: wildlife–wildlife interactions

During the wet season, wildlife interactions involving ungulates increased at longer distances to houses (H1, model: CESS_wH1; Table 3), and in areas with lower road densities, for the species pair CE_VV (H1, model: CEVV_wH1). Human disturbance, through human presence, also had a negative effect on the abundance of wildlife interactions in this season: in this case between wild boar and red fox (H1, model: VVSS_wH1). Furthermore, wildlife interactions – encompassing VV_SS and CE_SS species pairs – increased in areas with higher landscape diversity (H2, model: VVSS_wH2) and when the temperature was lower (H4, model: CESS_wH4). In the dry season, wildlife interactions also increased as a function of low road densities, specifically for the SS_MM species pair (H1, model: SSMM_dH1), while

interactions between the red fox and wild boar increased at reduced distances from houses (H1, model: VVSS_dH1). Furthermore, wildlife interactions – involving badger and red deer – increased in rainy periods (H4, model: CEMM_dH4). Overall, with a one-point increase in animal abundance (wildlife), the number of interactions would be expected to increase by an average IRR of 2.52 (sd = 0.39), holding all variables constant. Ecological predictors, linked to the study hypotheses, had a lesser pronounced effect size. Positive predictors had an average IRR of 1.26 (sd = 0.27), while predicators exhibiting a negative relation with the number of wildlife interactions had an average IRR of 0.80 (sd = 0.149), meaning that a one-point increase in a given predictor would be expected to result in a decrease in the rate ratio for the number of interactions.

4.4 Discussion

Pathogen transmission at shared interfaces is a heterogeneous and dynamic process, significantly dependent on spatial and temporal processes. Despite being overlooked in certain TB risk areas, characterizing spatial-temporal variation in interaction patterns, addressing all relevant hosts, is essential to properly understand pathogen transmission dynamics in complex animal communities.

We demonstrated that: 1) wildlife–cattle and wildlife indirect interactions occur frequently. All the target species contributed to the network of disease-relevant interactions yet, wild boar, red deer, and red fox were the wildlife hosts mostly involved in indirect interactions across seasons. Regardless of the group considered, species pairs interactions were generally higher in the wet season; 2) the rates of indirect interaction involving wildlife–cattle were higher than the interactions between wildlife species, in both seasons; 3) several hypotheses influenced indirect interactions, although responses differed among groups and seasons. Wildlife–cattle interactions were more frequently related with the natural food and water hypothesis (H5), while wildlife indirect interactions were more associated with the human disturbance hypothesis (H1).

4.4.1 Wildlife–cattle and wildlife interaction patterns

Interspecies direct interactions were rare, as previously documented in other studies (Cowie et al. 2016, Lavelle et al. 2016, Martínez-Guijosa et al. 2021). This highlights that even

generalist species, with similar ecological requirements, tend to partition resource use and habitat exploitation spatially and temporally (Curveira-Santos et al. 2017, Zanni et al. 2021, Teixeira et al. 2023). On the other hand, wildlife–cattle and wildlife indirect interactions were frequent and widespread throughout the study area. Such results are consistent with previous findings reported in Mediterranean ecosystems, supporting the hypothesis that *M. bovis* transmission (and other multi-host pathogens with similar excretion routes) is mainly indirect through contaminated shared environments (Allen et al. 2021, Varela-Castro et al. 2021, Gortázar et al. 2023). Agroforestry systems like *Montado* – known as *Dehesa* in Spain – are highly complex structures often considered as high nature value farming systems, supporting high levels of biodiversity (Pinto-Correia et al. 2011). Human activities (e.g. hunting interests), along with other ecological and social factors, have been shaping these interfaces, promoting a notable overlap between wildlife (e.g. big game hunting) and cattle faming. Consequently, *Montado* interfaces have become increasingly interconnected, requiring improved management practices, as shared space is expected to favour interspecies disease transmission. Indeed, the long-term excretion and viability maintenance of *Mycobacterium tuberculosis* complex bacteria (MTBC) in environmental substrates (Pereira et al. 2023) increase animal exposure risk, particularly in animal aggregation areas that are asynchronously used by different species. In Mediterranean Spain, host species richness has been correlated with increased community competence to maintain and transmit MTBC, oppositely to other epidemiological settings where biodiversity could favour a "dilution effect" and moderate pathogen transmission (Barasona et al. 2019).

Wild boar, red deer and red fox were the wildlife hosts more frequently involved in indirect interactions, as shown in previous studies conducted in similar environments (Carrasco-Garcia et al. 2016, Payne et al. 2016, Triguero-Ocaña et al. 2020a, Varela-Castro et al. 2021). The positive relationship between wildlife/cattle abundance and the number of interactions is notable, with significant effects observed in all tested models. This pattern is compatible with a density-dependent mechanism, a hypothesis previously suggested in the context of animal interactions within disease systems (Manlove et al. 2022), including TB (Carrasco-Garcia et al. 2016, Ferreira et al. 2023). Thus, higher interaction events involving ungulates and red foxes are expected, as they are more abundant in our study area. On the other hand, the low number of indirect interactions involving badgers could be related to their lower local abundance, in contrast to other Iberian environments (e.g. Asturias, Northern Spain)

and other European TB contexts (e.g. UK), known to have higher badger population densities and where significant shared space between badgers, cattle, and other wild mammals has been documented (Drewe et al. 2013, Varela-Castro et al. 2021). From an epidemiological perspective, these results highlight that reservoir hosts (wild boar and red deer) potentially play a key role in disease transmission in the study region and should therefore receive increased attention (Santos et al. 2022). Wild boar has been identified as a TB maintenance host in most study sites across the Iberian Peninsula. In the context of multi-pathogen networks (study conducted in Spain), wild boar is considered as the key and most connected species of the system community, bridging several hosts relevant to the epidemiology of MTBC (Santos et al. 2022, Barroso et al. 2023). Also, TB prevalence in wild boar and the red deer was considered an important factor positively linked to TB in cattle farms of Iberian regions (LaHue et al. 2016, Herraiz et al. 2023). Nevertheless, additional research (e.g. pathogen excretion patterns and burden) is needed, including for other non-reservoir hosts, given their potential to indirectly interact with various species, as the case of the red fox in our study. The red fox is a generalist carnivore that can exploit a variety of habitats, including farm-related sites (Tolhurst et al. 2011), and was recognized as a spillover host in certain regions (Richomme et al. 2020). However, despite recent insights about MTBC environmental contamination in the Iberian Peninsula (Martínez-Guijosa et al. 2020, Pereira et al. 2023), the relative importance of certain TB hosts – including the red fox – to environmental contamination remains poorly understood in TB risk areas.

The higher rates of interactions during the wet season may be due to different factors (e.g. species-specific behaviours, animal density; Carrasco-Garcia et al. 2016, Triguero-Ocaña et al. 2019), but are mostly driven by two. First, the higher availability and abundance of resources during the wet season (e.g. autumn). While summer periods tend to drive species aggregation around spatially limited resources (e.g. water sites), the wet season is characterised by high availability and abundance of natural food and water sites. This could attract species to new areas, resulting in indirect shared space across landscapes, which can be significant when considering common and generalist species as the case of red deer, wild boar, and red fox. Second, in our study area, cattle are confined to fewer grazing plots during the dry season when compared to the wet season. This may also be a plausible explanation for the lower rates of interactions involving cattle in the dry season (less sites where animal hosts may engage), and contradicts other studies that referred to a generalised increase in

indirect interactions in dry periods (but also in autumn periods) (Kukielka et al. 2013, Cowie et al. 2016).

4.4.2 Differences between wildlife–cattle and wildlife interaction rates

Wildlife–cattle indirect interaction rates were almost two times higher than wildlife interaction rates in both seasons. Triguero-Ocaña et al. (2020a) have also found that wildlife–cattle interactions involving red deer, fallow deer (*Dama dama*) and wild boar were more frequent than interactions between wildlife species. Such patterns could be related to how species partition resources across the landscape and to species-specific behaviour traits, which may differ between the two groups. The response of wildlife to cattle presence (e.g. behavioural effects) can be heterogeneous when considering different animal species and landscape contexts (Huaranca et al. 2022). Although some studies have shown that cattle presence had a negative influence on space use by some carnivore host species (e.g. badger and red fox; Mullen et al. 2013, Woodroffe et al. 2016, Alexandre et al. 2020), others have shown that cattle presence was positively associated with wildlife occurrence, namely for the wild boar and red deer in agroforestry areas (Carrasco-Garcia et al. 2016, Martínez-Guijosa et al. 2021). Regarding the spatial-temporal profiles of wildlife species, some studies demonstrated that even habitat-generalist carnivores (e.g. red fox and badger) may exhibit contrasting habitat preferences at a small-scale in agroforestry systems (Curveira-Santos et al. 2017); and mesocarnivore co-occurrence is limited by landscape homogeneity (Linck et al. 2023), a trait observed to some extent in our study area. In addition, species (e.g. ungulates) can segregate in terms of space and time to avoid competitive and agonistic encounters (Zanni et al. 2021). Therefore, in Mediterranean ecosystems characterised by multifunctional landscapes, interspecies avoidance through shared resources between cattle and wildlife should be smaller (but see Carvalho et al. 2018) than between nocturnal wildlife species with more similar activity rhythm periods, sizes, and diets (Payne et al. 2016, Vilella et al. 2020). In turn, animal co-occurrence patterns may dictate indirect interaction between hosts through shared environments, and thus having considerable influence on animal TB epidemiology.

4.4.3 Ecological hypotheses driving wildlife–cattle and wildlife indirect interactions

The abundance of natural food and water resources (H5) markedly influenced wildlife–cattle indirect interaction patterns, particularly those involving red deer, wild boar, and red fox. Our results indicate that, in the wet season, wildlife–cattle interactions increased in less productive areas (e.g. forested areas with high shrub cover), and around control sites; while during the dry season, wildlife–cattle indirect interactions were associated with more productive areas and occur significantly more at sites with higher water content. Water and food resources (natural and artificial) have been previously identified as key components, highly used by both cattle and wildlife at shared interfaces, thereby favouring interspecies transmission of *M. bovis* (Kukielka et al. 2013, Lavelle et al. 2016, Campbell et al. 2019). The seasonal patterns evidenced in our work may be related to changes in resource availability and abundance throughout the year. In the wet season (mainly autumn and early winter), acorns (important for ungulates) and pastures (important for cattle, ungulates and carnivores) are abundant in the study area and more water sites are available. Oppositely, water and natural food resources tend to be scarce and more spatially limited in the dry season. Given that, in the wet season, although lower levels of wildlife–cattle interactions are expected at specific sites (due to the use of different resources), spatial co-occurrence between cattle and wildlife continues to take place outside key resource areas in different habitats, as documented in other studies (Varela-Castro et al. 2021). On the other hand, highly productive natural food areas and water sites become more attractive to numerous animal hosts in the dry season. This leads to spatial aggregation of hosts at specific sites, increasing the probability of indirect interactions around key resources, as shown in previous studies (Barasona et al. 2014, Lavelle et al. 2016).

The tested hypotheses also revealed that the wildlife–cattle interactions increased in areas with low human presence (H1), more dense vegetation (H2; e.g. Forest), and in periods of low temperature (H4) during the wet season; and, during the dry season, wildlife–cattle interactions increased in areas with lower road densities (H1), in more open areas (H2; i.e. less tree cover) and during rainy periods (H4). The effect of land use (Barasona et al. 2014) and human disturbance (e.g. hunting effects; Martínez-Guijosa et al. 2021) on species interactions have previously been suggested in other Mediterranean areas. In addition, weather effects (H4) can also play a role in interactions involving cattle, since wildlife

movement behaviour on farms can be affected by temperature and rain (e.g. red fox and badger; O'Mahony 2015). Overall, our results indicate that the critical conditions for animal interactions, depending on the season, are shaped by several ecological components. This highlights the importance to consider a broad range of different ecological factors when determining *when* and *where* disease transmission can occur.

Effects associated with human disturbance hypothesis (H1) were observed for wildlife interactions as well, which have been largely understudied in the context of TB until now. During the wet season, wildlife interactions were negatively related with road density and human presence, and positively related with the distance to houses. In the dry season, lower road densities and increased distances from houses were also found to be key conditions where transmission of *M. bovis* may be favoured between wildlife species (i.e. high rates of indirect interactions). Studies have demonstrated that wildlife occurrence is strongly affected by different anthropogenic factors, such as roads (e.g. ungulates and carnivores in relation to dirt roads; D'Amico et al. 2016, Pita et al. 2020), human presence (e.g. ungulates; Rosalino et al. 2022) or even human settlements (e.g. carnivores; Tolhurst et al. 2009). We hypothesised that in the study area, wildlife species (both carnivores and ungulates) tend to avoid unpaved roads – they are frequently used by local workers and hunters throughout seasons – and areas close to houses (particularly interactions involving the red deer). By adopting such behaviours, species reduce the probability of disturbance, which, as expected, results in lower abundance of indirect interactions through common space use in those areas. In the dry season, the higher probability of wildlife interactions in areas close to houses could be explained by the characteristic behaviour of the species involved, namely the red fox and wild boar. These are opportunistic species that can take advantage of resources close to human settlements when those resources are scarce elsewhere, as documented in other Mediterranean areas and habitats (Alexandre et al. 2020). This may also explain why wildlife indirect interactions involving those species increase in more heterogeneous areas (H2), but in this case evidenced during the wet season when various resources are often available across different habitats. Finally, models showed that wildlife interactions were influenced by weather conditions (H4; ungulates in relation with temperature and red deer and badger in relation to rain). We hypothesised that during the wet season, species home range could increase as a function of temperature, as documented for ungulates and some carnivores (Rivrud et al. 2010, Main et al. 2020). As a result, this can lead species to use different spatial resources, likely reducing the abundance

of interactions under these circumstances. On the other hand, species can boost their activity during the dry season in rainy periods (very infrequent events), which could be linked to increased prey activity and/or immediate water availability, for instance. Because resources are more limited in the dry season, such patterns can result in negligible spatial segregation, and thus probably increase indirect interactions between wildlife species, particularly at specific resource sites (e.g. water sites).

Overall, improving our understanding of the ecological and environmental drivers underlying disease-relevant interactions at the wildlife–cattle interfaces is likely to provide valuable insights into the real nature of pathogen transmission events. This knowledge can help refine and guide effective control actions in risk areas wherein disease still persists. Currently, TB surveillance in wildlife in Portugal almost exclusively relies in veterinary inspection of hunted large game animals in specific areas with endemic circulation of *M. bovis*. Moreover, conventional biosecurity measures can be particularly difficult to implement in animal extensive production systems, posing a considerable challenge for controlling multi-species pathogens. Still, additional preventive measures could be considered for disrupting *M. bovis* transmission chains. One example could involve implementing selective fencing and gating systems in specific areas where wildlife and cattle frequently share space, and where increasing interaction rates are expected (e.g. water sites in the dry season; Barasona et al. 2013). Data from the present study may guide future actions as it could help refine disease risk maps, which presently mainly rely on data from disease breakdowns in cattle herds. Furthermore, wildlife densities – given their role in our study – should be closely monitored, along with environmental sampling to assess contamination of natural substrates, particularly in areas highly used by different hosts.

4.4.4 Study limitations and future perspectives

We identified three main aspects that should be further scrutinised by researchers in the multi-host TB context: first, in our study, the even distribution of cameras across the landscape, encompassing different land uses, enhances the representation of features influencing animal detection proportionally to their availability. However, this does not eliminate the overall detection bias arising from the landscape, which can influence the field of vision of camera traps (e.g. reduced detection field in dense environments compared to open areas). Future studies on interaction patterns should integrate new tools (e.g.

occupancy models) to address imperfect detection of individuals. Additionally, exploring animal-based metrics (e.g. via REM- Random Encounter Model) that consider the collective viewsheds of a camera array could improve animal detection rates and related estimates (e.g. interactions) across varying spatial gradients and external drivers (Curveira-Santos et al. 2017, Gilbert et al. 2020); second, host behaviour may determine the relative importance of a host within a disease system. Even if not very abundant, the behavioural repertoire could favour an increased contact with other hosts through shared environments (Webster et al. 2017, Dougherty et al. 2018). For instance, certain risk behaviours (e.g. wallowing, drinking) can promote frequent and prolonged contact with various infection sources and affect infection outcome and excretion patterns per host. This topic needs further research as it remains poorly understood in the Iberian context; third, since indirect transmission depends on *M. bovi*s survival time in environments, the use of CTW is crucial for generating reliable estimates. However, as *M. bovis* can survive for extended periods, depending on climate, substrates, and others (Fine et al. 2011), important questions arise: where should the baseline (CTW, in time axis) be established in a given context? Should the infectious period be based on the average environmental persistence of *M. bovis*? Should we examine the frequency of indirect interactions that occurred within a plausible range of CTW´s, according to hosts, to better define baselines? Should different CTW estimates based on *M. bovis* survival be considered across various substrates associated with sampling sites? (Payne et al. 2017). Progress in addressing these important questions has been made, with a few studies pioneering the implementation of CTW´s through different approaches to define host interactions (Lavelle et al. 2016, Martínez-Guijosa et al. 2021). Adopting similar frameworks, with environmental survival as a gold-standard metric, will improve integration and comparison of results across studies. Nevertheless, researchers will also benefit from studies exploring multiple CTW´s as a function of interaction gradients, as well as the definition of CTW´s according to sampling spatial conditions (Cowie et al. 2016). This is key to developing general theory on this topic, also applicable to other infectious diseases at the wildlife–cattle interface.

4.5 Conclusions

This is the most comprehensive study carried out in Portugal focusing on species indirect interactions in an endemic TB context, and identifying the most likely key ecological factors

driving these interactions across shared environments. Our study confirmed that the availability of natural food and water was a main driver of wildlife–cattle interactions, while wildlife indirect interactions were more associated with human disturbance. However, other ecological hypotheses influenced indirect interaction patterns, suggesting that the conditions favouring the complex transmission of *M. bovis* are determined by multiple factors, depending on the host species and season. Future studies should combine interaction data with the extent of environmental contamination with *M. bovis* to properly assess transmission risk in multi-host communities. Furthermore, the composition and structure of multi-host communities determining complex interaction patterns in space-time axes should also be considered when establishing priority measures for disease control in shared environments.

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4.7 Author contributions

Eduardo M. Ferreira, Elsa L. Duarte, Mónica V. Cunha, António Mira, and Sara M. Santos conceived the study. Eduardo M. Ferreira collected the data, analysed the data, and wrote the first manuscript draft. Eduardo M. Ferreira, Renata Gonçalves, and Tiago Pinto processed the data. Eduardo M. Ferreira and Sara M. Santos developed the analysis protocol. All authors contributed substantially to revisions and gave final approval for publication.

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4.9   Supplementary material B

4.9.1   Supplementary Figures and Tables
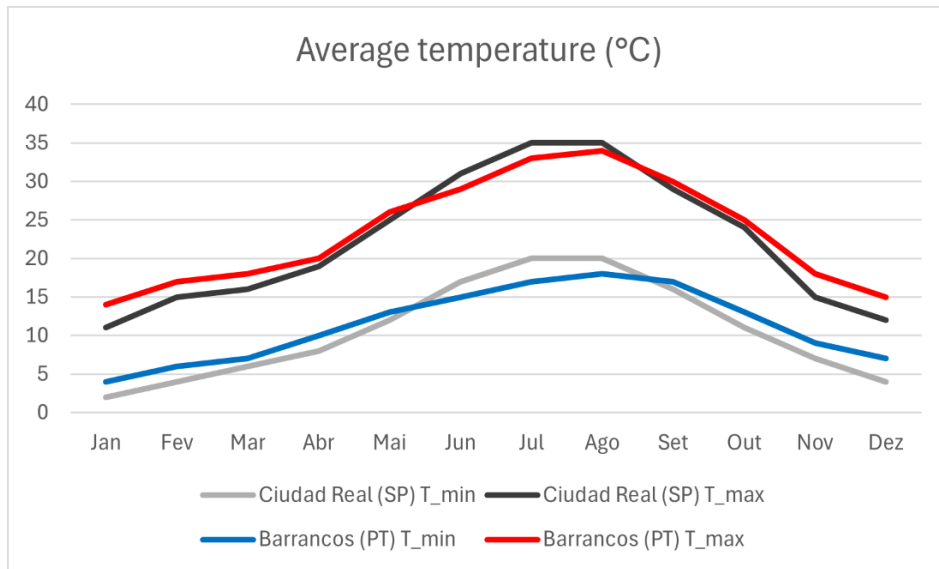
Figure S4-1. Comparison of monthly average maximum (T_max) and minimum (T_min) temperatures between Ciudad real (Spain; black and grey lines, respectively) and Barrancos (Portugal; red and blue lines, respectively). Sources: https://nomadseason.com/climate/spain/castille-la-mancha/ciudad-real.html; https://nomadseason.com/climate/portugal/beja/barranco
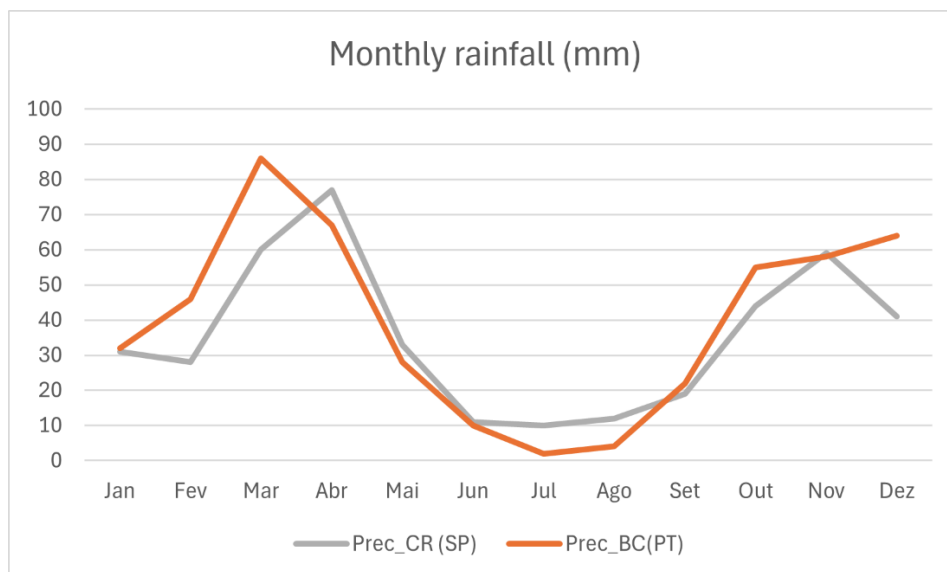
Figure S4-2. Comparison of monthly average rainfall between Ciudad real (Spain; Prec_CR; grey line) and Barrancos (Portugal; Prec_BC; orange line). Sources:

https://nomadseason.com/climate/spain/castille-la-mancha/ciudad-real.html;

https://nomadseason.com/climate/portugal/beja/barranco
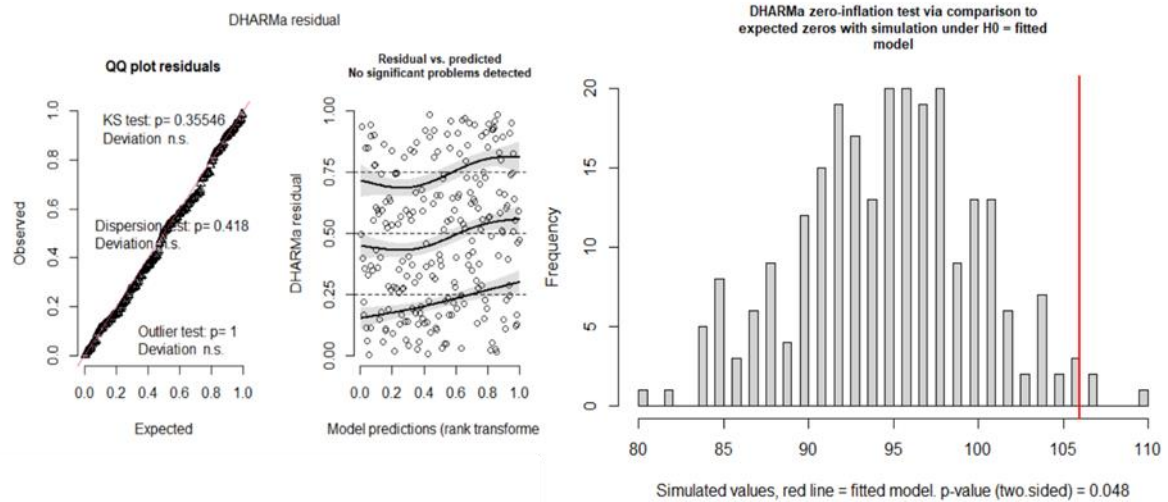
Figure S4-3. DHARMa diagnostic plots for the BTCE_wH5 model (species pair: BT_CE; season: wet, hypothesis: H5) showing residual, dispersion, outliers, and zero-inflation fits.
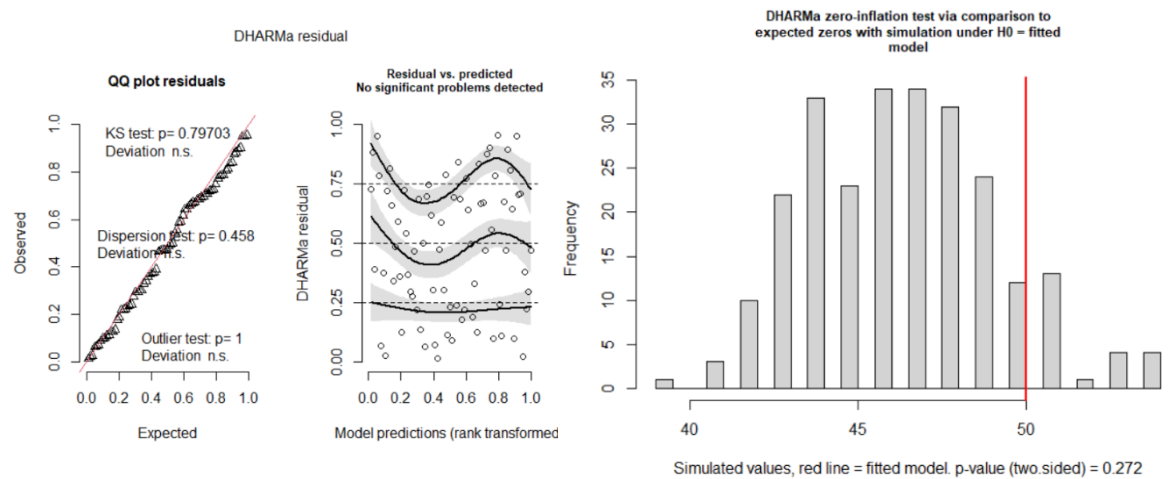
Figure S4-4. DHARMa diagnostic plots for the BTVV_dH2 model (species pair: BT_VV; season: dry, hypothesis: H2) showing residual, dispersion, outliers, and zero-inflation fits.
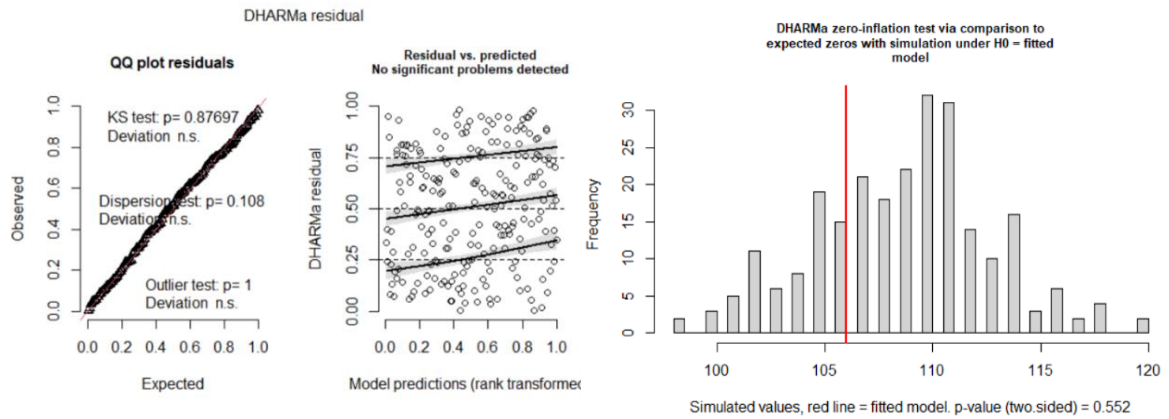
Figure S4-5. DHARMa diagnostic plots for the VVSS_dH1 model (species pair: VV_SS; season: dry, hypothesis: H1) showing residual, dispersion, outliers, and zero-inflation fits.
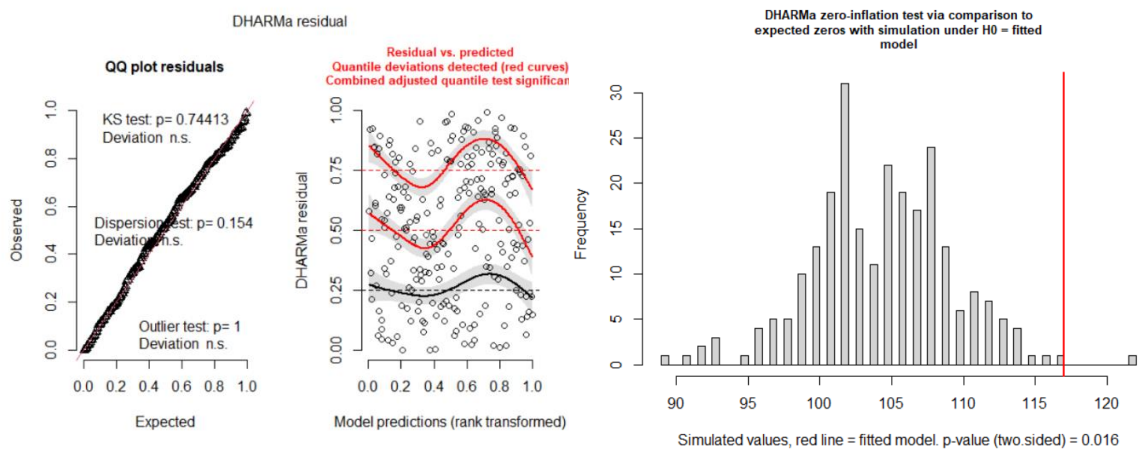
Figure S4-6. DHARMa diagnostic plots for the VVSS_wH2 model (species pair: VV_SS; season: wet, hypothesis: H2) showing residual, dispersion, outliers, and zero-inflation fits.

The examples above not only illustrate the diverse range of patterns observed across all tested models, but also represent the most representative models, considering both studied animal groups (wildlife–cattle and wildlife), seasons and tested hypotheses. In approximately 35% and 20% of the models generated for wildlife–cattle and wildlife groups, respectively, we identified zero-inflation, indicating a poor fit (see example in Figure S4-6). These models were then compared to models incorporating a zero-inflation formula correction [ZIP] (using the argument = ~ animal abundance A and/or animal abundance B (Brooks et al 2017, Santon et al 2023). While the corrected models demonstrated significant improvements in terms of

AICc and no violation of model assumptions, they exhibited instability in the zero-inflation component, with high standard errors associated with the ziformula-variables (likely due to the high complexity of the model structure). Considering that the trends and significance of the predictors (conditional part) remained consistent with or without the correction ZIP formula, we retained the uncorrected and simpler models for further analyses.

4.9.2 Supplemental References

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Chapter 5

Mapping high-risk areas for *Mycobacterium tuberculosis* complex bacteria transmission: linking host space use and environmental contamination

Eduardo M. Ferreira, Mónica V. Cunha, Elsa L. Duarte, António Mira, Daniela Pinto, Inês Mendes, André C. Pereira, Tiago Pinto, Pelayo Acevedo, Sara M. Santos (*submitted*) Mapping high-risk areas for *Mycobacterium tuberculosis* complex bacteria transmission: linking host space use and environmental contamination

Chapter 5 – Mapping high-risk areas for *Mycobacterium tuberculosis* complex bacteria transmission: linking host space use and environmental contamination

Abstract

In many Mediterranean ecosystems, animal tuberculosis (TB), caused by *Mycobacterium bovis*, an ecovar *of Mycobacterium tuberculosis* complex (MTBC), is maintained by multi-host communities. It is hypothesised that interspecies transmission is mainly indirect via shared contaminated environments. Therefore, identifying spatial areas where MTBC bacteria occur and quantifying space use by susceptible hosts might help predict the spatial likelihood of transmission across the landscape. Here, we aimed to evaluate the transmission risk of MTBC (as a proxy for *M. bovis*) in a multi-host system involving wildlife (ungulates and carnivores) and cattle (*Bos taurus*). We collected eighty-nine samples from natural substrates (water, soil, and mud) at 38 sampling sites in a TB endemic area within a Mediterranean agroforestry system in Portugal. These samples were analysed by real-time PCR to detect MTBC DNA. Additionally, host-specific space use intensity maps were obtained through camera-trapping covering the same sampling sites. Results evidenced that a significant proportion of samples were positive for MTBC DNA (49%), suggesting that the contamination is widespread in the area. Moreover, they showed that the probability of MTBC occurrence in the environment was significantly influenced by topographic features (i.e. slope), although other non-significant predictor related with soil conditions (SMI: soil moisture index) incorporated the MTBC contamination model. The integration of host space use intensity maps with the spatial detection of MTBC showed that the red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) exhibited the highest percentages of high-risk areas for MTBC transmission. Furthermore, when considering the co-occurrence of multiple hosts, transmission risk analyses revealed that 26.5% of the study area represented high-risk conditions for MTBC transmission, mainly in forest areas.

Keywords

camera-trapping, transmission risk; *Mycobacterium tuberculosis* complex (MTBC), environmental contamination, *Mycobacterium bovis*, wildlife–cattle interface, space use

5.1 Introduction

With the encroachment of human activities into wildlife habitats, an exponential growth of animal interactions across wildlife-livestock interfaces has been anticipated, with important implications in infectious disease emergence and transmission worldwide (Jones et al. 2013). Pathogens shared by wildlife and livestock have devastating consequences to livestock industry, biodiversity, and public health (Webster et al. 2017, Hassell et al. 2021). Animal tuberculosis (TB), caused by *Mycobacterium bovis* or other ecovars of the *Mycobacterium tuberculosis* complex (MTBC), is one of the most prevalent and challenging health issues of cattle farming in many countries worldwide. In the European Union (EU), the eradication of TB in bovine has been a central priority. Despite all efforts made to date, some countries in the EU, including Portugal, have been unable to obtain the officially tuberculosis-free (OTF) status (Hardstaff et al. 2014, Pereira et al. 2020, Reis et al. 2020a, Report 2023). TB can persist in cattle farms due to interactions of cattle (*Bos taurus*) with several wildlife hosts that share the same areas and resources and are usually not under surveillance programmes (Varela-Castro et al. 2021, Herraiz et al. 2023). Specifically, some wildlife species that detain a significant role in TB epidemiology are considered reservoirs, maintaining the pathogen in ecosystems, and transmitting infection to cattle, decreasing the success of eradication programmes (Duarte et al. 2008, Palmer 2013, Canini et al. 2023, Gortázar et al. 2023).

Over the last years, numerous studies have been addressing TB dynamics and disease risk across different wildlife–cattle interfaces through varying ecological and epidemiological lenses (Acevedo et al. 2019, Pereira et al. 2023b). From an ecological perspective, different approaches (e.g. camera-trapping, proximity collars) have been used to characterise interaction networks within multi-host TB communities in farming systems (Kukielka et al. 2013, Drewe et al. 2013). The integration of ecological tools in TB epidemiology has improved our understanding of the likely transmission pathways of MTBC considering different eco-epidemiological scenarios (Wilber et al. 2019, Triguero-Ocaña et al. 2020a), with interactions between susceptible hosts being recognized as crucial determinants of transmission (Triguero-Ocaña et al. 2019, Ferreira et al. 2023).

Interaction patterns between susceptible hosts have been used as a reasonable proxy to discuss pathogen transmission risk, since it is very difficult to record real transmission events (e.g., Cowie et al. 2016, Campbell et al. 2019, Triguero-Ocaña et al. 2019). Nevertheless,

pathogen transmission "*occurs at intersections of host and pathogen movement trajectories*" (Manlove et al. 2022). This means that pathogen transmission does not only occur directly when an uninfected susceptible host crosses paths with an infected host, but also indirectly when it comes into contact with the pathogen that has been spread in the environment by an infected host (Ferreira et al. 2023). In this sense, transmission risk depends not only on the frequency of interactions and host densities through space use, but also on the extent of environmental contamination (Dougherty et al. 2018). In fact, previous findings in different TB settings support the hypothesis that M. *bovis* transmission is mainly indirect through asynchronous use of contaminated shared environments by different host species (Allen et al. 2021, Triguero-Ocaña et al. 2021, Gortázar et al. 2023). An increased risk of transmission is expected during the dry season (the limiting season in Mediterranean environments) when natural food and water resources tend to become scarce, leading to the aggregation of multiple hosts at specific sites (e.g. supplementary feeding locations or at farm water sites) (Kukielka et al. 2013, Barasona et al. 2014). Despite the lower MTBC concentrations described in this season (Santos et al. 2015b), animals are more likely subjected to infection risks at spatially limited sites under harsh climate conditions (e.g. high temperature) where they might interact closely. A growing attention has been given to the potential role of the environment in animal TB epidemiology, particularly through attempts to evaluate the presence and distribution of MTBC in shared environments. Contamination of environmental substrates (e.g. water and soil) with MTBC DNA have been evidenced in various Mediterranean TB settings across the Iberian Peninsula (Santos et al. 2015b, Barasona et al. 2016, Pereira et al. 2023a, Herrero-García et al. 2024). Given the prolonged excretion of bacteria by infected hosts and viability of MTBC in the environment, animals might be exposed to increased risk for extended time periods (Vicente et al. 2013, Triguero-Ocaña et al. 2020a, Pereira et al. 2024). This is of particular concern in Mediterranean systems where MTBC is able to cause disease in multiple wildlife species (not only reservoir hosts) that occur in sympatry, along with cattle, originating complex multi-host communities (Santos et al. 2012, Matos et al. 2014, Gortázar et al. 2023).

Despite the evident need to close the gap between animal ecology and disease epidemiology fields, multi-disciplinary analyses are still uncommon, representing an under-explored avenue for investigation of disease systems at the wildlife–livestock interfaces (Dougherty et al. 2018, Manlove et al. 2022). To date, attempts to integrate ecological data on host space use with environmental exposure to TB remains scarce. Linking these components is crucial

for accurately predicting the spatial likelihood of transmission risk in multi-hosts communities and identifying specific hotspot areas with high-risk exposure (Barasona et al. 2016). Such knowledge can guide and refine disease control actions according to time-space-host axes in risk areas where disease persists. Hence, this work focuses on the transmission risk of MTBC during the dry season within a multi-host system involving wildlife (ungulates and carnivores) and cattle. We targeted a TB endemic area within a Mediterranean agroforestry system in Portugal with the following goals: 1) evaluate the extent of environmental contamination with MTBC; 2) identify environmental drivers influencing the occurrence of contamination and estimate the probability of MTBC occurrence across the landscape; and 3) predict potential high-risk areas for MTBC transmission, considering environmental contamination and host space use intensity.

5.2 Material and Methods

5.2.1 Study area

This study was carried out in Barrancos, located in southeast of Portugal (Alentejo region), near the Spanish border (38˚08' N; 6˚59' W) (Figure 5-1). This area is recognized as a hotspot for TB in cattle and wildlife, and is included in the official epidemiological TB risk area where big game species (red deer *Cervus elaphus* and wild boar *Sus scrofa*) are the subject of a monitoring scheme that implies the initial examination in the field of hunted animals by a credentialed veterinarian to search for TB-compatible lesions (Cunha et al. 2011, DGAV 2011, Santos et al. 2018). Ungulates are abundant in the region (wild boar density = 3-4 individuals/km^2; red deer density = 4-8 individuals/km^2) (Santos et al. 2022). The dominant land use in Barrancos is the *Montado* (holm oak *Quercus rotundifolia* open woodland, with varying tree density) with extensive husbandry of cattle. Other land uses, albeit less abundant, include agricultural land, olive groves and scattered shrub areas. The topography is characterized by gentle to moderate undulating terrain, with altitude ranging between 160 and 350m above sea level. The climate is Mediterranean, characterized by mild and wet winters and hot and dry summers. Mean winter temperatures (January) range from 5 °C to 14 °C, while mean summer temperatures (July) range from 15 °C to 34 °C (IPMA 2023). The mean temperature during the research period was 25.5°C in July.

Herd TB prevalence was estimated at 1.83% for the Alentejo region in 2022, higher than the national mean prevalence (Portugal mainland and Azores, 0.65%) (DGAV 2023). Also, during the field work (2021 to 2022), outbreaks were confirmed in Barrancos. Regarding wildlife, the few recent available studies point towards low TB rates (3.1% and 1.8% for red deer and wild boar, respectively; Costa 2015) in Barrancos. However, at a national scale, a meta-analysis estimated the pooled TB prevalence as 27.5% and 13.3% for red deer and wild boar, respectively (Reis et al. 2020b).
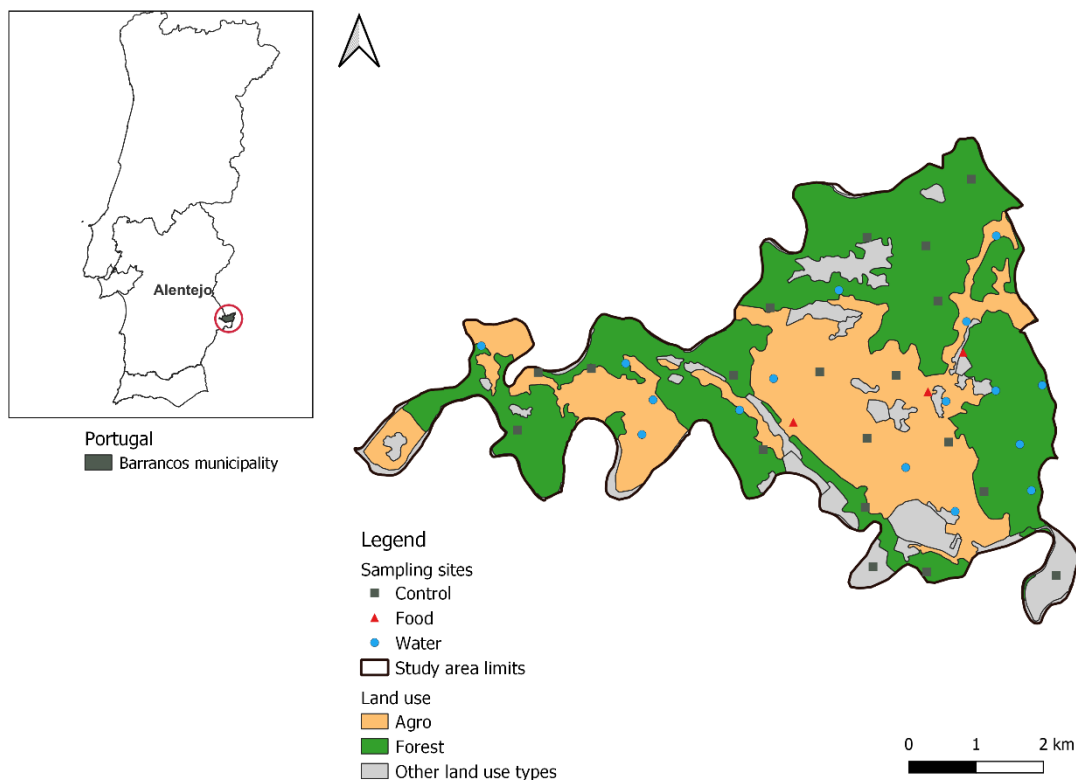
Figure 5-1. Study area location in Barrancos region, Portugal, showing sampled sites and main land uses. Agro: holm oak stands with low or absent shrub cover due to grazing and other pastoral activities; Forest: holm oak stands or mixed woodland patches with high shrub cover.

5.2.2 Study design

We selected five free-ranging adjoining farms with similar management practices, comprising an area of ~3048 ha (farm size ranging from 148 ha to 980ha), with an average of 136 adult cows per farm. A total of 38 sampling sites (Figure 5-1) were defined: 16 water sites (natural water sources and water trough), three food sites (hay feeders), and 19 control sites (without any water sources or supplementary food, e.g. forest animal path). Minimum

distance between sampling sites averaged 686 m (range: 350 m to 1300 m). These sites were defined and sampled with camera traps for monitoring animal visitation and interaction rates within a previous work (see below the description on the inference of transmission risk maps; more details in Supplementary Material C: Camera-trap design).

A total of 89 environmental samples were collected in September 2022 (dry season) (at least 2 samples collected per sampling site). Samples included water (n = 10), mud (n = 17) and soil (n = 62). They were collected into sterile propylene flasks (1000 mL) and kept at 4ºC during transportation. Samples were then frozen until laboratory analysis were performed.

5.2.3 Sample processing, DNA extraction and MTBC detection by qPCR

Samples were processed and analysed as described in Pereira et al. (2023). Briefly, collected mud and soil samples were subjected to homogenization by stirring. Subsequently, 250 grams of each sample were resuspended in 50 mL of cell recovery solution, comprising 1x PBS, 0.05% Tween®80, and 0.01% sodium pyrophosphate, and incubated at 28°C for 30 minutes with continuous shaking. Following incubation, the sample suspensions underwent centrifugation at 150 x g for 5 minutes and the supernatant was collected. For collected water samples, a 10 µm pore size filter was employed, and the resulting filtrate was centrifuged at 3220 x g for 30 minutes. The cell pellet obtained was then resuspended in 10 mL of 1x PBS. Processed, resuspended soil and water were centrifuged at 3220 x g for 30 minutes. The supernatants were discarded.

DNA extraction was conducted using 250 mg of sediments from the previous step and the DNeasy PowerSoil Pro kit (Qiagen, USA), adhering to the manufacturer's instructions. Subsequently, DNA quantification was performed using Qubit™ dsDNA Quantification Assay Kits (ThermoFisher Scientific), following the manufacturer's guidelines. The presence of MTBC was assessed using real-time PCR with IS6110-specific primers and probe, as previously described (Costa et al. 2014). In brief, NZYSupreme qPCR Probe Master Mix (NZYtech, Portugal) was utilized along with 0.4 µM of each primer and 0.2 µM of the probe. Five microliters of 10-fold diluted total DNA were added to the reaction mix. Amplification consisted of an initial denaturation step of 3 minutes at 95°C, followed by 45 cycles of 5 seconds of denaturation at 95°C and 30 seconds of extension at 60°C. Thermal cycling and fluorescent signal acquisition occurred in a Bio-Rad CFX96 thermocycler (Bio-Rad, USA), with reactions performed in triplicate.

Negative results were confirmed by testing 5 µL of undiluted samples to detect low MTBC burden. Reactions were initially performed in duplicate for all samples, except in cases of disagreement between duplicates, where a triplicate was performed. It is noteworthy that *M. bovis* BCG Pasteur has a single copy of IS*6110*; however, other members of the MTBC may possess up to 16 copies (Comín et al. 2022). Positive, negative, and blank controls were included in each PCR batch.

5.2.4 MTBC occurrence: predictor selection

We considered a total of 16 environmental predictors that might influence the occurrence of MTBC in environmental matrices from the study area (Table 5-1) (Walter et al. 2014, Martínez-Guijosa et al. 2020, Allen et al. 2021, Pereira et al. 2023a).

Table 5-1. Description of the environmental predictors used for modelling the occurrence of MTBC.

Predictor group	Predictor code	Predictor type	Description	Original spatial resolution
Topography	Altitude	numeric	Terrain altitude within 100, 250 and 500 m spatial scales around sampling sites (m).	30 m
	Slope	numeric	Topographic slope within 100, 250 and 500 m spatial scales around sampling sites (degrees).	30 m
	Hillshade	numeric	Hillshade – light reflectance from a terrain surface – expressed within 100, 250 and 500m spatial scales around sampling sites (degrees).	30 m
Landscape composition	Agro	numeric	Percentage of agroforest land (holm oak stands with low or absent shrub cover due to grazing and other pastoral activities) within 100, 250 and 500 m spatial scales around sampling sites (%).	
	Forest	numeric	Percentage of forest (holm oak stands or mixed woodland patches with high shrub cover) within 100, 250 and 500 m spatial scales around sampling sites (%).	
	Dist_ForestEdge	numeric	Distance of sampling sites to the nearest edge of forest patches (m).	30 m
	TreeD	numeric	Percentage of tree cover density within 100, 250 and 500 m spatial scales around sampling sites (%).	30 m

Predictor group	Predictor code	Predictor type	Description	Original spatial resolution
	Dist_waterlines	numeric	Distance of camera sites to the nearest water line (m).	30 m
Abiotic components (remote sensing)	LST	numeric	Radiative temperature of land surface (°C) within 100, 250 and 500 m spatial scales around sampling sites.	30 m
	SMI	numeric	Soil moisture index within 100, 250 and 500m spatial scales around sampling sites.	30 m
	EVI	numeric	Enhanced Vegetation Index within 100, 250 and 500m spatial scales around sampling sites.	30 m
	NDWI	numeric	Normalised Difference Water Index within 100, 250 and 500m spatial scales around sampling sites.	30 m
Soil texture	Clay	numeric	Percentage of soil clay expressed in a 500m spatial scale around sampling sites (%).	500 m
	Silt	numeric	Percentage of soil silt expressed in a 500m spatial scale around sampling sites (%).	500 m
	Sand	numeric	Percentage of soil sand expressed in a 500m spatial scale around sampling sites (%).	500 m
Ecological factors	Wild_div	numeric	Wildlife diversity at sampling sites.	

Regarding topographic predictors, we estimated elevation from a 30-m Digital elevation Model (DEM), and derived slope and hillshade metrics from the DEM using Quantum GIS v. 3.0.3 (QGIS 2022). For landscape composition-related predictors, we computed the percentage of land cover, considering the main land uses (Agro and Forest) occurring in the study area. The Shannon landscape diversity index and the Euclidean distance of sampling sites to forest edges were also computed. Those metrics were obtained from the Corine Land Cover (2018) dataset (European Union, Copernicus Land Monitoring Service, European Environment Agency) and were retrieved from the 'landscapemetrics' R package (Hesselbarth et al. 2019). In addition, tree cover density was derived from the Tree Cover Density (2018) dataset (Copernicus Land Monitoring Service, European Environment Agency). Remote-sensing data were derived from the LANDSAT 8 image collection (level 2, Tier 1) to the period of sample collection (September 2022), with a 30 m spatial resolution, and processed in Google Earth Engine (Gorelick et al. 2017). Only high-quality images (with ≤ 5% of cloud cover) were considered (Pinto et al. 2023). Soil texture predictor variables were extracted from the European Soil Data Centre (ESDAC, http://esdac.jrc.ec.europa.eu/;

Panagos et al. 2012). The Shannon wildlife diversity index was calculated using the visitation rates (the number of detections of each species at each sampling site in a month/(number of active camera days/number of days of a given month) of the target species derived from camera trap monitoring, considering averaged values for the dry season.

A multi-scale modelling approach was carried out to maximise accuracy of predictors. Continuous predictors not based on distances (Altitude, Slope, Hillshade, Agro, Forest, TreeD, LST, SMI, EVI, NDWI) were stacked in a 30 m spatial resolution multi-raster layer. We then applied the following spatial scales of analysis: 90, 240 and 510 m focal-radius moving window as a proxy for 100, 250 and 500 m scales of analysis (Ferreira et al. 2024). Mean was used to summarize the raster values within each spatial scale.

5.2.5 Ecological modelling of MTBC

We modelled MTBC DNA presence in environmental samples to identify drivers of environmental contamination and predict patterns of transmission risk. We calculated the proportion of positive samples per sampling site (*prop*) by dividing the number of positive samples by the total number of samples analysed for each site. Afterwards, we defined a binomial response variable (MTBC occurrence; *occurrence_bin*) based on prop to be used in modelling. When *prop* was => 0.5, we considered a sampling site as potentially contaminated (coded as 1; n = 24); otherwise, the site was considered non-contaminated (coded as 0; n = 14). Generalized linear models (GLM´s) were applied to test the effects of predictors on MTBC occurrence. These models were chosen for their suitability for binary prediction and frequently used for disease mapping (de Oliveira et al. 2022, Li et al. 2022, Ndolo et al. 2022).

We first ran univariate models to identify likely relevant predictors. As such, fitted univariate models testing one predictor at a time were compared with the null model using AICc, Akaike's Information Criterion adjusted for small sample sizes (Burnham & Anderson 2002). A predictor variable was considered informative when: 1) the 95% confidence intervals (CI 95%) of the predictor coefficient being tested did not include zero; and 2) a delta AICc > 2 was obtained when comparing the univariate model with the null model (Burnham & Anderson 2002, Stephens et al. 2005). If highly correlated informative predictors (r > |0.7|) were identified, we only retained the one producing a lower AICc to be included in the multivariate model. Multivariate models were built testing all possible combinations of the

informative predictors using dredge in the 'MuMIn' package (Bartoń 2022). When several models had ΔAICc < 2, all associated predictors were included in a single best multi model (eg. Humphrey et al. 2023). Prediction performance of the best model was assessed using the area under the curve (AUC) of the receiver operating characteristic [ROC], combined with model accuracy and Cohen's kappa coefficient through Leave One Out Cross Validation (LOOCV) procedure (Morris et al. 2016, Xia et al. 2019, Deka 2022). We obtained the potential occurrence of MTBC in the study area by applying the predicted probability of the best model to the entire study area.

5.2.6 Development of transmission risk maps

To evaluate the transmission risk for each animal host, two components were combined: (1) the potential occurrence of MTBC in the study area and (2) host-specific space use intensity maps. Space use intensity maps for each host (cattle, wild boar, red deer, red fox *Vulpes vulpes*, and badger *Meles meles*) were obtained on the same sampling sites (Ferreira et al. 2024). For each of the sampling sites, a camera-trap was installed during the dry season 2021 (June to September; Kukielka et al. 2013, Cowie et al. 2016) to measure visitation rates by domestic cattle and wildlife species. Busnhell Trophy Cam HD Aggressor or Reconyx Hyperfire cameras were used and placed 30-50 cm above the ground. No bait of any kind was used. We programmed cameras to operate 24 hours a day, taking three pictures per trigger with a 30-second delay between consecutive triggers (Kukielka et al. 2013, Triguero-Ocaña et al. 2020b). Visitation rates were calculated for each sampling site and each host species, considering 15 min as the time to independent observations (Kukielka et al. 2013, Carrasco-Garcia et al. 2016, Martínez-Guijosa et al. 2021). Visitation rates were first calculated as the number of detections of each species at each sampling site in a month/(number of active camera days/number of days of a given month). We then calculated the mean visitation rate (VR), discriminated by species, for the dry season at each sampling site by averaging visitation estimates across all sampled months (see supplementary material C: Host space use intensity maps and Table S5-1).

Species-specific space use intensity maps were generated based on averaged visitation rates by inverse distance weighted interpolation (IDW) (e.g. Sarmento et al. 2011, Curveira-Santos et al. 2019), thus producing spatial interpolation surfaces for the entire study area. We tested different combinations of IDP (inverse distance power) and nmax (the number of nearest

observations for prediction) values. The chosen values were based on a balance between statistical accuracy (lower RMSE [Root Mean Square Error]) and spatial coherence (considering land-uses and species ecological traits [e.g. minimal vital areas]). Predicted space use intensity maps for each species are available in the supplementary material C, Figures S5-1 – S5-5).

We reclassified the host space use intensity maps using quartile intervals as follows: low (VR < 2Q, i.e. second quartile), medium (2Q =< VR < 3Q) and high (VR >= 3Q). Similar reclassification was applied to MTBC contamination map based on the predicted probability, grouping it into three categories: low (MTBC occurrence probability < 0.5), medium (MTBC occurrence probability >= 0.5 & < 0.75) and high (MTBC occurrence probability >= 0.75). After, transmission risk maps were built for each target host species based on reclassified MTBC contamination and host space use intensity maps. A high-risk transmission level was assigned to a given area when both maps indicated high conditions, or when high and medium conditions were combined (Supplementary material C: Table S5-2). Areas classified as medium-risk resulted from either the convergence of two medium conditions or the combination of high and low conditions. The remaining areas were designated as low-risk transmission using similar criteria, based on the intersection of low with medium conditions, and low with low conditions. A final multi-host transmission risk map was also generated by overlaying high-risk transmission areas shared between wildlife and cattle hosts using R packages 'raster' and 'geoR' (R Core Team 2022). Specifically, a combined map was built based on high-risk areas considering a gradient of hosts: areas associated with just one host, two hosts, and with three or more hosts, serving as proxy for multi-host TB scenarios.

5.3 Results

5.3.1 Environmental contamination with MTBC

From a total of 89 samples collected across 38 sites, 49% were positive for the presence of MTBC DNA. Similar percentage of positive samples were registered for mud (53%) and soil (56%) matrices. No positive samples (0%) were recorded for water matrices. Hence, considering MTBC occurrence, 63% of sampling sites were considered contaminated by MTBC, with 29% having all samples testing positive for the presence of MTBC.

5.3.2 Environmental drivers influencing MTBC contamination

To uncover the predictors of MTBC contamination, a GLM model was built. The MTBC contamination GLM model had good fit, with an estimated accuracy of 0.79 and a Kappa value of 0.55. Additionally, it had good discriminating ability (AUC = 0.82). The best model included Slope (scale 250 m) and SMI (scale 500 m) as predictor variables. Slope had a positive and significant effect on the probability of MTBC occurrence (coef = 1.409, CI 95% [0.156; 2.662]). A positive relation was also detected between SMI and MTBC occurrence, although not statistically significant (coef = 0.478, CI 95% [-0.561, 1.518]). According to the predicted map, 26.9% of the study area is categorized as low risk for MTBC occurrence (probability < 0.5) while 73.1% is considered as medium to high-risk (probability >= 0.5) (Figure 5-2). The high-risk areas for MTBC occurrence (49% of the study area) occur across all the SA but are predominantly concentrated in the Northeast section.
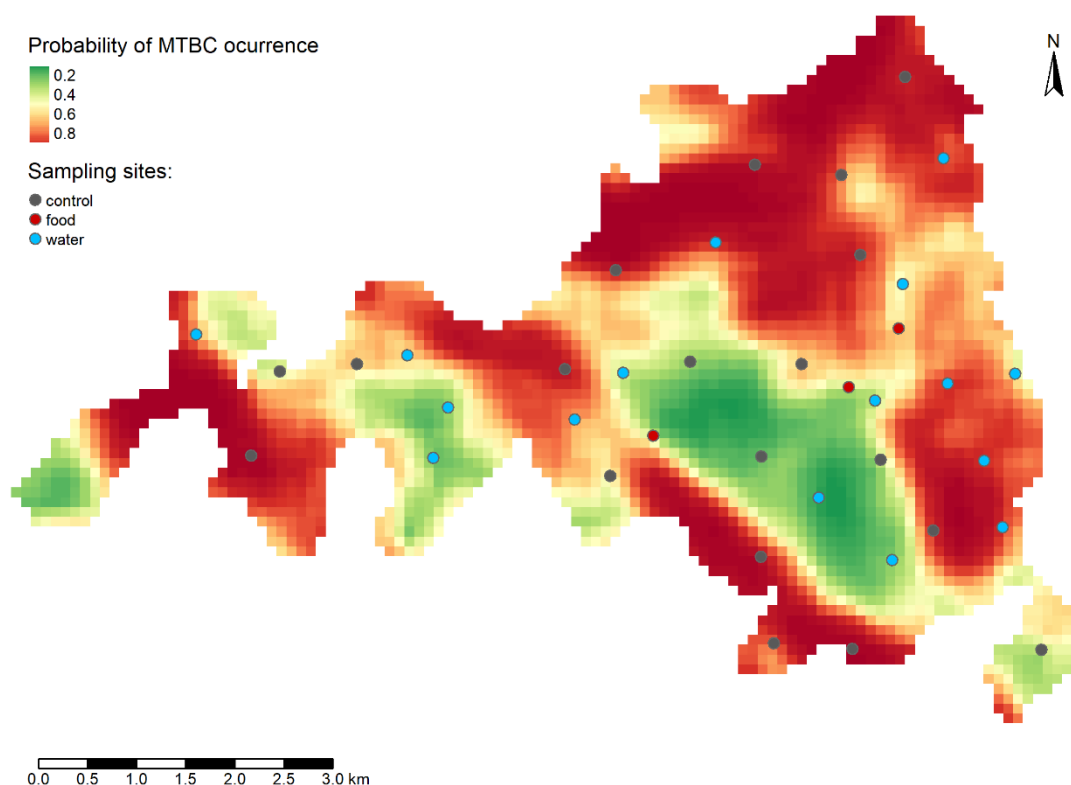
Figure 5-2. Risk map of MTBC occurrence in the study area, Southeast of Portugal, layered with sampling sites.

5.3.3 Prediction of potential high-risk areas for MTBC transmission

Overall, transmission risk analysis revealed that the SA is dominated by medium-risk areas for MTBC transmission (43 % of the study area extension; sd = 8). Low and high-risk areas came in second, equally represented, each comprising 29 % of the study area (sd = 4 and 5, respectively) (Table 5-2).

Table 5-2. Percentage of area occupied by low, medium, and high-risk areas for MTBC transmission in the study area according to the target hosts (red deer, wild boar, red fox, badger, and cattle).

Host	% low risk areas	% medium risk areas	% high risk areas
Red deer	34.4	30.2	35.4
Wild boar	29.3	39.4	31.3
Red fox	27.3	46.6	26.1
Badger	26.6	47.6	25.8
Cattle	25.2	50.7	24.1
mean	28.5	42.9	28.5
sd	3.6	8.2	4.7

The red deer and the wild boar were associated with the highest percentages of high-risk areas (Table 5-2; Figure 5-3, and Figure 5-4, respectively). Transmission risk maps for these species exhibited similar spatial trends, with main high-risk areas concentrated in the southeast, north, as well as in the west-central sections of the SA. Although less represented (see Table 5-2), high risk areas for cattle shows a substantial degree of overlap with wild ungulates high-risk transmission areas, particularly in the east and west-central sections, albeit with slightly different spatial configurations (Figure 5-5).

Figure 5-3. MTBC transmission risk map for red deer in the study area, Southeast of Portugal, layered with sampling sites.

Figure 5-4. MTBC transmission risk map for wild boar in the study area, Southeast of Portugal, layered with sampling sites.
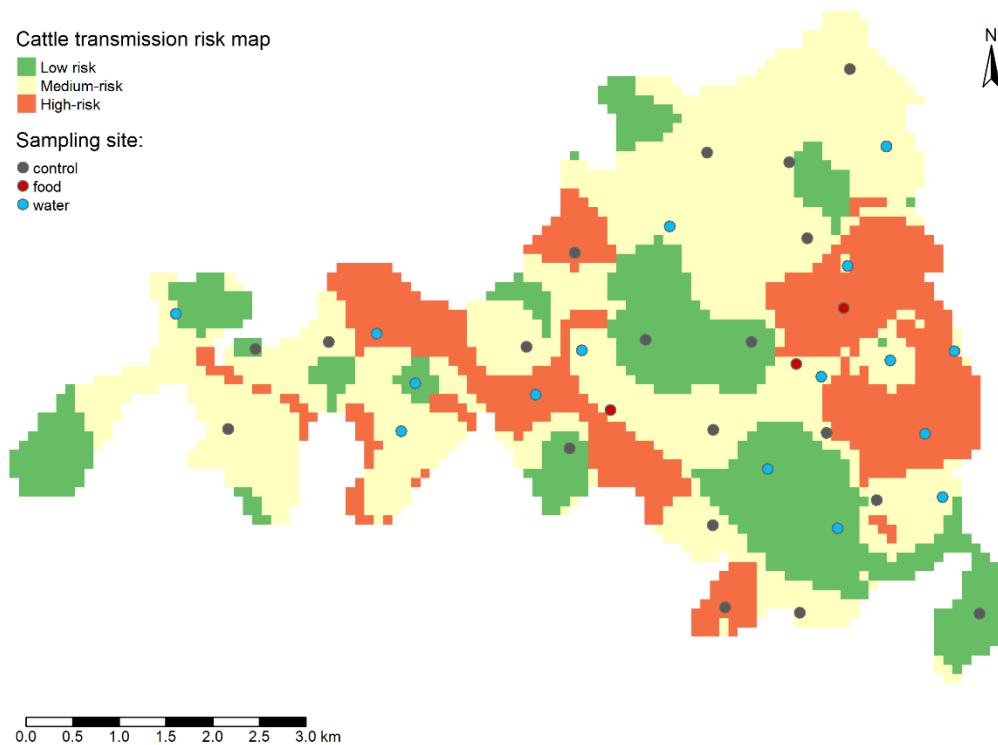
Figure 5-5. MTBC transmission risk map for cattle in the study area, Southeast of Portugal, layered with sampling sites.

High-risk areas for MTBC transmission associated with red fox and badger are concentrated in the northern section of the SA (Figure 5-6 and Figure 5-7, respectively). Smaller and more fragmented high-risk areas are present in the western section of the SA as well. Much of these areas are concentrated in forest areas, but covering small portions of agro land use, and including water, control, and food sites.

When examining the overlap of high-risk areas across various host settings (involving one, two or more), results indicated that 26.5% of the SA is designated as high-risk when considering multi-host conditions (Figure 5-8). There are three main core areas of high-risk distributed along the southeast to northeast axis, with two additional areas located in the western section of the SA. High-risk areas involving multi-host conditions included five water sites (5/16; 31%), one food site (1/3; 33%), and two control sites (2/19; 11%). Furthermore, the transmission risk map indicated that 18.9% of the SA poses a high risk for MTBC transmission for a single TB host, whereas only 11% are deemed high-risk when considering the co-occurrence of two hosts combined.
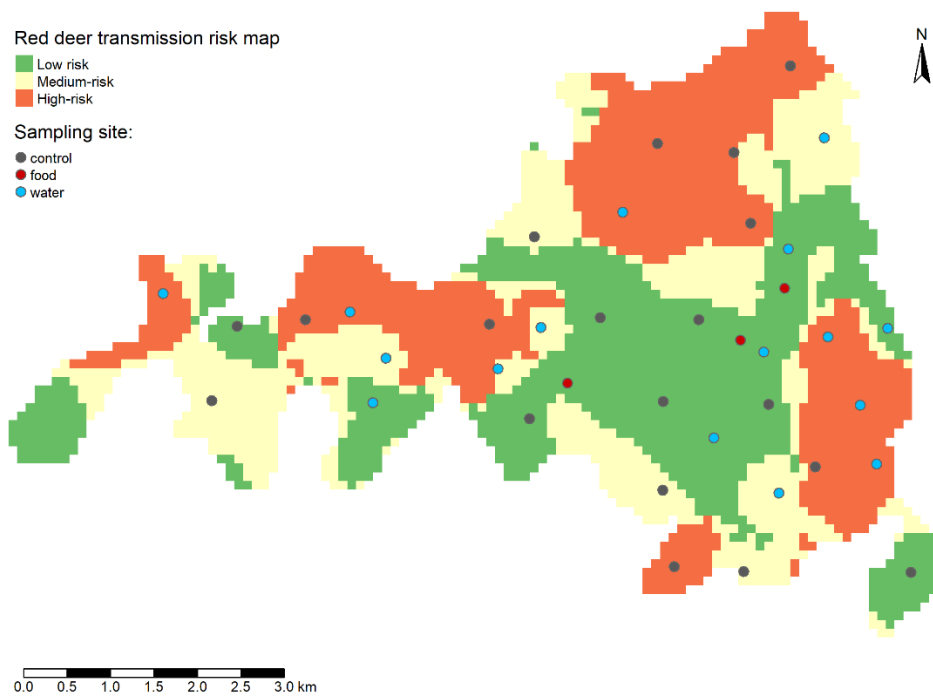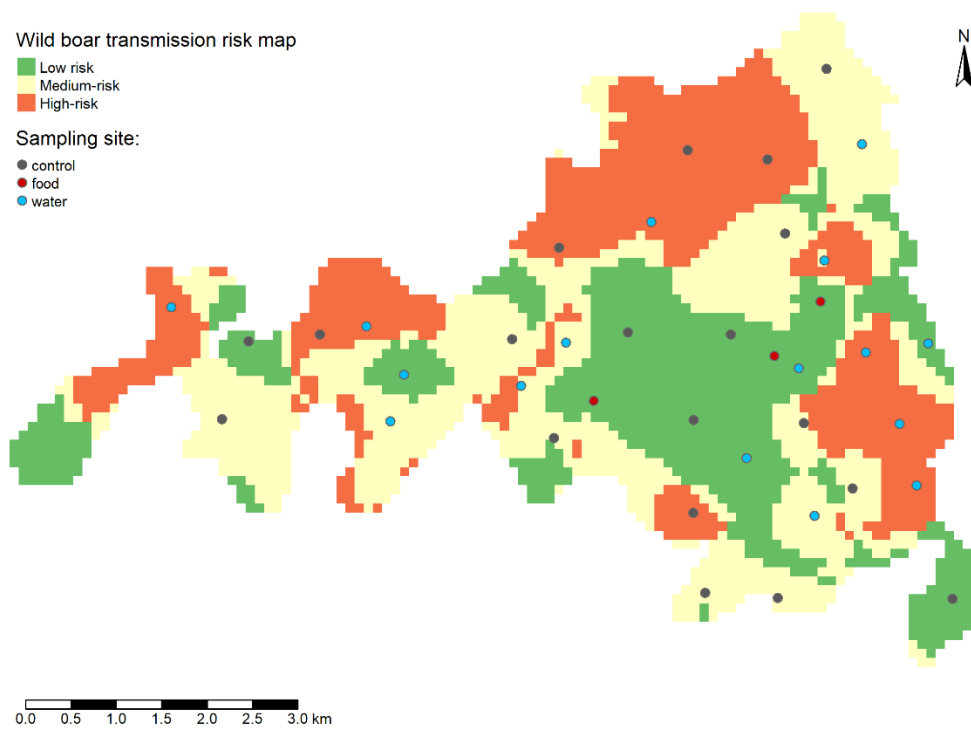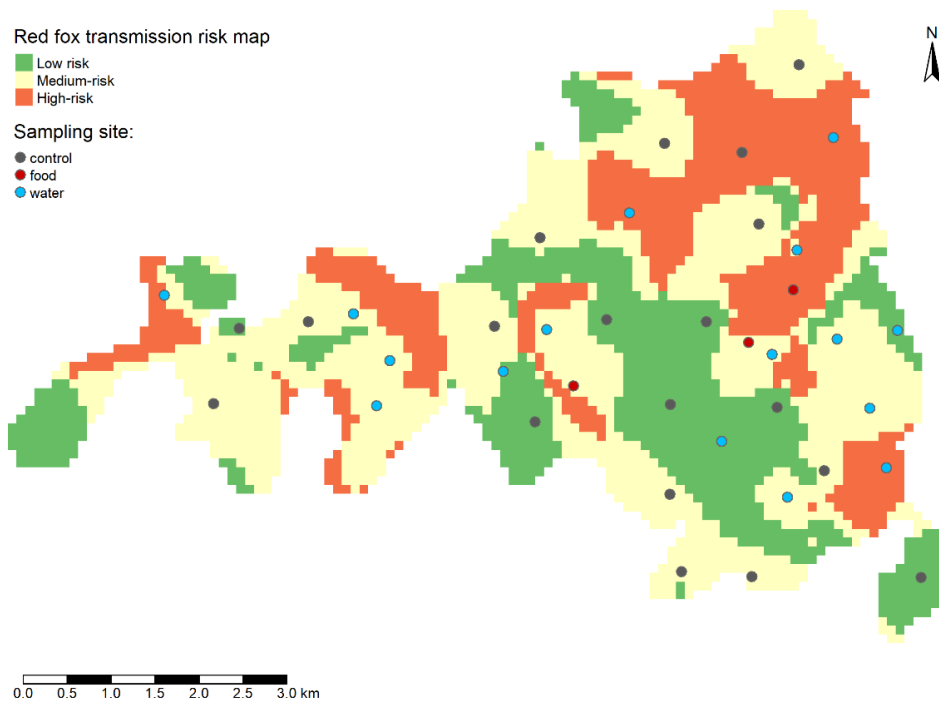
Figure 5-6. MTBC transmission risk map for red fox in the study area, Southeast of Portugal, layered with sampling sites.
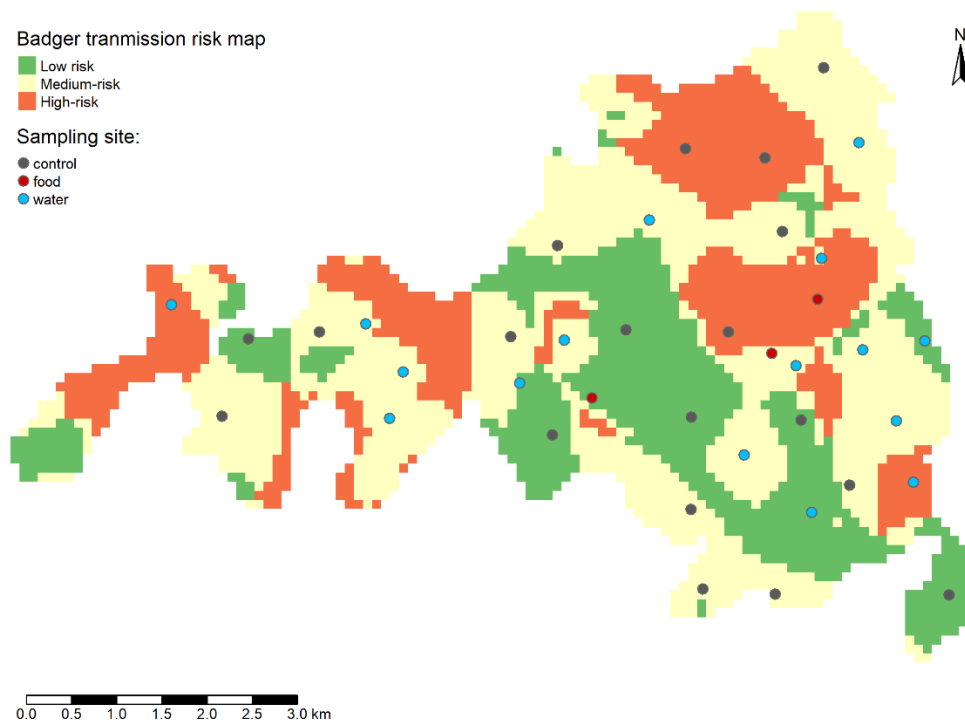
Figure 5-7. MTBC transmission risk map for badger in the study area, Southeast of Portugal, layered with sampling sites.
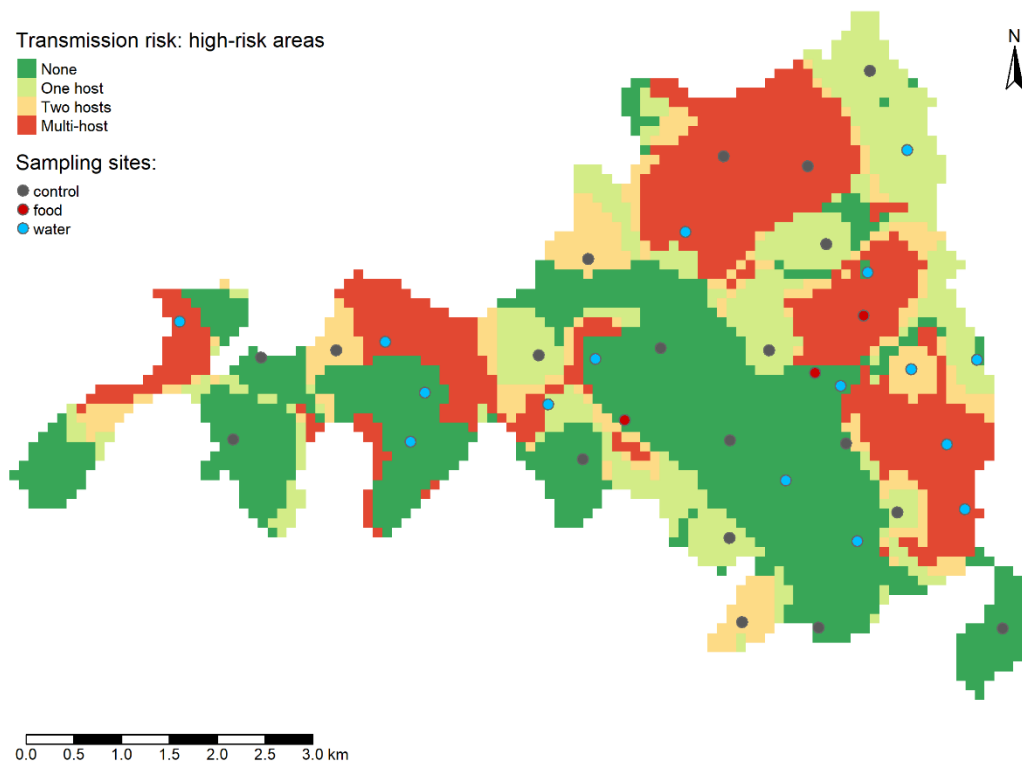
Figure 5-8. MTBC multi-host transmission risk map covering high risk-areas according to different host species compositions, including a multi-host scenario, in Southeast of Portugal, layered with sampling sites.

5.4 Discussion

Incorporating data on host space use into disease models can improve predictions of transmission dynamics, thus aiding in the definition of priority areas for effective disease control (Morris et al. 2016, Dougherty et al. 2018). While the complex interplay between host ecology and transmission pathways (direct and indirect) for animal TB has been studied in some depth (Payne et al. 2016, Varela-Castro et al. 2021), significantly less is known on how host ecology and spatial gradients of MTBC occurrence influence transmission risk across the landscape.

In this study, we demonstrated that: 1) environmental contamination with MTBC is widespread in different types of environmental matrices in the study area; 2) the probability of MTBC occurrence significantly increased in areas with higher slope values; 3) transmission risk analyses provided valuable insights into the spatial distribution of high-risk areas associated with different MTBC hosts. Red deer and wild boar presented the highest

percentages of high-risk transmission areas, with a significant overlap with cattle-related areas. Furthermore, results suggested that a substantial proportion of the study area (26.5%) could be at high-risk when considering the co-occurrence of multiple hosts. Regardless of the host considered, high-risk areas are primarily concentrated in forest areas (dense shrub cover) but also encompass small portions of agro land use (reduced or absence shrub cover). They not only include recognised aggregation points, such as water and artificial food sites, but also encompass control sites (e.g. random sites such as animal trails, pastures) where animal encounters are less likely to occur.

5.4.1 Environmental contamination with MTBC

In our study area, a total of 49% of tested samples were positive for the presence of MTBC DNA and 63% of sampling sites were deemed contaminated. This pattern is in agreement with other recent studies conducted in epidemiological risk areas across the Iberian region, also characterised by multi-host communities. In Idanha-a-Nova, nearby the International Tagus Natural Park region (Portugal), Pereira and colleagues found that the majority of samples (54%) contained metabolically active or dormant MTBC cells (Pereira et al. 2023a). Similarly, in the Alentejo region, Santos et al. (2015) confirmed the widespread environmental contamination with MTBC in a TB infected area, with 32% of samples testing positive for MTBC DNA. In Spain, where wildlife–cattle interfaces share many ecological and environmental characteristics with Portugal ecosystems, up to 55.8% of sampling sites – mud samples collected at water sites – tested positive for MTBC DNA (Barasona et al. 2016). Additionally, studies conducted elsewhere also demonstrated the occurrence of MTBC in the environment (i.e. badger setts and latrines in cattle farms), such as in the UK, a non-officially free country where badgers are considered a reservoir host (Courtenay et al. 2006).

In our study, three types of environmental matrices were examined, with higher rates of positivity recorded in mud and soil matrices (56% and 53%, respectively), whereas MTBC DNA was absent from water samples. Barasona et al. (2016) and Pereira et al. (2023a) also recorded higher rates of positivity in mud samples collected from water sites (48% and 53%, respectively). Contrary to our findings, they detected MTBC DNA in water samples, albeit lower proportions of positive samples were recorded (ranging between 8.9% to 19%). Similarly, Santos et al. (2015) documented significantly lower positivity rates in water samples from dams when compared to other sample types, regardless of the season.

Nevertheless, water sites, and even running water, could become contaminated with MTBC from cattle or from wildlife excretions, and thus also constitute an infection source (Allen et al. 2021). In addition, this is likely to be a relevant issue during the dry season, when various species aggregate around limited water sites as described in shared interfaces across Mediterranean environments (Kukielka et al. 2013, Triguero-Ocaña et al. 2019). On the other hand, given the lower rates of positivity in water samples, we hypothesized that surface water is unlikely to be significant in the transmission of MTBC in this ecosystem, in opposition to mud samples. Overall, reported MTBC prevalence rates in mud samples tend to be high (around 50%) in TB multi-host systems (Barasona et al. 2016, Pereira et al. 2023a). Additionally, high prevalence rates of MTBC/*M. bovis* have been detected in sediment samples, although marked heterogeneity was observed, depending on the sediment type, study system and season (Santos et al. 2015b, Martínez-Guijosa et al. 2021).

Regardless of the sample type considered, host space use intensity and host behaviour are likely key factors that render a given site more prone to MTBC contamination and persistence. Sites that are more attractive to numerous wild species are expected to be at a higher risk of contamination with MTBC because more animals may shed MTBC into the environment. Furthermore, host behaviour might influence the length of contact time with the environment and the number of pathogens shed. For example, wild boar and even red deer tend to wallow in water sites (Carrasco-Garcia et al. 2016), leading to prolonged and significant physical contact with the environment. This may increase the likelihood of environmental contamination, particularly in sediments-like mud, due to the excretion from infected animals that tends to occur through various routes (e.g. oronasal, urinary) (Santos et al. 2015a, Barasona et al. 2017).

5.4.2 Slope and soil moisture index are predictors of MTBC environmental contamination in the study area

When analysing environmental drivers of MTBC occurrence, the GLM model indicated that the probability of occurrence in the SA was positively related with slope and soil moisture index, yet only slope demonstrated a significant effect. Although the drivers of MTBC occurrence across spatial scales is still a relatively poorly studied topic, other authors have also shown connections between environmental features and the presence of MTBC in Iberian contexts. For instance, Martínez-Guijosa et al. (2020) demonstrated a greater risk of

detecting MTBC DNA on farms at higher altitudes. We hypothesised that areas with pronounced slopes – mainly associated with forests in the study area – may feature specific conditions (e.g. greater heterogeneity of shadows, moist conditions, and humidity) that could reduce the effects of extreme temperatures and direct sunlight. These factors are known to be critical for MTBC survival (Rodríguez-Hernández et al. 2016, Barbier et al. 2017, Allen et al. 2021). Previous studies have also demonstrated that topography-related factors were important in predicting the abundance distribution of soil bacteria and even bacterial community composition (Liu et al. 2020, Mod et al. 2021). Our findings highlight the need to account for topographic and also edaphic factors in future forecasts of MTBC occurrence, as specific environmental requirements (e.g. niches) are still being uncovered.

We found no support for the effect of other tested predictors on MTBC occurrence. However, recent studies have demonstrated that, depending on the disease-ecological system, MTBC occurrence can be affected by land use factors (Pereira et al. 2023a), configuration of water sites, soil-related factors (e.g. soil temperature) (Santos et al. 2015b) and the presence of wildlife cachectic animals (Barasona et al. 2016). Future studies should aim to encompass larger sample sizes across diverse geographical areas and explore different sets of potential drivers. This can offer new opportunities to identify specific environmental signatures related with MTBC, thereby improving predictive accuracy of modelling approaches.

5.4.3 Prediction of potential high-risk areas for MTBC transmission confirms the central role of red deer and wild boar on TB epidemiology

Wild boar and red deer are considered the most important animal TB reservoir hosts in the Iberian Peninsula: infection is maintained in eco-epidemiological scenarios where any of these species acts as a single reservoir (most often the wild boar) to a facultative multi-host situation (Gortázar et al. 2012, Santos et al. 2022). Our transmission risk analyses demonstrated that red deer and wild boar presented the highest percentages of high-risk transmission areas, thereby supporting their key role on TB epidemiology in the study area. This hypothesis can be supported by three main premises: first, high abundance of wild ungulates that coexist in the same space. Animal density is recognised as a key element in pathogen transmission (Manlove et al. 2022). In our case, both species are highly abundant in the study area – as reported in other regions across the central-southwestern section of

the Iberian Peninsula – and thus likely enhance pathogen transmission and maintenance (Vicente et al. 2013, Santos et al. 2022); second, specific ecological traits in terms of space use can favour transmission in shared environments. Results of camera-trapping surveys support previous findings that both species extensively explore a variety of spatial sites (Laguna et al. 2021b, a). However, a significant proportion of high-risk areas (for both species) is associated with forest areas (indicated by higher slope values), being predominantly concentrated in the Northeast section of the study area. This crucial aspect proves that two main conditions are linked: areas that may provide favourable conditions for pathogen survival (e.g. higher shade in stepper areas) and spatial sites frequently used by ungulates. Therefore, hosts with territories encompassing these characteristics, are expected to foment environmental contamination and have a higher transmission risk; third, wild boar and red deer populations include "super-shedders" individuals. These can develop extensive lesions and excrete considerable amounts of mycobacteria through several routes, occurring intermittently from early stages of the disease (Santos et al. 2015a). In this regard, ungulates are central hosts that could influence environmental contamination and within-host persistence in Mediterranean multi-host systems, as we hypothesised in our study. Consequently, cattle, by sharing areas with ungulates (e.g. ecotone zones between forest and agro land uses), can be exposed to an increased infection risk.

High-risk areas for MTBC transmission associated with red fox and badger, overall, exhibit similar patterns to those of ungulates when considering their spatial distribution. However, high-risk areas are smaller and more fragmented, and particularly less prevalent in the eastern section of the study area. In our study, carnivores displayed a higher intensity of space use more frequently in spatial sites located in agro land use, rather than deep within forest areas. A similar and more pronounced pattern can be observed with cattle, which tend to avoid large, forest patch areas. As a result, transmission risk maps do not designate the northern area of the study area (the largest contiguous forest area) as high-risk, in opposition to wild hosts. Regardless of the host considered, it should be noticed that medium-risk areas for MTBC transmission were the most dominant in the study area. Nevertheless, when considering potential control measures *in situ* to target multiple hosts in complex communities, the identification of critical areas (high-risk areas) should be a priority (Barasona et al. 2013, Triguero-Ocaña et al. 2019, Gortázar et al. 2023). Decisions about where to act (e.g. site selection) are challenging when considering varying transmission risk gradients that arise from distinct ecological backgrounds of hosts (De

Garine-Wichatitsky et al. 2021). We took a further step in this direction by identifying transmission risk areas across multiple hosts. Twenty-six-point five percent of the SA is considered high-risk for MTBC transmission when considering multi-host conditions. Accordingly, there are five main core areas primarily associated with forest land use but also encompassing marginal portions of open areas (e.g. agro land use). Disease control measures should focus on these areas, encompassing specific spatial sites (e.g. artificial food sites and water sites) that tend to promote host aggregation, but also natural areas (e.g. pastures) widely distributed across the landscape.

5.5 Conclusions

Our findings quantified transmission risk gradients in a TB multi host system involving ungulates, carnivores, and cattle. Our predictions, by combining host space use maps with the spatial occurrence of MTBC, provide, for the first-time, risk maps useful for targeting priority areas for MTBC surveillance and control. We demonstrated the presence of MTBC in the environment, specifically in soil and mud matrices, wherein topographic features (i.e. slope) may play a key role. Although red deer and wild boar presented the highest percentages of high-risk areas regarding MTBC transmission risk, our results indicated a potential for high-risk areas when considering the co-occurrence of multiple hosts. Thus, management of disease within multi-host systems may require focusing on such areas, as pathogen abundance depends on the cumulative presence of all relevant hosts involved. Our approach can be applicable to other disease systems that are likely mediated through shared environments, informing and guiding risk assessment plans for control and management actions.

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5.7   Author contributions

Eduardo M. Ferreira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - Original Draft, Writing – Review & Editing, Visualization. Mónica V. Cunha: Conceptualization, Methodology, Validation, Investigation, Resources, Writing – Review & Editing, Supervision, Funding acquisition. Elsa L. Duarte: Conceptualization, Methodology, Validation, Writing – Review & Editing, Supervision. António Mira: Writing – Review & Editing, Funding acquisition. Daniela Pinto: Investigation, Writing – Review & Editing. Inês Mendes: Investigation, Writing – Review & Editing. André C. Pereira: Investigation, Writing – Review & Editing. Tiago Pinto: Writing – Review & Editing. Pelayo Acevedo: Writing – Review & Editing. Sara M. Santos: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – Review & Editing, Supervision, Funding acquisition.

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5.9   Supplementary Material C

5.9.1   Camera-trap design

Camera-trapping was initially set up to estimate interaction patterns involving wildlife and cattle hosts. However, other related metrics, such as visitation data, were available and were used in this study. Data was collected in the dry season (June to September) in 2021 (Kukielka et al. 2013, Cowie et al. 2016). Besides cattle, we used as target species the TB reservoir hosts described for Portugal (red deer and wild boar), and two other susceptible species that occur in the region: the red fox and the badger (Santos et al. 2012, 2022, Matos et al. 2014, Bencatel et al. 2019).

A 1 km grid was overlaid on the study area (SA) (Curveira-Santos et al. 2017, Zanni et al. 2021). One camera was installed on each 1 km^2 cell, to assure land cover representativeness. From this grid, we first selected key sites (water and food sites; Kukielka et al. 2013) – known as important aggregation points between species – prioritizing sites located in different grid cells (Triguero-Ocaña et al. 2020), and an even distribution across farms. The remaining empty cells were defined as control sampling sites, and camera-traps were placed on their centroids. Each sampling site consisted of a single camera-trap (Busnhell Trophy Cam HD Aggressor or Reconyx Hyperfire) placed 30-50 cm above the ground, attached to trees or artificial stakes. At water and food sites, the cameras were facing towards areas highly used by cattle and wildlife to maximise the detection of different species. At control sites, we prioritised animal trails or other areas (e.g. resting sites) potentially used by cattle and wildlife in suitable habitats. No bait of any kind was used. We programmed cameras to operate 24 hours a day, taking three sequential pictures per trigger with a 30-second delay between consecutive triggers. On average, every 10-15 days, we visited camera sites for battery and memory card replacement.

5.9.2   Host space use intensity maps

In this study, we focused on the spatial occurrence of hosts to be used in the assessment of transmission risk. Previous findings have shown that during the dry season, regardless of the host considered, indirect interactions among MTBC hosts are primarily influenced by the abundance of the hosts involved, rather than by ecological and environmental components

(Ferreira et al. 2024). Moreover, other some studies have evidenced similar results, supporting the hypothesis that animal interactions likely follow a density-dependent mechanism at the wildlife–cattle interfaces in TB context (Carrasco-Garcia et al. 2016, Ferreira et al. 2023). Given that, we decided to use host space use intensity – expressed as visitation rates of hosts across sampling sites – as a proxy for interspecies indirect interactions. With this approach, we were able to create and infer individual transmission risk maps for each host. All space use intensity maps generated based on distance weighted interpolation (IDW) were created using a spatial scale of 100 meters.

5.9.3 Supplementary Figures and Tables

Table S5-1. Descriptive results regarding visitation rates (VR) used in the interpolation process are presented, including mean (Mean), standard error (SE) and minimum-maximum (Min-Max) values. These results are categorized by the host species considered in this study: cattle, wild boar, red deer, red fox, and badger.

Host	Mean	SE	Min – Max VR
Cattle	53.6	15.9	0.0 – 434.1
Wild boar	4.5	0.6	0.0 – 12.4
Red deer	8.3	1.6	0.0 – 36.0
Red fox	9.7	2.3	0.0 – 66.4
Badger	2.2	0.6	0.0 – 17.2
Overall mean	15.6	4.2	2.2 – 53.6

Table S5-2. Schemes of the map's combination process and risk-level generation based on conditions of MTBC contamination and host space use intensity maps.

MTBC contamination	Host space use intensity	Transmission risk
Low	Low	Low
Low	Medium	Low
Low	High	Medium
Medium	Low	Low
Medium	Medium	Medium
Medium	High	High
High	Low	Medium
High	Medium	High
High	High	High
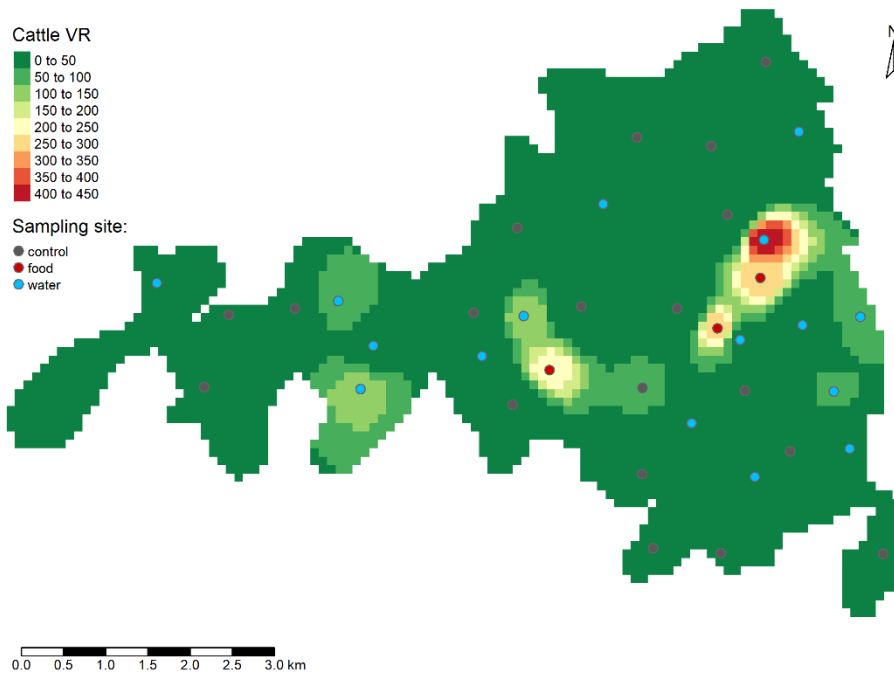
Figure S5-1. Cattle space use intensity map in the study area, Southeast of Portugal, layered with sampling sites.
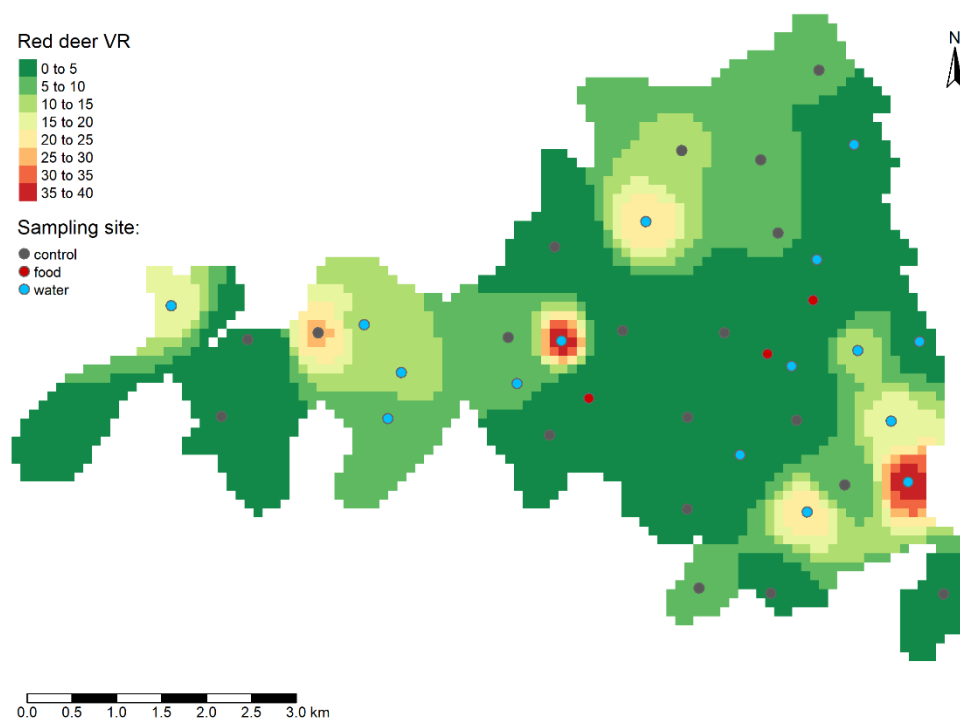
Figure S5-2. Red deer space use intensity map in the study area, Southeast of Portugal, layered with sampling sites.
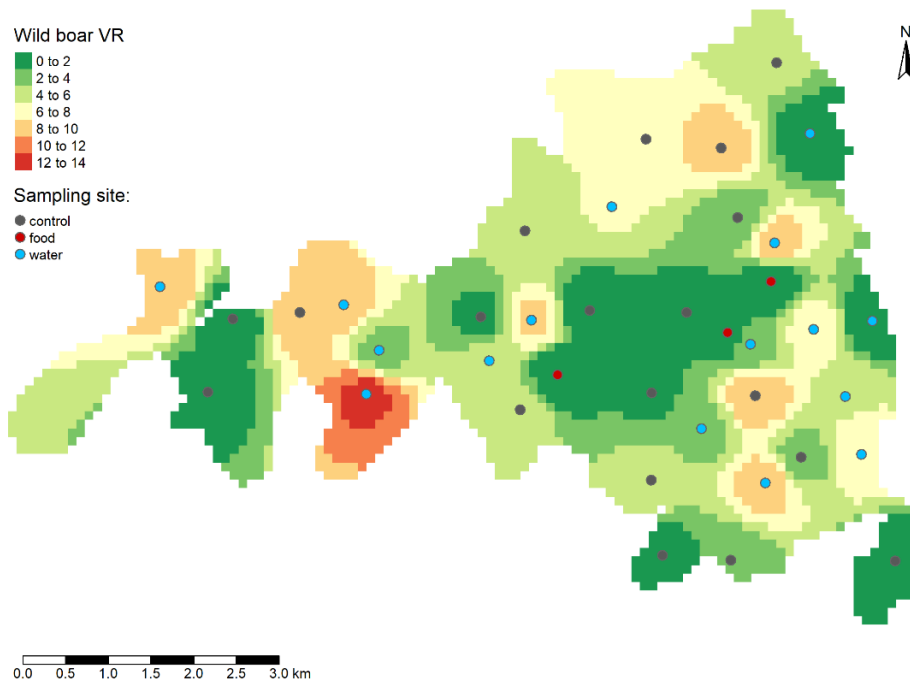
Figure S5-3. Wild boar space use intensity map in the study area, Southeast of Portugal, layered with sampling sites.
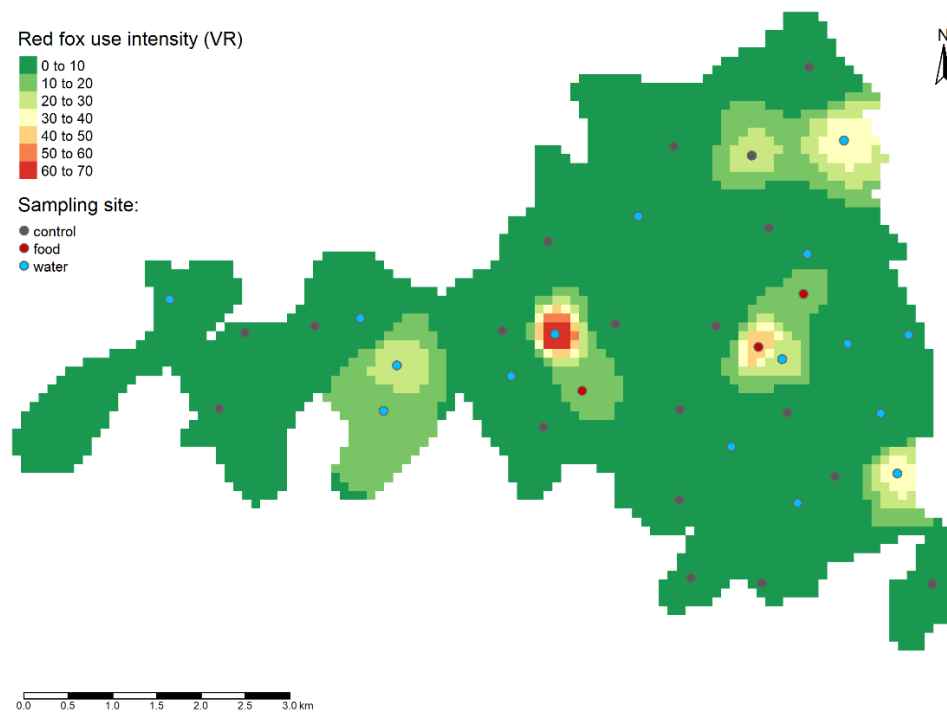
Figure S5-4. Red fox space use intensity map in the study area, Southeast of Portugal, layered with sampling sites.
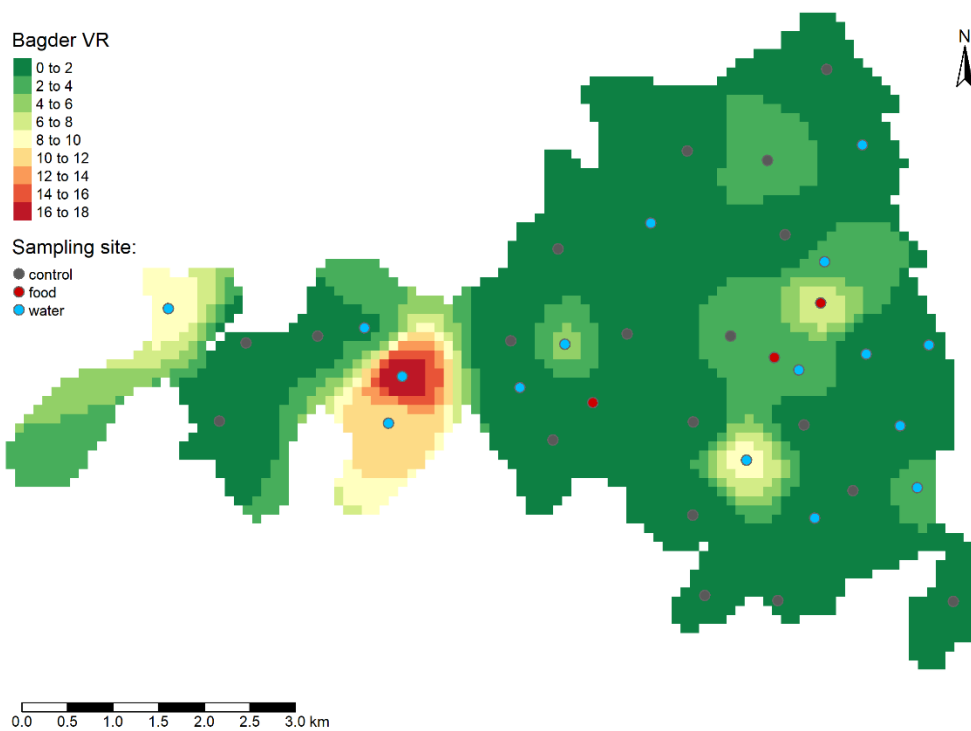
Figure S5-5. Badger space use intensity map in the study area, Southeast of Portugal, layered with sampling sites.

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Chapter 6

General discussion

Chapter 6 – General discussion

6.1 Summary of main results and conclusions

Animal interfaces have become more interconnected, and both livestock and wild mammals nowadays share habitats and resources, thereby increasing opportunities for the transmission of pathogens (Karmacharya et al. 2024). More sustainable animal production systems that aim to promote welfare and reduce environmental impacts pose considerable challenges for infectious diseases control. In Mediterranean agroforestry ecosystems, livestock can be reared in extensive farming systems, sharing space with wildlife, which is the case for the *Montado* and the study area of this thesis. The hazards and impacts derived from emerging (or re-emerging) diseases – including animal TB – have never been so real, demanding research efforts at all levels, particularly in recent decades (Wiethoelter et al. 2015, Hassell et al. 2017, Conteddu et al. 2024). Today, there is an urgent need to address knowledge gaps regarding transmission pathways and intervention opportunities, and to develop new solutions/tools to alleviate the burden of shared diseases in various eco-epidemiological contexts. A major challenge in the coming years will be the incorporation of the ecological dimension into infectious disease epidemiology (Dougherty et al. 2018, 2022). Ecological modelling approaches and the use of big data on wildlife/cattle interactions and behaviour empowers both ecologists and epidemiologists to pose new questions and expand our understating of host-pathogen dynamics. Ultimately, this is crucial for protecting animal health, ensuring food security.

In **Chapter 3**, we conducted a systematic review to identify approaches (criteria definition and methodological approaches) available to assess wildlife–cattle interactions relevant to TB epidemiology. This chapter also provided an opportunity to evaluate through a meta-analysis the relative weight of direct and indirect inter-specific interactions on TB dynamics at a global level.

- We synthesised two main criteria to define direct interactions and five criteria to define indirect interactions between wildlife and cattle through camera-trapping, proximity loggers and Global Positioning System collars (thesis goal no. 1).
- We estimated that wildlife–cattle indirect interaction rates – encompasing various wildlife hosts – were 154 times more frequent than direct interactions; and indirect

interaction rates significantly increased with increasing wildlife density (<u>thesis goal no. 2)</u>.

In **Chapter 4**, we applied a methodological approach outlined in Chapter 3 to describe, *in situ,* local interspecies interaction patterns involving cattle and wildlife within a multi-host community in a high-prevalence TB area located in southern Portugal. In addition, we identified key ecological drivers potentially favouring indirect transmission considering wildlife-wildlife and wildlife–cattle relations.

- Our findings showed that indirect wildlife–cattle and wildife–wildlife interactions occur frequently, with rates of interactions generally higher in the wet season (<u>thesis goal no. 3</u>).
- The wild boar, red deer and red fox were the wildlife hosts mostly involved in the disease-relevant interaction network (<u>thesis goal no. 3</u>).
- Rates of indirect interaction involving cattle were higher than those involving different wildlife species (<u>thesis goal no. 3</u>).
- The abundance of interspecies interactions was influenced by different ecological backgrounds, depending on the host and season: wildlife–cattle interactions were primarily influenced by natural food and water availability, whereas wildlife indirect interactions were more frequently associated with human disturbance factors (<u>thesis goal no. 4).</u>

In **Chapter 5**, building upon the study area covered in Chapter 4**,** the extent of environmental contamination by *M. bovis*, using MTBC as a proxy, was evaluated. Additionally, by integrating host space use data from Chapter 4 with predicted areas of MTBC occurrence, we determined spatial gradients of transmission risk, considering both single-host conditions and multi-host scenarios encompassing multiple species simultaneously.

- We demonstrated the presence of MTBC DNA in a high proportion of sampling sites (63%) across the study area (<u>thesis goal no. 5</u>).
- We confirmed the presence of MTBC DNA in mud and soil samples, whereas MTBC DNA was absent from water samples (<u>thesis goal no. 5</u>).
- The probability of MTBC occurrence significantly increased in areas with higher slope values (<u>thesis goal no. 6</u>).

- The highest percentages of high-risk areas for MTBC transmission were associated with the space use intensity of red deer and wild boar, while other hosts presented smaller and more fragmented high-risk areas (<u>thesis goal no. 7</u>).

- One quarter of the study area was classified as high-risk transmission when considering multi-host conditions (<u>thesis goal no. 7</u>).

- Wild boar and red deer, being abundant and highly interactive within the network community, are likely responsible for MTBC environmental contamination and can play a key role in TB epidemiology in the study area. Consequently, these ungulates can be a potential source of infection for cattle (<u>thesis goal no. 8</u>).

6.2 Defining disease-relevant interactions: a global overview of criteria

The study of host interactions has garnered attention in epidemiology to hierarchise likely transmission pathways within multi-host communities. Various methodological tools from ecology are available for studying interaction patterns involving wildlife and cattle within disease contexts, including TB (Böhm et al. 2009, Kukielka et al. 2013, Triguero-Ocaña et al. 2019). However, the complexity underlying methodological approaches and study systems has led to considerable variation in how criteria are applied to define disease-relevant interactions between hosts. Although defining interactions poses challenges (Eames et al. 2015), literature reviews are powerful tools that can help frame the current state of knowledge (e.g. Bernardino et al. 2018, Rees et al. 2021), thereby helping to develop comprehensive synthesis and flexible frameworks on the topic (Bacigalupo et al. 2020).

In **Chapter 3**, we provided a systematic literature review on the criteria used to define wildlife–cattle interactions relevant to *M. bovis* transmission, covering the main methodological approaches (camera-trapping; proximity loggers and Global Positioning System collars) across different eco-epidemiological contexts. We demonstrated that only a few studies (10 %) used specific criteria to define direct and indirect interactions between wildlife and cattle, although varying definitions and related specificities (e.g. features related with parameterisation) were available. These findings are consistent with previous research in the field, which has revealed a lack of consensus in defining interactions and highlighted the need to develop unified approaches sufficiently flexible to apply to a wide range of

wildlife and livestock species for non-vector borne diseases (Bacigalupo et al. 2020). Drawing from the definitions covered in this chapter, we synthesised the main criteria used to express a wildlife–cattle interaction according to interaction type (direct and indirect) and method of data collection (camera-trapping, proximity loggers and global positioning system [GPS] collars). Overall, direct interactions included physical contact between animals, as well as close proximity between individuals as a surrogate of that interaction; for indirect interactions, a general baseline considered the use of shared environments by individuals at different times. These results significantly complement the existing literature, not only by providing a comprehensive overview but also by framing the current knowledge according to methodological approaches and interaction type, filling a missing link. As a result, they serve as a guide easily accessible to researchers (Eames et al. 2015, Bacigalupo et al. 2020). Incorporating standardised and commonly accepted definitions in future studies will enhance consistency in datasets and facilitate the integration of results across various approaches and experimental designs. Finally, by establishing standards in this field, we can create room for promoting a data repository at different spatial scales (e.g. national and European levels), as has been applied to data collection on wildlife species occurrence through similar methodological approaches (e.g. camera trapping) (Casaer et al. 2023, Ruiz-Rodríguez et al. 2023). We conclude **Chapter 3** with recommendations for future studies on how to define interaction data and improve the reporting of results (rates and raw data of interactions), as well as other key parameters (e.g. cattle/wildlife density, TB prevalence) associated with study systems. Additionally, in this research, we formulated a framework that smooths raw interaction data taking into consideration different sampling efforts inherent to data collection methods. Given that, we advocate for rigorous sampling efforts (i.e. number of camera-trapping stations, monitoring sites or collared animals) and appropriate study duration estimates to be considered in data analysis and results expression.

6.3 Patterns and ecological drivers of interspecies interactions in TB systems

In **Chapter 3**, based on published literature, we quantified wildlife–cattle direct and indirect interactions on a global scale. We compiled interaction data primarily from studies from Europe (84%) and, to a lesser extent, North America (16%), with camera-trapping emerging

as the most frequently used method. Building on this, **Chapter 4** shifted focus to a local level. It documented, for the first time, the composition of the network of interactions in a multi-host community within a Mediterranean ecosystem, focusing on an area well-known as a TB hotspot for both wildlife and cattle in Portugal mainland. Three essential inputs for **Chapter 4** are derived from **Chapter 3**: first, how to define host interactions through camera-trapping, the selected methodological approach for this study; second, how to standardise interaction data as a function of sampling effort, reporting all summary results and other important parameters; and third, the importance of considering the potential influence of varying ecological predictors on patterns of host interactions.

We demonstrated that wildlife–cattle direct interactions are infrequent both globally and locally. On the other hand, indirect interactions are significantly more frequent, thereby underscoring the importance of shared environments in TB epidemiology across multiple eco-systems (Barasona et al. 2016, Allen et al. 2021, Gortázar et al. 2023). In terms of rates, wildlife–cattle indirect interactions were 154 and 517 times more frequent than direct interactions at global and local scales, respectively. Likely influenced by a multitude of factors, but mostly driven by behavioural effects, these results support the idea that, even in shared environments, wildlife and cattle do not tend to engage in the same spatial site at the same time. Nevertheless, even though such events are rarely observed, the likelihood of infection involved in direct host-to-host interactions should not be neglected when considering TB dynamics (Wilber et al. 2019). Given this, we hypothesise that, when considering interspecies interactions involving cattle, the transmission of *M. bovis* is likely mostly indirect through asynchronous space sharing, which could occur in distinct eco-epidemiological scenarios (Walter et al. 2014, Gortázar et al. 2015, Allen et al. 2021). This is particularly relevant in the case of ecosystems affected by TB due to *M. bovis* ability to survive in the environment for extended periods (Fine et al. 2011, Allen et al. 2021). Therefore, frequent shared space use may facilitate indirect transmission via multiple sources of infection, such as water and soil matrices, thereby increasing the risk to infect multiple sympatric host species (Barasona et al. 2016, Rodríguez-Hernández et al. 2016, Allen et al. 2021). Indeed, recent findings demonstrated, for the first time, the environment as a potential source of new infection at shared interfaces (Pereira et al. 2024). We also found that the wildlife species more frequently involved in wildlife–cattle indirect interactions are generalist species, namely the wild boar and red fox at both global (particularly in Europe) and local scales, with the red deer specifically prominent in the

Portuguese context (local scale). We hypothesise that the higher number of interactions involving these species are likely related with local species abundances (Carrasco-Garcia et al. 2016, Campbell et al. 2019, Manlove et al. 2022). Wild boar and red fox can occur in a variety of habitats and exploit a wide range of resources in different landscape contexts (Tolhurst et al. 2011, Main et al. 2020, Laguna et al. 2021, Castañeda et al. 2022); additionally, ungulates and red foxes are common in the study area, and thus higher rates of indirect interaction involving these hosts are expected. The role of animal abundance on this topic is supported by the fact that wildlife–cattle interactions (analysed as rates in **Chapter 3** and abundance in **Chapter 4**) were positively influenced by increasing wildlife abundance (a proxy for wildlife density). Host density is a key component in spatial transmission dynamics (Hu et al. 2013, Manlove et al. 2022). Overall, our results support the hypothesis that, in the TB context, indirect interactions are likely modulated by a density-dependent mechanism, encompassing different hosts. Despite not evidenced in **Chapter 3**, results from **Chapter 4** also confirm that, regardless of the host and season considered, the abundance of wildlife–cattle indirect interactions was significantly mediated by cattle abundance (positive relation). This is particularly expected in scenarios where cattle are reared outdoors year-round in larger herds, as it is the case in the study area. In contrast, in other TB endemic countries (e.g. United Kingdom), where cattle are kept indoors during certain periods of the year, or in dairy farms (e.g. Campbell et al. 2019), this can potentially minimise the effect of cattle abundance on shared space and subsequent interspecific interaction patterns. In short, these findings highlight the role of host abundance in interaction patterns regarding farming systems. In this sense, we advocate for the need to re-think surveillance and control programmes upon this issue. At the very least, monitoring wildlife abundance should be prioritised, as it could inform complementary surveillance measures.

It is worth mentioning that, in **Chapter 3**, poor reporting of interaction data (e.g. summary statistics and raw data) and information regarding potential predictors (e.g. study area size, animal densities) posed significant difficulties, limiting data extraction, and ultimately leading to the exclusion of some studies from the meta-analysis. In this sense, the conclusions about the effect of the predictors examined should be viewed as a first exploration attempt, prompting further investigation to validate the proposed hypotheses. Thus, based on the results, limitations and recommendations from **Chapter 3**, the **Chapter 4** aimed to investigate interaction patterns and ecological links across spatial and temporal scales in greater detail. Accordingly, in **Chapter 4**, we expanded our research on TB

epidemiology by evaluating wildlife interspecies interactions. Interspecies transmission not involving cattle is equally an important component to understand animal TB maintenance in multi-host communities (Triguero-Ocaña et al. 2020, Gortázar et al. 2023). By addressing multiple hosts and the interspecific routes of interactions that likely contribute to pathogen transmission, research conducted in **Chapter 4** helps to depict the complexity of multi-host communities, such as those found in Iberia (Reis et al. 2020, Gortázar et al. 2023).

Our work also confirms the widespread occurrence of wildlife–wildlife indirect interactions in *Montado* landscapes. However, we demonstrated that wildlife–cattle interactions are more frequent than interspecies wildlife interactions. Additionally, in **Chapter 4**, for the majority of the species pairs analysed, we demonstrated that indirect interactions are more frequent in the wet season, contrasting with other studies conducted in Mediterranean environments that have shown a peak of interspecies indirect interactions during the dry season (e.g. July-September), and sometimes during autumn periods as well (Kukielka et al. 2013, Triguero-Ocaña et al. 2019). Such findings suggest that disease-relevant interaction occur throughout the year period, even outside key resources areas (e.g. water sites) during the resource-deficient dry season, which may have been somewhat overlooked (Varela-Castro et al. 2021b). Although seasonal patterns of interactions can be influenced by various factors (e.g. species-specific behaviours), differences in interaction rates among seasons are primarily driven by the availability and abundance of resources, which vary markedly between dry and wet periods in Mediterranean ecosystems (Serrano et al. 2021).

Moving forward, in **Chapter 4**, we also observed that wildlife–cattle indirect interactions were frequently associated with natural food and water resources. The ecological associations between wildlife–cattle interactions and natural resources underscore the hypothesis that in shared environments, cattle and wildlife indeed compete for resources and space (Jori et al. 2019, Vercauteren et al. 2021, Cravino et al. 2024). To mitigate potential conflicts related to disease transmission, it is essential to incorporate dynamic and species-specific management actions focused on shared resources for both cattle and wildlife (Barroso & Gortázar 2024a, b). Integrated strategies can improve the harmony of *Montado* interfaces, preserving biodiversity and production systems. On the other hand, the abundance of interspecific wildlife indirect interactions was mainly supported by human-related factors. Currently, there is a lack of empirical evidence on how human stressors can affect host interactions and disease dynamics (Conteddu et al. 2024). Given our findings, this

topic warrants further investigation. Wildlife–cattle interfaces are particularly susceptible to human interference (e.g. hunting, forestry, agriculture), which can either favour or hinder wildlife populations in terms of space use and animal abundance (Laguna et al. 2021, Cravino et al. 2024). We hypothesised that the negative relation observed between the abundance of wildlife indirect interactions and human disturbance factors may be attributed to the strong avoidance behaviour displayed by some wildlife species in response to anthropogenic stressors (Alexandre et al. 2020, Rosalino et al. 2022). However, opportunistic species (e.g. wild boar) can also benefit from certain anthropogenic interventions (e.g. land use conversion to forestry or agriculture), taking advantage of the opportunities presented by these changes (Giménez-Anaya et al. 2020). Consequently, increased interactions through shared environments may occur in areas with a higher human footprint (e.g. predominantly agricultural and semi-urban areas). Lastly, in **Chapter 4**, we confirmed that interspecies indirect interactions were also influenced by other ecological factors, specifically related with landscape composition (e.g. percentage of open areas [agro land use]) and weather (e.g. minimum and maximum temperatures). Therefore, this work also suggests that indirect interactions between sympatric species at *Montado* shared interfaces are determined by different ecological backgrounds, depending on the host and season (Carrasco-Garcia et al. 2016, Triguero-Ocaña et al. 2020b, Varela-Castro et al. 2021b). We can infer that *M. bovis* transmission patterns within this interface are likely influenced by a multitude of ecological factors operating within the multi-host community across spatial and temporal scales.

6.4 Transmission risk gradients: the interplay of cattle, wildlife and the environment

M. bovis tends to exhibit geographically structuring as a function of environmental and ecological gradients that support hosts and, potentially, biological conditions favouring mycobacteria survival (Santos et al. 2018, Reis et al. 2020, Martínez-Guijosa et al. 2020, Pereira et al. 2023b). **Chapter 5** has the ultimate goal of evaluating the transmission risk of MTBC (as a proxy for *M. bovis*) for the study area by incorporating host ecology data [data derived from **chapter 4**]) with environmental contamination. Initially, we determined the extent of environmental contamination in the study area through molecular approaches (Real-Time PCR). Our findings show that a significant proportion of the study area is contaminated with MTBC, with contamination confirmed only in soil and mud samples. Mud

samples could be particularly relevant in Mediterranean systems as they are associated with water sites wherein wildlife and cattle frequently gather (Kukielka et al. 2013). Other authors have also demonstrated significant prevalence rates in mud samples from forested shared interfaces, including areas with high TB prevalence in wildlife (Barasona et al. 2016, Pereira et al. 2023a). Moreover, behaviours such as wallowing or drinking at these sites can increase risk exposure. Host behaviour likely determines the type and length of contact with environmental matrices, thus influencing the time during which individuals shed bacilli and the number of bacilli shed (Herrera & Nunn 2019, Silk & Fefferman 2021). When considering a broader perspective, once more, our results reflect the importance of the environment in TB epidemiology and emphasise the need to improve knowledge on this theme.

In **Chapter 5**, we tested a set of environmental factors potentially influencing MTBC contamination in the study area. The results indicated that the probability of MTBC occurrence in the landscape was positively related with slope and soil moisture index, although only slope demonstrated a significant effect. These findings suggest that areas with higher slope values (steep areas), primarily associated with forest land use (high tree and shrub cover), can offer a repertoire of conditions (e.g. shading) conducive to MTBC occurrence and persistence (Allen et al. 2021). Recent studies have demonstrated, for the first-time, the relations between ecological and environmental factors and MTBC occurrence in multi-host systems across Iberia (Santos et al. 2015b, Barasona et al. 2016, Pereira et al. 2023a). Indeed, Pereira et al. (2023a) demonstrated that in another endemic animal TB setting in Portugal (Idanha-a-nova, also near the Portuguese-Spanish border), eucalyptus forest and pasture cover are potential major factors driving the occurrence of viable MTBC cells in natural matrices. The presence of viable and dormant *M. bovis* cells makes indirect transmission via environmental contamination plausible. In multi-host scenarios, environmental and animal genomes can be highly intertwined and often distribute similarly into the same *M. bovis* lineages (Pereira et al. 2024). This indicates that environmental contamination originates from *M. bovis* excretion by infected animals (livestock and wildlife). Thus, host presence must be considered a likely key factor driving the spatial occurrence of MTBC in shared interfaces (Barasona et al. 2016). However, to date, we still lack a clear understanding of the general mechanisms driving MTBC contamination across varying spatial and temporal scales. In this regard, our results are a significant addition to the present body of knowledge.

Finally, after modelling MTBC occurrence and uncovering its related drivers, a novel contribution to the study of TB epidemiology at the wildlife–cattle interface brought by this work is the simultaneous assessment of transmission risk considering wildlife and cattle presence, and environmental contamination. Transmission risk analyses integrated host space use intensity – based on visitation rates collected through camera-trapping in **Chapter 4** – into MTBC spatial mapping. Indeed, this marks a novel perspective, as, to the best of our knowledge, the spatial integration of these elements, in these terms, has never been attempted (but see Barasona et al. 2016). We decided to use host space use intensity – expressed as visitation rates of hosts across sampling sites – as a proxy for interspecies indirect interactions. With this approach, we were able to create and infer individual transmission risk maps for each host. Our results confirmed that space use of red deer and wild boar showed the most overlap with MTBC occurrence, resulting in the highest percentages of high-risk areas for MTBC transmission. These findings are in consonance with recent epidemiological evidence collected at the Iberian level, highlighting the key role of wild boar and red deer as TB maintenance hosts in different epidemiological scenarios (Santos et al. 2022). Likewise, results support the hypothesis that both species are likely the main source of environmental contamination in the study area. Ungulates can shed pathogen through multiple routes (e.g. saliva and faeces) (Santos et al. 2015a, Justus et al. 2024), and are often found in forest areas, where they share space and resources with other wild species, as well as with cattle, as demonstrated in **Chapter 4**. Additionally, the larger body mass of ungulates (relative to carnivores in the study area) may also contribute to a higher number of pathogens excreted in the environment (Justus et al. 2024). Overall, it is thus expected that abundant species like ungulates, which can exhibit larger home ranges, likely play a central role in the transmission pathways within this multi-host system (Gortázar et al. 2006, Varela-Castro et al. 2021a). Not only they contribute to the environmental contamination, but can also increase the risk of exposure of other species over wide areas, including spillback to cattle. Nevertheless, we cannot dismiss the possibility of infection sources originated from infected cattle and subsequently spread among wildlife through shared space (Romero et al. 2008, Réveillaud et al. 2018). Regardless of the direction of transmission – not covered in this thesis – interaction and transmission risk analyses confirm that key potential epidemiological connections in the study area are grounded on red deer, wild boar, and cattle. The results of **Chapter 5** also confirmed smaller high-risk transmission areas for carnivores. However, special attention should be given to the red fox, as it has been

identified as one of the most interactive hosts in **Chapter 4**, indirectly sharing space with both cattle and other wildlife species, ungulates included. Red foxes must, therefore, be considered of particular concern for the transmission of *M. bovis*, and further investigation is required (Justus et al. 2024). Since MTBC infection can spread across multiple layers within animal communities, the results obtained when considering the co-occurrence of various hosts (multi-host scenario) may represent a pioneering and accurate approach to mapping transmission risk. TB control is unlikely to be achieved if interventions only target a subset of the hosts involved, normally the reservoirs; instead, a comprehensive understanding of the entire potential host community is necessary, both as a group and individually, along with the role of the environment (Santos et al. 2020, Gortázar et al. 2023). Bearing this in mind, our approach, which encompasses cattle, wildlife, and the environment, demonstrated that one-quarter of the study area exhibits conditions conductive to high-risk MTBC transmission. We successfully identified and spatially delineated five main core areas with high-transmission risk. These areas are spatially separated and mainly associated with forest land use, but they also encompass open areas (i.e. agro land use). Identifying critical areas for pathogen transmission has the potential to redefine our classical approach to disease control, likely providing valuable insights for designing effective monitoring and control programmes. By informing biosecurity and biocontainment in these areas, we aim to reduce indirect transmission at the host community level, while optimising limited resources and efforts. Furthermore, this approach, with the necessary adjustments, can be tested in other disease systems, particularly those where environmental contamination plays a central role on disease spread and maintenance.

In conclusion, this thesis provides an overview of the potential transmission dynamics of *M. bovis* in the national context through an eco-epidemiological framework. Direct interspecies interactions are rare events in this study system, while the indirect sharing of space between wildlife and cattle, as well as among different wildlife species, is very frequent. Second, we have demonstrated the presence of MTBC in the environment, covering a large portion of the study area. Hence, indirect interspecies transmission, facilitated by contaminated environments, likely occurs here, wherein the wild boar and red deer likely play a key role in TB dynamics. Overall, thesis results highlight the importance of considering complex and dynamic multi-host communities where cattle, various wild species, and the environment contribute to pathogen transmission and disease dynamics.

6.5 Future perspectives and contributions for animal TB control

Animal tuberculosis is regarded as an emerging disease in wildlife populations in endemic regions such as Barrancos. It is crucial to prevent further ecological and geographical spread and mitigate prevalence increase, which is especially challenging when multi-host communities are involved. We must contemplate new, integrated approaches, as ongoing control and eradication measures in cattle have proven insufficient. Based on our findings, we suggest recommendations for future studies at distinct spatial scales and actions to refine existing programmes, considering general wildlife–cattle shared interfaces, and along three main axes: cattle, wildlife, and the environment (Table 6-1). These recommendations should be envisaged within a multi-actor approach involving policy-makers, regulatory bodies, academia, farmers, and other related stakeholders.

Table 6-1. Suggestions for future research and recommendations building on knowledge acquired in this thesis, focusing on general wildlife–cattle interfaces in the context of animal tuberculosis, and specific actions related to cattle, wildlife, and environment components.

Research topic	Potential recommendation	Rationale|Goal
Wildlife–cattle interfaces		
Obtain a comprehensive overview of TB dynamics through ecological perspectives within Portugal mainland territory.	• Assess host spatial-temporal interaction patterns, following standardised sampling and data reporting protocols, in other TB hotspot areas, as well as within other epidemiological risk gradients surrounding high-risk areas. • Compare local study findings and assess common features associated with transmission events on a broader scale (e.g. regional level).	Although the official area for TB surveillance in large game species in Portugal encompasses diverse eco-epidemiological contexts, the composition of host interactions is still insufficiently known at local and regional scales and warrants further research. Risk mitigation strategies can be developed for specific scenarios, but these will depend on the local cattle and wildlife ecological and epidemiological conditions. Therefore, conducting local studies is a primary necessary approach to accurately assess transmission pathways. In turn, this is essential to better evaluate transmission risk at a

national scale, encompassing varying geographic gradients.

Cattle		
Management and biosecurity actions upon cattle	• Limit the access of cattle to large forest areas. • Limit the access of cattle to a smaller number of grazing plots in the wet season whenever possible. • Limit the herd size in areas associated with forest land use areas. • Install appropriate fencing systems, such as selective barriers (e.g. "cattle-only"), at water sites designated for cattle, while ensuring that other water sources remain available for wildlife in surrounding areas. • Reduce the amount of suitable and attractive habitats (e.g. shrub cover) for wildlife around sub-fenced units where cattle spend significant amounts of time year-round.	Given the role of forest land use areas as common spaces where cattle, wildlife, and environmental contamination my converge, it is important to develop strategies aimed at segregation space use. By limiting the sharing of resources and space between cattle and wildlife, we minimise the abundance of interactions between hosts and the probability of disease spillover from wildlife to cattle and vice-versa.
Wildlife		
Management and biosecurity actions upon wildlife	• Estimates of ungulates abundance should be closely monitored and integrated into wildlife surveillance programmes. • Consider the possibility of installing game fencing in key grazing areas, associated with open areas (e.g. agro land use), minimizing contact between cattle and wildlife. • Perform additional targeted surveillance of TB in red foxes, for example, using samples from hunted/road-killed animals in areas associated with high TB risk. • Take advantage of active surveillance of large game species to inform farmers on the presence of wildlife with suspect/confirmed TB	A deeper understand of disease epidemiology and transmission within wildlife communities at shared interfaces would enable better prediction and control TB outbreaks. Our results indicate that wild boar, red deer, and, possibly the red fox, are likely the main wildlife species to consider at the wildlife–cattle interface in the Barrancos region. Regardless of the wild species considered, wildlife density is a key factor driving indirect interspecies interactions, requiring further attention.

	lesions in the vicinities of their farms.	
Environment		
Management and biosecurity actions upon environment	• Assess the extent of environmental contamination in other TB hotspot areas encompassing multi-host communities. • Assess ecological drivers of MTBC environmental contamination, including ecological and environmental factors (e.g. host densities, terrain features). • If wildlife and/or cattle are confirmed to be infected in a given farm, assess environmental contamination with MTBC in the surrounding geographical area, particularly focusing on mud and soil samples from suspected shared environments (e.g. water sites). • Use spatial maps of environmental contamination to guide management and biosecurity measures (i.e. temporally fencing off a contaminated water site to prevent animal access).	Many aspects of MTBC environmental contamination and related transmission mechanisms (environment-host) require further research. A broad characterisation of TB high-risk areas in terms of environmental contamination would enable the optimisation of recommendations and surveillance efforts based on geographical contexts and the extent and patterns of contamination.

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