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TESIS DOCTORAL:

Ecology of rodent outbreaks and zoonotic diseases: common voles in the farmland of northwest Spain

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Abstract

Some rodent populations fluctuate in abundance by several orders of magnitude and are characterized by multi-annual "boom-bust" dynamics. The temporal overabundance of rodents favours the spreading of individuals in humanized landscapes, which causes damage to agriculture and/or forestry producing significant economic losses. Noteworthy, such irruptive spatial-temporal spreading of rodents also contributes to the amplification and spill-over of zoonotic pathogens of risk to humans, pets, livestock or other wildlife. Identifying the causative mechanisms and factors behind unstable rodent populations remains an enduring challenge to population ecology, largely motivated by rodent-borne socio-economical and public health impacts. Studying the dynamics of rodent-borne zoonotic diseases, identifying the key hosts, reservoirs and vectors involved in their transmission routes, is thus crucial for a better understanding of epidemiological cycles of zoonoses in nature. Moreover, examining the dynamics of habitat use by fluctuating rodent hosts in humanized landscapes contributes to a better understanding of the spatial-temporal patterns of spill-over processes of zoonotic diseases in the environment, which in turn contributes to more precise surveillance and disease prevention efforts.

In this thesis, I studied wild populations of common vole (*Microtus arvalis*) from northwest Spain (Castilla-y-León region) that have recently invaded (<30 years) irrigated agricultural areas where large boom-bust periodic outbreaks are now endemic. To better understand the impacts of such temporal alternating density scenarios, that is, lots of voles present everywhere during outbreaks to virtually none between outbreaks, I studied: (i) the "contraction-expansion" dynamics of habitat use by voles in recently-colonised farmlands analysing space use patterns, (ii) the role of these unstable vole populations in the processes of amplification and spill-over of zoonotic diseases of risk to humans in the environment, and (iii) the dynamics and nature of ecological interactions between key irruptive hosts like voles and their parasitic arthropod-vectors, and their relative role in the transmission cycles of zoonotic micro-parasites (pathogens). The combination of these study approaches, incorporating the ecological context, intends to contribute new knowledge for the vole control and outbreak management, and disease prevention by providing key aspects about how zoonotic pathogens circulate through wildlife in farmlands.

I specifically explored: (i) how common voles are distributed in intensively-farmed ecosystems and which crops (cereals and alfalfas) or semi-natural habitats (fallows and field margins) act as reservoirs during low density phases, and which are more prone to be colonized by common voles at high density phases, (ii) the main zoonotic pathogens of bacterial origin carried by voles, and the

density-dependent relationship between pathogen prevalence (specifically of *Francisella tularensis* and *Bartonella* spp.) and host abundance, (iii) the role of voles as reservoirs and spill-over agents in the epidemiological cycle of *F. tularensis* and the associations between vole outbreaks and tularemia cases in humans, (iv) whether a rodent host (common vole) and its main arthropod-vector (fleas) share the same zoonotic pathogens, and (v) the density-dependent relationship between flea burdens and common voles, and how fleas can affect key aspects of the dynamics and numbers of this irruptive rodent host (i.e., body condition, reproduction, population growth rate).

I showed that common vole habitat use was dynamic, with a greater overall abundance of voles in field margins and alfalfas and an invasion process of cereal crops from the field margins during population increases. Spill-over of voles through the farming landscape is thus density dependent and originates in a matrix of linear semi-natural habitats interconnecting crops. I also found that tularemia cases in humans coincided in space and time with common vole outbreaks, and that *F. tularensis* prevalence in voles increased with vole density, highlighting that voles act as amplifiers and spill-over agent of the bacterium in the environment. I also contribute to propose a conceptual model based on my data in which fluctuating mammalian host populations have a key role in the epidemiology of tularemia across Europe. Other zoonotic pathogens found at a high prevalence in the studied common vole populations were *Bartonella* spp. Different species of *Bartonella* can be found among voles, showing different seasonal dynamics and associations with vole density. The main ectoparasites of common voles in intensive farmland were fleas, which also carried *F. tularensis* and *Bartonella* ssp., suggesting a potential role as vectors of both pathogens. Flea burden on voles varied with vole density in a delayed density-dependent manner. Temporal variations in flea burden can be explained by a dilution effect, as fleas concentrate on fewer hosts during population declines. Greater flea burdens were associated with reduced reproduction outputs and vole population growth rate, suggesting that fleas could contribute to maintain low density phases of common vole populations. I discuss the benefits of considering ecological interactions to better understand the dynamics of rodent fluctuations and prevent their impacts, such as crop damages and zoonotic outbreaks, as well as the need to consider the dynamic interactions between host, vectors and pathogens to improve predictions of disease emergence, disease control programs and bio-control initiatives.

Resumen

Algunas poblaciones de roedores son capaces de fluctuar en su abundancia en varios órdenes de magnitud siguiendo una dinámica plurianual de "auge y caída" (*boom-bust dynamic*). La sobreabundancia temporal de roedores favorece la propagación de los individuos en los ambientes humanizados, provocando daños en la agricultura y/o en la silvicultura, lo que genera significativas pérdidas económicas. Hay que destacar que la propagación irruptiva de roedores en el espacio y en el tiempo contribuye también a la amplificación y la propagación de patógenos zoonóticos de riesgo para las personas, los animales de compañía, el ganado y otros animales silvestres. La identificación tanto de los mecanismos como de los factores causantes de dichas dinámicas inestables en las poblaciones de roedores sigue siendo un gran desafío para la ecología de poblaciones, que, en gran medida, está motivado por los impactos que los roedores generan a nivel socio-económico y de salud pública. Tanto el estudio de la dinámica de las enfermedades zoonóticas transmitidas por roedores como la identificación de los principales hospedadores, reservorios y vectores involucrados en sus rutas de transmisión son, por lo tanto, aspectos cruciales para entender de una manera más eficaz los ciclos epidemiológicos de las enfermedades zoonóticas en el medio ambiente. Por otro lado, con el fin de obtener un conocimiento más preciso de cómo tiene lugar la propagación espacio-temporal de las enfermedades zoonóticas en los hábitats humanizados es necesario estudiar cómo es la dinámica del uso del hábitat de los roedores con poblaciones que fluctúan. Sin duda, este conocimiento contribuye a una mejor vigilancia y prevención de las enfermedades en el medio ambiente.

En esta tesis, he estudiado las poblaciones naturales del topillo campesino (*Microtus arvalis*) en el noroeste de España (región de Castilla y León). En esta región agrícola altamente intensificada, el aumento de los cultivos de regadío, principalmente de los cultivos de alfalfa, ha favorecido que el topillo campesino haya invadido rápidamente esta región, en menos de 30 años. Durante este proceso de colonización, grandes explosiones demográficas de “auge y caída” han ocurrido periódicamente, pasando a ser endémicas. Con la intención de entender mejor los impactos que provocan las fluctuaciones poblacionales en la densidad del topillo campesino, es decir, un número elevado de topillos en cualquiera de los hábitats del medio agrario cuando se producen explosiones demográficas o brotes y, un bajo número de topillos entre dichas explosiones demográficas, he estudiado: (i) cómo ocurre la dinámica de "contracción-expansión" del uso del hábitat por el topillo campesino en el medio agrario con el

fin de analizar los patrones de uso del espacio, (ii) el papel que tienen estas poblaciones inestables de roedores en los procesos de amplificación y de propagación de enfermedades zoonóticas de riesgo para los seres humanos en el ambiente, y (iii) la dinámica y la naturaleza de las interacciones ecológicas entre hospedadores irruptivos (topillo campesino) y sus vectores artrópodos parásitos (pulgas), así como su papel en los ciclos de transmisión de microparásitos zoonóticos (patógenos). La combinación de estos enfoques de estudio, que incorporan un contexto ecológico, tiene la intención de aportar nuevos conocimientos para el control del topillo campesino, así como en la gestión de las explosiones demográficas y en la prevención de enfermedades, proporcionando aspectos clave sobre cómo los patógenos zoonóticos circulan a través de la fauna silvestre en los ecosistemas agrarios.

Específicamente, en esta tesis he explorado: (i) cómo el topillo campesino se distribuye en los ecosistemas agrarios intensificados y qué cultivos (cereales y alfalfas) o hábitats seminaturales (barbechos, perdidos y márgenes de los cultivos) actúan como reservorios durante las fases de baja densidad, y cuáles son más propensos a ser colonizados por el topillo en las fases de alta densidad, (ii) los principales patógenos zoonóticos de origen bacteriano transportados por los topillos, y la relación denso-dependiente entre la prevalencia de los patógenos (específicamente, *Francisella tularensis* y *Bartonella* spp.) y la abundancia del hospedador, (iii) el papel del topillo campesino como reservorio y agente dispersante en el ciclo epidemiológico de *F. tularensis* y las asociaciones entre las explosiones demográficas de topillo campesino y los casos de tularemia declarados en humanos, (iv) si el hospedador (el topillo campesino) y su principal vector artrópodo (las pulgas) comparten los mismos patógenos zoonóticos, y, finalmente, (v) la relación denso-dependiente entre la carga parasitaria de pulgas y la densidad del topillo, así como el efecto de las pulgas en aspectos claves de la dinámica poblacional del hospedador, es decir, cómo afectan las pulgas a la condición corporal, a la reproducción y a la tasa de crecimiento poblacional del topillo campesino.

A lo largo de esta tesis he demostrado que el uso del hábitat por el topillo campesino es dinámico, con una mayor abundancia global de topillos en las lindes de los cultivos y en las alfalfas, y que la invasión de los cultivos de cereales desde las lindes de los mismos ocurre cuando la población de topillos crece. La propagación de los topillos dentro del paisaje agrícola es, por lo tanto, denso-dependiente y se origina en una matriz de hábitats lineales seminaturales que interconectan los cultivos. También he encontrado que existe una coincidencia espacio-temporal entre los casos de tularemia en humanos y las explosiones de topillo campesino, y que la prevalencia de *F. tularensis* en los topillos aumenta con la densidad poblacional del roedor. Esto sugiere que los topillos actúan como amplificadores y agentes de propagación de la bacteria

en el ambiente. Basándome en mis datos, he propuesto un modelo conceptual en el que las poblaciones fluctuantes de mamíferos hospedadores tienen un papel fundamental en la epidemiología de la tularemia en toda Europa. Además, he detectado una alta prevalencia de otros patógenos zoonóticos en las poblaciones de topillo, como *Bartonella* spp. En concreto, he podido detectar varias especies de *Bartonella* entre los topillos estudiados con diferentes dinámicas estacionales asociadas a la densidad de topillos. Las pulgas son el principal ectoparásito del topillo campesino en la zona de estudio y también albergaban *F. tularensis* y *Bartonella* ssp., lo que sugiere que las pulgas tienen un papel potencial como vectores de ambos patógenos. La carga parasitaria de pulgas en los topillos cambiaba con la densidad de topillos de una manera denso-dependiente retrasada. Estas variaciones temporales en la carga parasitaria podrían explicarse por un "efecto dilución", ya que las pulgas se concentraban en un menor número de hospedadores cuando la población del roedor decrecía. Una mayor carga parasitaria se podía asociar con una reducción en el rendimiento reproductivo y en la tasa de crecimiento poblacional del topillo, sugiriendo que las pulgas podrían contribuir a mantener las fases de baja densidad en las poblaciones de este roedor. Por último, he discutido los beneficios que tiene considerar las interacciones ecológicas para entender cómo funciona la dinámica de las poblaciones de roedores que fluctúan con el fin de poder prevenir sus impactos, es decir, los daños a los cultivos y las epidemias zoonóticos. También discuto la necesidad de considerar las interacciones dinámicas entre el hospedador, los vectores y los patógenos para mejorar las predicciones de la emergencia de enfermedades, los programas de control de enfermedades y las iniciativas de control biológico.

GENERAL INTRODUCTION

1. General context: boom-bust population dynamics

Some species of insects, rodents and birds show *boom–bust dynamics*, a term used in ecology to describe populations that increase rapidly from low numbers, but also decline rapidly from high to low numbers over time. The boom-bust dynamic can be classified as *solitary* (Fig. 1.a) or *recurrent* (Fig. 1.b) (Strayer et al., 2017). In a *solitary boom-bust dynamic*, population size undergoes a rapid and fast increase from a low baseline to a high value (the boom), then drops (the bust) to, and persists at, values substantially lower than the boom, possibly even zero, and then, the population may not recover. In *recurrent boom-bust populations*, the population size undergoes repeated episodes of boom and bust over time (Arthington and Balcombe, 2011) that may repeat *cyclically*, i.e., the booms occur at more or less regular intervals, or *irregularly*, i.e., booms occur without any clear periodicity. For example, populations of snowshoe hares in Canada (*Lepus americanus*) often fluctuate with a period ranging from 8 to 13 years, with an average of 9-10 years (Keith, 1990). In Europe, most common vole (*Microtus arvalis*) populations fluctuate in abundance every 3-5 years (Jacob and Tkadlec, 2010).

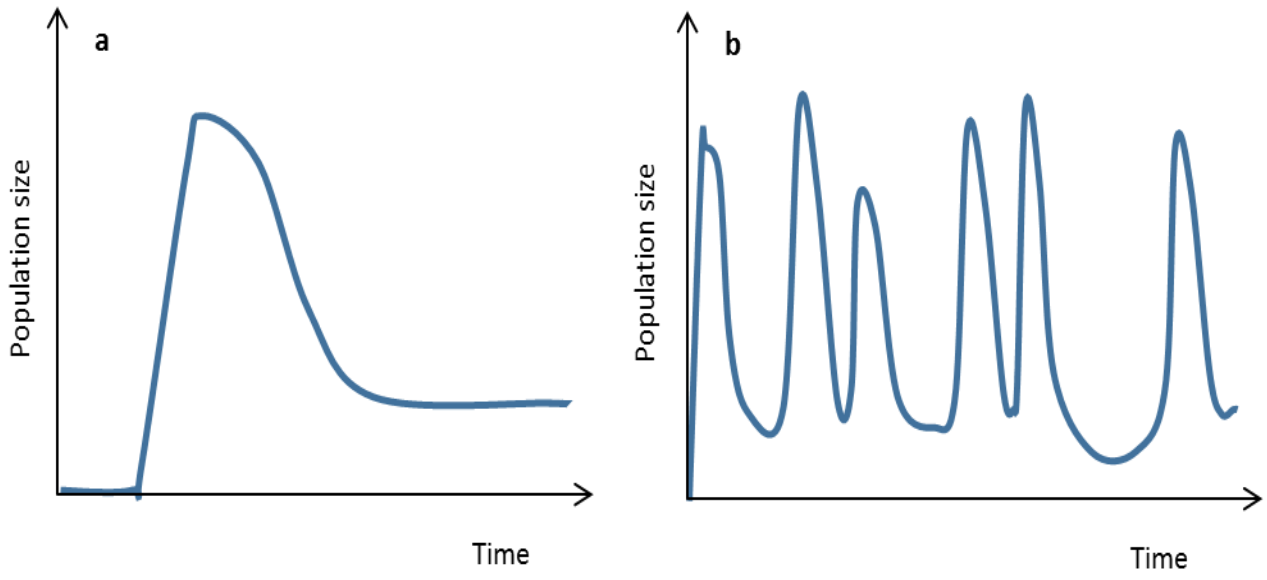

Figure 1. Examples of solitary (a) and recurrent (b) boom-bust population dynamics. Figure adapted from Strayer et al., 2017.

Natural populations increase or decrease in response to changes in the factors that restrict their growth. Many factors influence population density and growth, and may lead to oscillations in population size over time. Past research has focused on identifying whether the regular fluctuations in numbers (*cyclic boom–bust* dynamics) that characterize certain animal populations (voles, insects,

grouse) are caused by (i) *extrinsic factors*, i.e., those factors that act from and originate outside the population, such as climate, weather, food resources and enemies (parasites, predators and diseases) (Krebs et al., 1995; Redpath et al., 2006); and/or by (ii) *intrinsic factors*, i.e., those factors that operate and originate within individual organisms or between organisms of the same species, such as dispersal and sociality, behaviour (Martínez-Padilla et al., 2013; Mougeot et al., 2003a, 2003b), kinship (Lambin and Krebs, 1993) or maternal effects (Bian et al., 2015; Sheriff et al., 2015, 2009). Both extrinsic and intrinsic factors often interact to produce population fluctuations, and many, if not most, populations likely experience their influence simultaneously, which makes it often difficult to determine the exact single factor or combination of them that regulates population growth rate (Krebs, 2013). According to this, Andreassen et al. (2013) used empirical data on experimental populations to propose a multifactorial causative model that integrates the interaction of intrinsic and extrinsic factors through the sequential increase (boom) and crash (bust) phases of the multi-annual abundance cycles of voles. They showed that, apart from predation, other factors of animal´s ecology, in particular, sociality and dispersal can play a key role in regulating vole population dynamics.

The growth of animal populations can vary in a density-dependent or density-independent way. *Density-dependent processes* refer to the influence of population density on vital rates and the subsequent effects of changes in vital rates on population growth rate. Such processes include events or conditions that change in severity as population size increases or decreases. Typical examples include organic ecological interactions such as predation, competition for limited resources, disease and parasitism (Begon et al., 2006). By contrast, *density-independent processes* are stochastic, non-organic, and fluctuate in time affecting all individuals in a population simultaneously, regardless of the overall population size. Such processes include weather and climate variation, e.g., storms, cold, drought, and its effect on environmental factors that also affect changes in vital rates, e.g., abundance or shortage of food or other limiting resources. Boom-bust dynamics expand during months and years, so their analysis requires a wide time perspective and long series of well-structured data. Time series analysis of population changes has been a method widely used in the studies of boom-bust species, such as small mammals. The approach of time series analysis is to fit an autoregressive model to the logarithms of annual indices or estimates of population size (Krebs, 2013). The number of time lags used in the regression is a key point to estimate the direct or delayed density-dependence. It is assumed that any factor that operates in a delayed density-dependent manner will have the potential for generating cyclic population dynamics, because these factors influence the fitness of individuals in a population and ultimately, determine the variation in population abundance through time. Unlike density-dependent processes, density-independent processes alone cannot keep a population at constant levels. Indeed, there is growing evidence that different processes are required to interact to

regulate populations and cause fluctuations in abundance. Cycles in snowshoe hares are caused by the interaction between predation and food supplies (Krebs et al., 1995); population dynamics of white-footed mice (*Peromyscus leucopus*) and deer mice (*P. maniculatus*) are shaped by the interaction between resource availability and infectious disease (Pedersen and Greives, 2008); and population cycles in red grouse (*Lagopus lagopus scoticus*) may be caused by intrinsic (territorial behaviour) and extrinsic (parasitation) mechanisms (Martínez-Padilla et al., 2013) that interact within natural populations (Mougeot et al., 2005; Seivwright et al., 2005). All these examples highlight the need to consider that multiple factors may drive oscillations in wild animal populations (Krebs, 2013), as it occurs with empirically-based modelling, which has also demonstrated that intrinsic and extrinsic factors interact to regulate population dynamics (Andreassen et al., 2013; Radchuk et al., 2016).

2. Rodent population dynamics

Multiannual population cycles have fascinated ecologists for a long time, and this is well illustrated by the studies of rodent population cycles (Boonstra et al., 1998; Korpimäki et al., 2004; Krebs, 1996; Krebs, 2013). Populations of small arvicoline rodents (e.g. lemmings, voles, muskrats) show an intrinsic propensity to fluctuate in numbers, and represent the best and most studied animal model in the field of population dynamics. In the Northern Hemisphere, multiannual fluctuations of population size of these small rodents are a common feature across forest and steppe-like ecosystems. There, the two most common intervals between oscillations are 3 to 4 years, in the case of lemmings (Stenseth, 1999; Wilson et al., 1999) and voles (Krebs, 1996; Ylönen et al., 2003), and 6 to 10 years, in the case of muskrats (Butler, 1962; Errington, 1954) and ground squirrels (Byrom et al., 2000). Notably, a large-scale dampening syndrome in cycle amplitude (maximum densities attained) has been observed among fluctuating populations of European voles during the last decades, and which has been related to changing climatic conditions that directly or indirectly affect rodent population dynamics (Cornulier et al., 2013). To explain vole cycles, both extrinsic (community level; e.g., predation and disease), and intrinsic (population level; e.g., dispersal and sociality) processes have been proposed to affect vole dynamics. Predation has probably received the most support from field experiments (Gilg et al., 2003; Hanski et al., 2001; Hanski and Korpimäki, 1995; Krebs, 1996), but also from modelling studies (Gilg et al., 2003; Hanski et al., 1991; Hanski and Korpimäki, 1995; Turchin and Hanski, 1997). Nevertheless, predation seems not to be a sufficient factor, as cycles are not affected by the removal of a key specialist predator in manipulative large-scale experiments (Graham and Lambin, 2002; Oli, 2003).

Some rodents undergo irregular boom and bust population dynamics, which are mainly related to climate conditions, while other rodents fluctuate with regular cycles. An example of rodent

populations that dramatically fluctuate over time comes from Australian desert rodents (*Pseudomys hermannsburgensis* and *Notomys alexis*) that fluctuate at an approximately 40-fold difference between periods of lowest and highest abundance. For these rodents, Predavec (1994) suggested that natural irruptions are triggered by rainfall and possibly rain-induced bursts of food availability (population growth rate is limited by water availability in arid systems). Other rodent species that widely fluctuate in numbers, but not on with cyclical pattern, are *Mastomys* rodents (multimammate mice), which are the most common muroid species in sub-Saharan Africa. Population outbreaks cause problems in agriculture and in public health (Gratz, 1988). For example, in 1989, an outbreak of *Mastomys* was reported in Tanzania, where rodent densities were estimated to reach 1,400 individuals per hectare, causing a yield loss of 48% in maize fields (Leirs et al., 2010). The changes in population numbers seem to be regulated primarily by bottom-up processes (Leirs et al., 1997, 1993). Abundant rainfall, especially early or late in the season, is hypothesized to cause outbreaks of multimammate mice (Leirs et al., 1996). In the case of rodents with regular dynamics (cycles), many hypotheses have been developed to explain regular multiannual fluctuations. These hypotheses include food resource limitation (Hörnfeldt et al., 1986), predation (Korpimäki and Norrdahl, 1991), vegetation cover (Jędrzejewski and Jędrzejewska, 1996), density-dependent breeding season length (Smith et al., 2006), breeding performance (Mihok et al., 1985), defence mechanisms from food plants (Massey et al., 2008), and disease outbreaks (Wolff and Edge, 2003).

The huge impacts that outbreaks of rodent directly produce on society are related to priority ecological issues worldwide: food security (crop damage) and global health (disease emergence). This has fuelled the large number studies addressing the causes of boom-bust dynamics in this mammal group. Another peculiarity of rodents is that despite their diversity, they just include a small proportion of species with widespread range distributions that become pests, i.e., when they cause damage to agriculture by feeding on high quantities of crops or stored food. The intrinsic inherence of these rodent populations to outbreak make them responsible of a variety of impacts in agriculture, urban areas, ecosystems, forestry and public health (see reviews in Jacob and Tkadlec, 2010; Singleton et al., 1999). Damage to farming crops and forest production includes plant damage and loss and decreased crop quality (Brown et al., 2007; Jacob and Hempel, 2003; Sullivan and Sullivan, 2009), which represent significant economic costs of rodent pests to agriculture (Singleton et al., 2010; Stenseth et al., 2003). In East Africa, for example, rodents are responsible for substantial damage to food and cash crops, structures and industrial and domestic property (Makundi et al., 1999). This is the case of *Mastomys natalensis*, which can cause considerable damage and economic losses (during outbreaks but also during years with lower population densities). In Europe, there are four widely distributed irruptive vole species showing boom-bust dynamics: common voles (*Microtus arvalis*), field voles (*Microtus*

agrestis), bank voles (*Myodes glareolus*), and water voles (*Arvicola* species), whose numerical outbreaks cause important crop damages and socio-economic impacts (Jacob and Tkadlec, 2010). For example, a large (regional scale) common vole outbreak occurred in 2007 in northwest Spain (Castilla-y-León), causing crop damages and complains from farmers. As consequence, costly compensations to farmers and vole control actions (use of rodenticides) reached 24 million € (Jacob and Tkadlec, 2010). Given the high fluctuations in densities of these rodents and the damage they can inflict on public health, agriculture and local economies, it becomes necessary and urgent to understand (1) the relationship between the fluctuating densities of voles and the variation of their damage to crops, and (2) all the factors promoting changes in their population dynamics, with the horizon of applying appropriate (sustainable) and scientifically-informed (ecologically-based) management measures to vole control.

3. Rodent outbreaks and their impacts on food production

Despite the problems caused by rodents to agriculture during outbreaks, they are an integral part of the farming ecosystem. Rodents contribute to many ecological interactions at community level, including the dispersion of seeds and spores, pollination, seed predation, energy and nutrient cycling, plant succession and composition, as well as soil ventilation. They also constitute a key food resource for other trophic levels including numerous mammalian and avian predators, some of them of conservation concern (Arlettaz et al., 2010; Aschwanden et al., 2005; Butet and Leroux, 2001; Šálek et al., 2010). The creation and maintenance of simplified and intensive agroecosystems can favour rodent populations, which could later become a problem in the case of irruptive populations. Human agricultural activities have been favourable for many rodent species. No-till farming can preserve soil horizons and water resources, which provides suitable and stable habitat (food and cover) for rodents (Witmer et al., 2007). Remnants of natural and semi-natural habitats, such as grassy strips, field margins or set-aside, are also beneficial for rodents, as well as high indexes of biodiversity in general (Briner et al., 2005; Macdonald et al., 2007; Tattersall et al., 1997). Grassy edges and fallow fields surrounding crop fields provide refuge for rodents and can then favour their spread into crop fields once crop plants grow to stages that produce abundant forage and cover. Wide landscape areas sown with single-type crops constitute ideal habitats for rodents and some provide better conditions and resources than others. For example in the USA corn fields support more rodents than soybean fields (Witmer et al., 2007). In Europe, traditional crops such as alfalfa and winter wheat are also important habitats for overwintering and reproduction in small mammals (Aschwanden et al., 2007; Heroldová et al., 2005). Noteworthy, the presence and abundance of rodents in agroecosystems is dynamic. The

stacks of cereals during the harvesting season can attract rodents, as well as harsh climate conditions make rodents to migrate from agrarian areas to close human settlement increasing the contact between rodents, livestock and humans (Kuceruk, 1963), which may subsequently favour the spread and transmission of zoonotic diseases from rodents to humans and livestock. Thus, a periodic monitoring of irruptive rodent populations in the field not only permits to know how density and population growth vary through time, but also to study how rodents are distributed in the ecosystem and use the habitat in order to identify which habitats act as sources and sinks of voles from an integrated spatial-temporal perspective. An integrated spatial-temporal community-level approach to study rodent dynamics and their boom-bust numeric outbreaks can help society to develop more efficient and sustainable rodent control practices in agricultural landscapes (Krebs, 2013). As irruptive and repetitive increases of rodents in the environment are the main factor triggering the impacts they produce, the technical monitoring of their populations changes in space and time can benefit the management of any derived impacts including plant damage but also those related to public health, such as the transmission of zoonotic diseases.

4. Rodent outbreaks and their impacts on public health

Rodents are an important public health issue worldwide (Meerburg et al., 2009) because they transmit zoonotic diseases to other wild animals, livestock, companion animals (pets) and humans. During outbreaks, rodents can play a key role in the amplification of many diseases in the environment by acting as both reservoirs and carriers of pathogens (Meerburg et al., 2009). Factors such as climate change, globalization and urbanization are contributing to increase disease emergence, but most of these factors are to some extent ultimately caused by humans. The rapid intensification of agricultural systems, especially of livestock indoor-keeping, and the increasing interactions between wild animals and humans has caused changes in habits and practices of the society, which have contributed to an increased frequency of contacts between wildlife and humans. For example, the recent increase of animal-friendly livestock production systems has led to increased prevalence of certain zoonotic diseases, such as toxoplasmosis, in some European agrarian areas (Kijlstra et al., 2004). The prevalence of zoonotic infectious diseases is rising worldwide and causing losses in human and animals, as well as large costs to society. The economic consequences of rodent-borne diseases are not well understood yet, despite their clear negative economic impacts from individual to national scale (Bonnefoy et al., 2008). Our understanding is still limited because zoonoses emerge from complex ecological interactions; this has led to an increased interest in identifying how pathogen interactions occur and understanding how pathogen transmission takes place between hosts, vectors and their environment.

The propensity of some small rodent species to be reservoirs of zoonotic diseases is due to their fast life history profile, characterised by an extraordinary high reproduction output, which contributes to rapid population turn-overs (Han et al., 2015). These features have made rodents to become the main reservoirs and amplifiers of a large number of human infectious diseases occurring in natural foci. In particular, rodents are reservoir of many human zoonotic pathogens, both bacteria and viruses, and among them at least 60 zoonotic diseases have been recognised as a serious threat to human health (Meerburg et al., 2009). For example, rodents can harbour bacteria such as *Leptospira* spp., *Rickettsia* spp., *Bartonella* spp., and blood parasites such as *Babesia* spp. (Kallio et al., 2014; Schmidt et al., 2014). Rodents also host viruses such as hantaviruses, orthopox viruses, lymphocytic choriomeningitis virus and other arenaviruses, tick-borne encephalitis virus and Ljungan virus (Meerburg et al., 2009; Schlegel et al., 2014; Ulrich et al., 2008). Recent model predictions have revealed that rodents are likely to be associated with novel zoonotic diseases in the near future and will generate new emerging disease hot-spots in diverse geographical regions of the planet (Han et al., 2015). One such prediction relates to outbreaking vole species with disease emergence across the densely populated European region. Of 2,277 extant rodent species at world scale, 217 species (9,5%) are known reservoirs harbouring up to 66 zoonoses caused by viruses, bacteria, fungi, helminths, and protozoa; among these, 79 species (3,5%) carry between 2 and 11 zoonoses. The real study and management target, in terms of public health interests, is therefore only a small proportion of all rodent species. Surprisingly, despite the medical significance of many of their pathogens, rodent-borne diseases are still greatly under-investigated (Bordes et al., 2015). This lack of knowledge should be addressed given the risk that rodent-borne diseases pose nowadays to humans. High rodent population densities fluctuating at landscape level can result in an increased transmission of rodent-borne diseases to humans. Different pathogens infecting rodents might also show important interactions among them and, therefore, affect the overall infection dynamics of zoonotic pathogens in studied systems (Telfer et al., 2010).

Understanding pathogen interactions at the level of the individual and the population, as well as their evolutionary consequences, has important implications to improve predictions of disease emergence, disease control programmes and bio-control initiatives (Lello et al., 2004; Thomas et al., 2003). Currently, the potential interactions between parasites and the processes that shape within-host parasite communities remain unclear in the case of natural rodent populations (but see Telfer et al., 2008). A long-term study conducted in natural populations of field voles in the UK investigated the individual infection risks for a community of microparasites consisting of cowpox virus (CPXV), *Babesia microti*, *Bartonella* spp. and *Anaplasma phagocytophilum* (Telfer et al., 2010). This study highlighted that all the pathogens interactions could be driven by effects on susceptibility and could have as much

impact on infection risk as more commonly considered factors such as host age and season. In addition, the transmission of some infectious pathogens from an organism to another requires a vector, for example an ectoparasite (tick, flea or mosquito). Thus, pathogen infections are not only dependent on the resources provided by the individual host, depending on its age, sex, body condition, or immune system, but also depend on vector features. Examining the relative impact of parasites on their host is of significant importance because parasite can be in turn reservoir and amplifier of many zoonotic pathogens. In a given system, clearly identifying the reservoirs, amplifiers and hosts will improve the understanding of the mechanisms underlying dynamic host-parasite relationships and our knowledge of the epidemiological cycle of many zoonotic diseases in nature.

Many vectors of zoonotic disease are blood-sucking arthropods that ingest etiological agents (pathogens) during a blood meal from an infected host (human or animal) and later inject them into a new host during a subsequent blood meal. Mosquitoes are probably the best-known disease vector of all, but other arthropod vectors include ticks, flies, sand flies, mites and fleas, and non-arthropod vectors include triatomine bugs and some freshwater aquatic snails. Ticks, fleas and sand-flies parasitize rodents and use rodent burrows as their main or unique habitat, passing the most important part of their biological cycles or even their whole lives in the burrows (Kuceruk, 1963). This relationship between ectoparasites and rodents suggests that both may play a key role in the transmission cycles of pathogens. Vector-borne diseases globally cause around 700,000 deaths every year, including these produced by malaria, dengue, schistosomiasis, human African trypanosomiasis, leishmaniasis, Chagas disease, yellow fever, Japanese encephalitis or onchocerciasis (WHO, 2017). Such alarming numbers reinforce the necessity to identify all key agents that participate in the epidemiological cycles of these pathogens.

Among arthropod vectors, fleas (Siphonaptera) are obligate hematophagous ectoparasites of, mainly mammals, but also birds (94% and 6% of known flea species, respectively). They are the most abundant and diverse ectoparasite group infecting small burrowing mammals and can alternatively occur on the body of their host or in their burrows or nests (Krasnov et al., 2002). Fleas directly feed of the blood vessels of the host and are themselves hosts to pathogens, which provide a natural way for pathogen dispersal. Pathogen transmission by fleas occurs by oral route through regurgitation of blood meals, or by faecal route, via contaminated faecal pellets (Bitam et al., 2010). Thus, fleas are of public health significance because of their bites, which can cause directly transmit diseases, cause considerable discomfort, secondary infections and allergic reactions. Additionally, some fleas are intermediate hosts of helminths, for example, *Dipylidium caninum* and *Hymenolepis diminuta*, that can parasitize humans, and be vectors of the etiological agents of several important zoonotic diseases

(Bitam et al., 2010; Eisen and Gage, 2012). Fleas are vectors of *Yersinia pestis* causing plague, *Rickettsia typhi* causing murine typhus, *Rickettsia felis* causing flea-borne spotted fever, *Coxiella burnetii* causing Q-fever, *Bartonella* spp. causing bartonelloses, Myxoma virus causing myxomatosis, *Francisella tularensis* causing tularemia, *Salmonella enteriditis* causing salmonellosis, and *Staphylococcus aureus* causing staphylococcal infection. Not all these infectious pathogens are always transmitted through vectors, and some, such as *F. tularensis*, can also be infective by other means.

Overall, the force of transmission of zoonotic pathogens from rodents to humans could vary positively with: (1) the population density of the rodent reservoir; (2) the frequency of infection (infection prevalence or seroprevalence) in the rodent reservoir; and (3) the density of infected individuals in the reservoir population (Fig. 2; Ostfeld and Mills, 2007). In this thesis, I evaluate the relationship between pathogen prevalence and rodent population density in order to know how the spill-over and amplification of diseases could occur between humans and rodents.

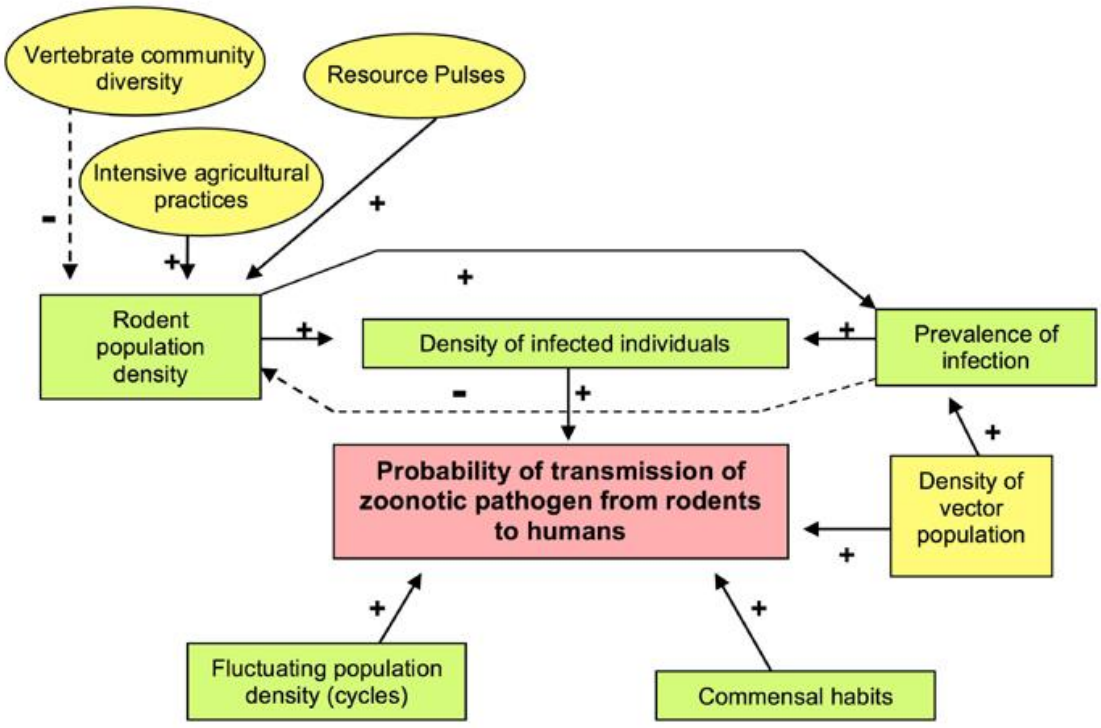

Figure 2. Selected factors known or suspected to affect the probability of transmission of a zoonotic pathogen from rodent hosts to humans. Positive signs near arrows indicate a positive effect on infection prevalence, and negative signs indicate a negative one. Dashed arrows indicate relationships suspected to occur but without strong empirical support, whereas solid arrows represent established relationships (Source: Ostfeld and Mills, 2007).

5. Parasites, pathogens and unstable host dynamics

Like predators and competitors, pathogens and parasites of rodents are likely to strongly influence the population dynamics of their hosts and contribute to regulate their populations, in some cases destabilizing them. The effects of pathogens at individual host level may influence host-parasite dynamics at the population level (Graham et al., 2007), although more research is necessary to understand the role of pathogens and parasites in rodent population dynamics. However, there is a detailed and broad study about natural host–parasite systems conducted in wild field vole populations in Kielder Forest in UK. This study examined the interactions between natural hosts and their parasites in order to know how parasites shape the cyclic population dynamics of field voles. The study integrated different approaches, i.e., genetic, evolutionary ecology, immunology and epidemiology, to better understand the biology of infectious diseases in wild populations (see review in Turner et al., 2014). In the case of pathogens transmitted by fleas, direct or indirect effects can affect the body condition of hosts, and ultimately on their fitness (survival and reproduction), thereby potentially affecting host population dynamics. For example, the effect of flea parasitism on common voles has been experimentally tested, showing that infestation of wild-derived common voles by fleas impairs host development (growth, energy consumption and immune response), survival probability and reproductive success (Devevey et al., 2008; Devevey and Christe, 2009). Flea parasitism also has long-term costs on individual voles increasing the metabolic rates in adults that have been previously parasitized by fleas in their early stage of life (Devevey et al., 2010). These experimental findings highlight that flea parasitism should be considered in studies of host population dynamics.

Early studies about rodent-parasite interactions focused on pathogens and disease as factors regulating rodent populations (Chitty, 1954; Elton et al., 1935, 1931). These studies were focused on understanding whether diseases could cause cycles in host population density. Fluctuations in host abundance can be the result of a delayed-density dependence between pathogen prevalence and population density or population growth rate of the host. However, many individual and environmental aspects may increase or decrease with density, but not be necessary to cause the multiannual change in the population (Krebs, 2013). Telfer et al. (2002) in a study about the effect of cowpox virus on survival in bank voles and wood mice (*Apodemus sylvaticus*) suggested that cowpox infection could increase rodent survival by reducing their dispersal and in turn, their predation risk, or by compensatory reduction of energy destined for reproduction in infected voles. By now, we know that diseases are possible causes of instability in the population dynamic of rodents, and that they should be considered as a relevant process when studying their population dynamics. Most of the studies of parasite-host dynamics have been focused on macroparasites, such as nematodes, but the

knowledge acquired with this model system not always applies to other parasites, such as ectoparasites (fleas, ticks, mice or lice), whose role in population fluctuations still need to be investigated. For example, Cerqueira et al. (2007) shown that the helminth community had a delayed density-dependent effect on water vole (*Arvicola terrestris*) populations, which may produce the cycles. But this effect should be tested on host survival and reproduction in order to know whether it is sufficiently large to cause the decline phase (Krebs, 2013). The role of endoparasites in host dynamics is based on the models of Anderson and May (Anderson and May, 1978; May and Anderson, 1978) that predicts three destabilizing features: (i) the existence of a time delays in endoparasite abundance in relation to host population size, (ii) a low aggregation level of endoparasites during phases of high host abundance, and (iii) endoparasites should induce reduction in host survival and/or fecundity. The theoretical models predict a positive relationship between parasite abundance and host density, although this generalization is complicated to test in natural systems and should be specific to each parasite taxon. For example, the lifecycles of endoparasites and ectoparasites are very different because, in the case of endoparasites, a host represents an ultimate habitat, providing it with a place for living, foraging, and mating (Krasnov et al., 2002). Moreover, when an infected host dies, its endoparasites typically die with it, but its ectoparasites may survive and find an alternative host. Ectoparasites are able to switch the hosts and consequently, the time that ectoparasites spend on the hosts may not reflect the real aggregation pattern in a host population. Thus, experimentation is necessary to test the role that ectoparasites have in their host dynamics by removing or adding ectoparasites to the hosts, and this should be complemented by empirical studies looking at patterns of variations in natural populations.

6. The case of common vole (*M. arvalis*) outbreaks in northwest Spain

Epidemiological studies have long been interested in rodent-borne zoonoses, but the main focus of studies has been largely on identifying and determining the primary reservoirs of zoonotic pathogens. A reservoir of an etiological agent is its natural habitat, which may include humans, animals and environmental sources (e.g., water). Some of these animal reservoirs may also be hosts, which provide a suitable place for an infectious agent to grow and multiply under natural conditions (Bonita et al., 2006). Identifying reservoirs, hosts and vectors is helpful but insufficient for assessing how host population dynamics influence transmission between the different agents involved and how this changes the risk of human exposure to zoonoses in nature (Walton et al., 2016).

The "*Eco-Health*" concept recognizes that humans are part of the ecosystems and that the ecology and health of all the species are highly interconnected, thus, multidisciplinary and collaborative approaches are required to understand the (re)-emergence and spread of zoonotic diseases in the ecosystems. In this thesis, I take a broad view of zoonotic disease by considering both the population perspective of a main rodent host characterized by boom-bust dynamic patterns, and the community perspective, focusing on the interactions of such dynamic host with zoonotic pathogens and vectors in the environment. The PhD thesis focuses on a key vertebrate from agroecosystems widely distributed across Europe, the common vole, a small rodent that often shows unstable population dynamics. I will consider how the study of the population dynamics and habitat preferences of this zoonotic host, and its interactions with vectors, can help to clarify how zoonotic pathogens are increased (amplified) and spread (spill-over) in the environment, and how such processes modulate infection risk to humans.

The common vole is a major European vertebrate pest for plant production that can cause important economic losses during outbreaks (Jacob and Tkadlec, 2010). Like most arvicolines, common vole populations often fluctuate in abundance, with a high amplitude during peaks and a prevailing cycle (frequency) period of 3-4 years in agricultural areas across Europe (Lambin et al., 2006; Luque-Larena et al., 2013; Tkadlec and Stenseth, 2001). A main biological feature of common voles provides the basis for the boom-bust dynamics of its populations: they have an extremely high reproductive potential and are considered amongst the most precocial breeders among mammals worldwide. Females mature extremely early in life and can mate at 2 weeks of age, and surprisingly the youngest mothers produced the first litters larger than those produced by old mothers (Tkadlec and Zejda, 1995). Females can have 5–6 pups per litter (up to 10), produced after a three-week gestation period, and with on average 4.5 litters produced per breeding season (Boyce and Boyce, 1988). The species inhabits primarily grasslands but is well adapted to steppe habitats where it occurs in meadows, set-asides, wildflower strips, grassy field margins and alfalfa crops (Jánová et al., 2008). It also occurs in many cropped lands of the intensive agricultural landscapes of Europe (Bonnet et al., 2013; Fischer et al., 2011; Jánová et al., 2011). The effects of agricultural intensification on abundance and habitat use of common vole have been surveyed in both northern and central Europe (Bonnet et al., 2013; Briner et al., 2005; Delattre et al., 2009; Fischer and Türke, 2016; Heroldová et al., 2018; Jánová et al., 2011, 2008), but not yet in southern Mediterranean Europe.

In the agricultural areas of northwest Spain (Castilla-y-León region), the common vole colonized new areas during the 1970-80s and invaded 5 million ha of previously unoccupied farmlands in less than 20 years (Luque-Larena et al., 2013). Initially, the distribution of the species was restricted

to the peripheral mountainous areas of Castilla-y-León up to the early 1970s. Ten years later, however, most of the lowland areas of Castilla-y-León were colonised. The presence of the species in the entire region was confirmed by 2002 and remained unchanged thereafter, reaching the 100% occupation in 2007. Vole expansion from the mountainous to plain areas has been facilitated by land use changes, namely an increase in irrigated herbaceous crops, such as alfalfa (Jareño et al., 2015; Luque-Larena et al., 2018). Ever since vole colonized the new farmland areas, recurrent large scale regional vole population outbreaks have occurred and had major impacts. During outbreaks, common vole density can reach 1,000 individuals/ha with unprecedented socioeconomic impacts, including significant crop damage episodes (Jacob and Tkadlec, 2010). During recent outbreaks, the vole control measures have been mainly based on the use of rodenticides (anticoagulants), which not only killed common voles, but also, direct and indirectly affected other species that cohabit with voles or feed on them. These measures lead to heated social conflicts between different collectives, such as farmers, hunters, and ecologists (Luque-Larena et al., 2013).

Under this new situation of common vole colonization in northwest Spain, there is a crucial need to understand how voles fluctuate in numbers and what vole population outbreaks mean for the farming landscape of the region. In this context, there is a need to study the habitat use by common voles, the diseases they harbour, the roles that voles may have in the spill-over and transmission of diseases, what relationship voles have with arthropod vectors and the role of these vectors in the pathogen-host system.

The monitoring of common vole populations based on the use of long temporal series of data provides an excellent opportunity to understand how the population fluctuates and how the increase in numbers correlates with the spread of the species through the environment. Consequently, studying the use of the space by common voles in recently-colonised agroecosystems give us the information about which habitats act as reservoir for voles at low densities, and which habitats have higher odds of being colonized by voles when they reach high densities. Better understanding of vole habitat use will be relevant to provide a better monitoring of the vole population dynamics, and for more precise and sustainable targeting of vole control measures aimed at preventing or reducing outbreak densities. From a zoonotic point of view, understanding habitat use helps to identify important vectors or alternative host sharing the same habitats, and, importantly, to evaluate potential disease spill-over (environmental contamination) during vole outbreak phases.

Throughout the four different chapters of this thesis, I explore host habitat preferences and the relationships between host (common vole) and parasites (pathogens and fleas) in intensive agricultural areas from northwest Spain.

I first identify the habitat preferences of the common vole comparing their densities in natural or semi-natural habitats (field margins and fallow lands) and in main crops (cereal and alfalfa), and how these varied in time, during and in-between vole outbreaks (**Chapter 1**). Understanding the ecology of the species, particularly its habitat use, is necessary in order to identify the risk that some crops have to be invaded by voles and then, be damaged by voles. I identify reservoir habitats in the same way as an epidemiologist identifies reservoir hosts. Indeed, particular hosts and environments may contribute disproportionately to parasite and pathogen transmission; thus, the challenge is to identify those habitats from which voles may spill-over in the environment when they are overabundant and point to specific targets where control measures could/should be applied. In turn, this knowledge can help to better understand the dynamics of disease transmission and infection risk at spatial and temporal scales.

Secondly, I investigated the role of common voles and their outbreaks in the epidemiology of tularemia, since northwest Spain is an endemic area for this infectious zoonotic disease affecting humans. *F. tularensis* is a zoonotic intracellular bacterium widely distributed in the Northern Hemisphere. There are two subspecies that cause clinical infections in humans: *F. tularensis* subsp. *tularensis* (type A), which is almost exclusively found in North America, and *F. tularensis* subsp. *holarctica* (type B), which occurs throughout the Holarctic region (Sjöstedt, 2007). The bacterium is highly virulent and has a wide host range (such as, rabbits, hares, voles and other rodents) (Kaysser et al., 2008; Mörner et al., 1988). The bacteria have been also detected from natural waters and mud, and from mosquito larvae collected in endemic areas (Broman et al., 2011; Lundström et al., 2011). Moreover, it is very likely that *F. tularensis* persists in natural waters, possibly in aquatic protozoa (Abd et al., 2003). The role of rodents in the transmission of tularemia is important because there is evidence of a correlation of tularemia outbreaks with preceding peaks of vole cycles in Finland, Sweden (Tärnvik et al., 1996), and Hungary (Gyuranecz et al., 2012). Furthermore, outbreak investigations suggest that high rodent population densities may trigger tularemia outbreaks in humans (Allue et al., 2008; Grunow et al., 2012; Reintjes et al., 2002). Therefore, there is a need to understand the role of common voles in the transmission of *F. tularensis* in northwest Spain. *F. tularensis* has been previously found to infect common voles in northwest Spain (Vidal et al., 2009). I study whether there is a temporal association between vole outbreaks and tularemia outbreaks in humans using monitoring data of species abundance and data on the number of officially declared cases of tularemia in humans (**Chapter 2.a**). I further investigated the role that common voles may play as hosts and reservoirs of this zoonotic disease, and whether voles have a role in the spill-over of the bacterium in the environment during outbreaks (**Chapters 2.b and 2.c**). To confirm this evidence, I tested for an association between vole density and *F. tularensis* prevalence in the common voles,

with the intention of knowing whether at high vole densities, the prevalence of the bacterium in voles is also high, i.e., whether there is a direct density-dependence in voles, confirming an amplification potential (**Chapter 2.b**). Due to its high infectiveness, *F. tularensis* has a very complex epidemiological cycle, and independent aquatic and terrestrial cycles have been suggested (Maurin and Gyuranecz, 2016), with different vectors (mosquitos, ticks, fleas) and hosts (small and medium sized wild herbivorous mammals: rodents and lagomorphs) (Fig. 3). Thus, it is important to know the dynamic role of hosts that show boom-bust dynamics in the epidemiology of a disease like tularemia, a disease of outbreaking nature in temperate regions, and which is the focus of **Chapter 2.c**. Rodents in general, and common voles in particular, can be reservoirs and hosts of many different pathogens (Han et al., 2016). I went on to evaluate the presence of other zoonotic pathogens of public health concern in common vole populations beyond tularemia, concentrating on zoonotic bacteria and spirochaetes (**Chapter 2.d**).

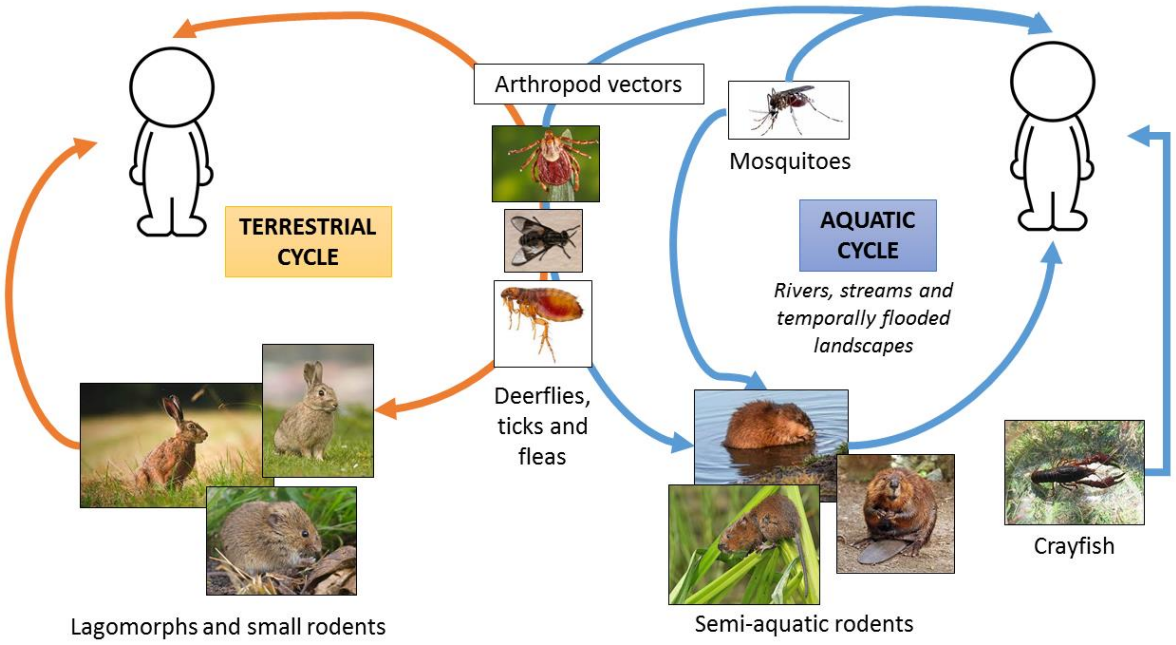

Figure 3. Lifecycles of *Francisella tularensis* - terrestrial and aquatic cycles. In the terrestrial cycle, the hosts and reservoirs of the bacteria are lagomorphs (e.g., rabbits and hares) and small rodents (e.g., voles); while in the aquatic cycle are semi-aquatic rodents (e.g., muskrats, beavers, water voles) and crayfish. Arthropod vectors, such as deerflies and ticks, are vectors in both cycles. Larval and adults of mosquitos take part as vectors in the aquatic cycle. The role of fleas in the transmission of the bacteria is understudied and unclear. Figure adapted from Akimana and Abu Kwaik (2011).

After looking at which pathogens occur in voles, I then looked at how pathogen prevalence varied with vole density during a complete vole boom-bust event (**Chapter 3.a**). I also investigated whether there was a direct or delayed density-dependence response of pathogen prevalence to vole density (**Chapter 3.a**). I expected pathogen prevalence to increase with vole density, because there would be a larger fraction of competent hosts, more contact between them, and consequently, a higher probability to spread the pathogen through the population. I also considered the potential effects of pathogens on vole population dynamics, which could cause the vole population to crash or maintain low densities following an outbreak.

Most pathogens need a vector to be transmitted between hosts, in particular blood-sucking arthropods. I therefore considered whether the pathogens that occurred in voles also occurred in their ectoparasites, which may act as vectors. I studied the prevalence of pathogens in the main ectoparasites of common voles, which were, in this study system, fleas (**Chapter 3.b**). Fleas have been highlighted to be potential vectors of many pathogens elsewhere.

Finally, ectoparasites such as fleas not only have a role in the transmission of diseases, but can also directly affect host fitness, reducing their reproduction and survival. I therefore evaluate the effects that fleas could have on common vole population dynamics. I specifically investigated how flea burden on voles varied in time, and its relationship (direct vs. delayed density-dependence) with vole density, its potential effect on vole condition, reproduction and population growth rate (**Chapter 4**). This vole-flea system offers the opportunity to test how fleas on individual voles may affect the vole dynamics in a natural population.

CHAPTER 1

DYNAMIC HABITAT USE OF HOST

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"Living on the edge": the role of field margins for common vole (*Microtus arvalis*) populations in recently colonised Mediterranean farmland.

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Abstract

Small rodents are common inhabitants of farmlands where they play key ecosystem roles but can also be major pests when overabundant, causing crop damages and significant economic losses. Agricultural landscapes are characterised by high fragmentation with remnant semi-natural habitats being typically restricted to narrow field margins. These linear habitats are key to maintaining local biodiversity, but can also harbour "irruptive pest" species, such as voles. The common vole *Microtus arvalis*, is a main vertebrate pest in continental European farmlands, and recently invaded the inland Mediterranean agricultural landscapes of NW Spain, where regular crop-damaging outbreaks now occur. Knowing how reliant common voles are on field margins in Mediterranean agricultural landscapes would be an important step forward for more targeted management. Here we report on common vole habitat use in Mediterranean European farmland and compare them with those found in northern latitudes, thus seeking for both general patterns as well as geographical differences. We conducted seasonal trappings over 6-years in the main habitats (cereal and alfalfa crops, fallows, and their margins). We show a strong edge effect, in the form of an exponential decay in vole abundance from the margin towards the inside of fields, and vole abundances 2.3 times higher in margins that inside fields. The magnitude of this edge effect varied depending on crop type, season and vole abundance (density-dependence). Cereal crops were characterised by a stronger edge effect than alfalfas or fallows (with abundance 8–10 times higher in margins than in fields during spring and autumn). Cereals appeared as the least optimal habitat for common voles, with important spill-over of voles inside the fields in summer when densities increased. Field margins, where vegetation characteristics hardly change seasonally, provide a limited (5% of the agricultural surface) but stable habitat and key refuge for common voles in Mediterranean farmlands. Our results suggest that targeting management actions in the field margins of cereal crops during spring and autumn and inside alfalfa fields during population increases should be considered in integrated control schemes of crop-damaging common vole outbreaks.

Keywords: Semi-natural habitats; Cereal; Alfalfa; Density-dependence; Outbreak management.

Highlights

_ Common vole abundance is 2.3 times higher in margins than inside fields.

_ Field margins and alfalfas are stable habitats and key refuges for voles.

_ Voles colonize cereal crops from the margins during population increases.

_ Vole outbreak management could target cereal field margins in spring or autumn.

1. Introduction

Current agricultural landscapes result from the removal, fragmentation and reduction of original natural habitats, leading to heterogeneous mosaics made up of large expanses of monoculture with scattered uncultivated areas of varying sizes and shapes (i.e., semi-natural habitats). In intensive agricultural landscapes, these semi-natural habitats are often reduced to linear features, such as hedges, field margins or grassy strips along watercourses, woods or roads (Tattersall et al., 2002) and non-linear habitats, such as set-asides, stubbles or fallows. Wild animals typically inhabit these uncultivated areas such that their conservation is crucial for maintaining habitat heterogeneity and biodiversity (Benton et al., 2003; Tscharntke et al., 2005). In general, wildlife in farmlands is reliant on remnants of natural or semi-natural habitats for persistence (Benton et al., 2003). Semi-natural habitats also act as dispersal corridors, which favour connectivity between patches, colonization and population maintenance (Duelli and Obrist, 2003; Fischer and Lindenmayer, 2007). Moreover, these habitats are considered as refuges for burrowing herbivores, such as Microtus voles, which play a keystone functional role within communities, but when irruptive, can also become an agricultural pest causing crop damage, economic losses and disease spill-over (Delibes-Mateos et al., 2015; Jacob, 2003; Renwick and Lambin, 2013).

The common vole (*Microtus arvalis*) is the most abundant burrowing herbivore in open agricultural European landscapes where grasslands, meadows, set-asides, wildflower strips, grassy field margins or alfalfa crops occur (Bonnet et al., 2013; Delattre et al., 1996; Fischer et al., 2011; Janova et al., 2011; Janova et al., 2008). Common vole population dynamics are characterized by multi-annual cyclic fluctuations, with population peaks occurring every 2-–5 years (Tkadlec and Stenseth, 2001; Lambin et al., 2006; Luque-Larena et al., 2013). Due to its ability to adapt to intensively cultivated areas, its irruptive population dynamics, and the damages to crops during outbreaks, this species is considered as a major rodent pest in many parts of its range (Jacob and Tkadlec, 2010; Jacob et al., 2014).

In NW Spain, the common vole recently invaded ca. 5 million ha of agricultural landscapes where the species was hitherto absent until the surface area of irrigated herbaceous crops including alfalfa steeply increased (Jareño et al., 2015; Luque-Larena et al., 2013). Ever since the colonization of agricultural areas, common vole population outbreaks have regularly occurred, causing significant economic losses to agriculture, as well as environmental impacts associated with the use of rodenticides for controlling vole populations (i.e., secondary poisoning of non-target fauna) and zoonotic outbreaks of tularaemia in humans (Luque-Larena et al., 2015, 2013; Sánchez-Barbudo et al., 2012; Vidal et al., 2009).

Agricultural habitats are seasonally dynamic and continuously modified by farming practices and crop phenology. Mechanical work in crop fields, such as ploughing, harvesting and mowing temporarily alters habitat suitability for burrowing rodents (Bonnet et al., 2013). The periodic alteration of soils greatly impacts vole populations in the cultivated portions of farming landscapes through habitat destruction, increased mortality, altered spatial behaviour, or reduced food availability (Brügger et al., 2010; Jacob and Hempel, 2003; Jacob, 2003). Field margins and fallows are not exposed to such frequent agricultural practises, and hence harbour a higher floral diversity and non-crop plant biomass than cropped fields (Heroldová et al., 2007). As such, field margins are known to provide relatively undisturbed and stable refuges for common voles in farmland areas of temperate Europe, where vegetation growth is not severely limited by rainfall (Bonnet et al., 2013; Jacob and Hempel, 2003; Jacob, 2003). In NW Spain, the inland Mediterranean climate is characterised by a period of strong hydric deficit during summer (i.e., summer droughts of variable duration and severity), which is critical for plant growth (Chaves et al., 2002). This consequently affects the availability of food, which in turn affects the reproduction and survival of upper trophic levels (i.e., herbivores such as voles) (Fernández-Salvador et al., 2005). The common vole is primarily a grassland species, so we would expect the semi-natural margins and fallows to be primary habitats and to act as refuges and sources of individuals for less optimal habitats during periods of low density. Alfalfa crops have also been pointed out as primary habitats for common voles in many European regions, owing to their long-term stability and suitability for vole colony formation (alfalfas remain unploughed for 5-–6 years) and provision of cover and high-quality food for voles (Jareño et al., 2015). By contrast, cereal crops represent the least stable habitats, since they are subjected to more vole-damaging tillage regimes, and thus are expected to be the least optimal habitats and potential sinks for vole populations. A better understanding of when and where voles are more abundant in the agricultural landscape, and of how reliant they are on field margins, would be an important step forward for more targeted management.

We report here on common vole habitat use in a novel farming landscape for this species. We studied the spatial and temporal variations in the use of semi-natural habitats (field margins and fallows) and of agricultural habitats (cereal and alfalfa crops) by common vole populations in recently-colonised Mediterranean farmland areas in the NW of Spain. Understanding the habitat use patterns by common voles in such recently-colonised agricultural landscapes would help us to: (i) understand patterns of habitat use and compare them with those found in temperate European farmlands, and (ii) infer specific management measures at regional level. We first identify the habitats harbouring more common voles at different phases of their population dynamics (two outbreaks and crash phases) according to the crop phenology (and associated variations in vegetation characteristics) and

seasonality. We predicted that fallows and field margins, which are not subjected to continuous farming practises, would act as refuges or source habitats, particularly during low-density phases and when availability of green vegetation is reduced (i.e., during summer drought periods). We thus expected to find an edge effect in the form of a decrease in vole abundance with increasing distance from the edge towards the inside of crop fields. We further expected this edge effect to vary depending on the habitat quality for voles inside fields (i.e., stronger edge effect in sub-optimal crops). Innovatively, we also investigated whether the proportional abundance of voles in margins (relative to fields) was density-dependent, expecting any spill-over of voles from the margins towards fields with increasing density (due to field margin saturation) being particularly marked in sub-optimal crops. Better understanding the links between crop colonisation and vole dynamics will allow for more timely and crop-specific management actions. Finally, we investigated whether the relative vole abundance in margins varied with vegetation characteristics in the margins and fields.

2. Materials and methods

2.1. Study areas

The study was carried out on a large intensive agricultural region of NW Spain (northern plateau, Tierra de Campos, Castilla-y-León region). Fieldwork was conducted in three study areas (40 km2 each) located in the provinces of Palencia (42°01'N, 4°42' W), Valladolid (41°34'N, 5°14' W) and Zamora (41°50'N, 5°36' W) (see Jareño et al. (2014) for a map of the region and more details on study areas).

The climate of Castilla-y-León is defined as "continental Mediterranean with cold winters", and is characterised by a wide seasonal temperature oscillation due to an elevated average altitude (regional mean: ca. 830 m.a.s.l.) and the limitation of Atlantic-buffering effects by peripheral mountain ranges that completely surround the region: summers are dry and hot with a variable drought period, while winters are cold and humid (Jareño et al., 2015; Rivas-Martínez and Loidi, 1999). Rainfall follows a Mediterranean pattern, with precipitation maximums during spring and autumn; the short spring and autumn seasons are thus critical periods for plant growth. Summer is the most stressful season for animals and plants due to the high evapotranspiration rates during this period and the little surface water available. Winter is relatively longer compared to coastal Mediterranean arid regions, and is characterized by frequent periods of frost (Blondel et al., 2010).

The farming landscapes of the study areas consist of a mosaic of crops dominated by non-irrigated cereals (mainly wheat and barley; ca. 48% of the agricultural surface), scattered with irrigated and non-irrigated alfalfa crops (ca. 10%) and other herbaceous crops, such as sunflower, sugar beet,

peas and maize (Jareño et al., 2015). These agricultural landscapes also include fallows (small and dispersed patches of uncultivated land, pastures or meadows; ca. 21% of the agricultural area) and a network of field margins (principally grassy or wildflower strips, but also linear patches of hedges or scrubs along field boundaries, tracks or roads) covering less than 5% of the agrarian surface (based on the average edge width in this study, and the average field size reported in Jareño et al. (2015)).

2.2. Vole trappings and abundance estimates

The monitoring of the three vole populations was conducted every 4-months (in March, July and November, hereafter referred as "spring", "summer" and "autumn trappings", respectively) from July 2009 to November 2014 (*n* = 17 seasonal trapping sessions). During each trapping session in a given season and study area, we sampled the three crop types that dominate the agrarian landscape: cereal, alfalfa (including irrigated and non-irrigated crops) and "fallows" (natural or semi-natural habitats, such as uncultivated lands, meadows, pastures or set-asides). For each seasonal trapping, we selected 12 fields (4 cereals, 4 alfalfas and 4 fallows) randomly within each area amongst all the available crops. Our trapping method was extractive, in order to collect samples and detailed information on vole condition and reproduction for other aspects of our research agenda. Removing voles from sampled fields could influence subsequent local vole abundance estimates through migration movements, but in order to avoid such effects we avoided repeated trappings at the same fields in consecutive seasons and always selected fields as further apart as possible from previously sampled ones within a given 40 km^2 study area. Within each field (hereafter "sampling unit"), we set-up a total of 35 live traps (8 cm × 9 cm × 23 cm; LFAHD Sherman©) spaced every 2 m and forming a "T"-shape (10 traps were placed along a 20-m transect line in the field margin, and 25 traps were placed along a 50-m transect line perpendicular to the field margin and going towards the field centre (Fig. 1)). Each trap was baited with apple or carrot, which provide both food and water for trapped individuals. When the temperatures were low (autumn), hydrophobic cotton was also provided inside traps to increase vole survival. Traps were set up in the morning, were inspected after 24 h and subsequently removed.

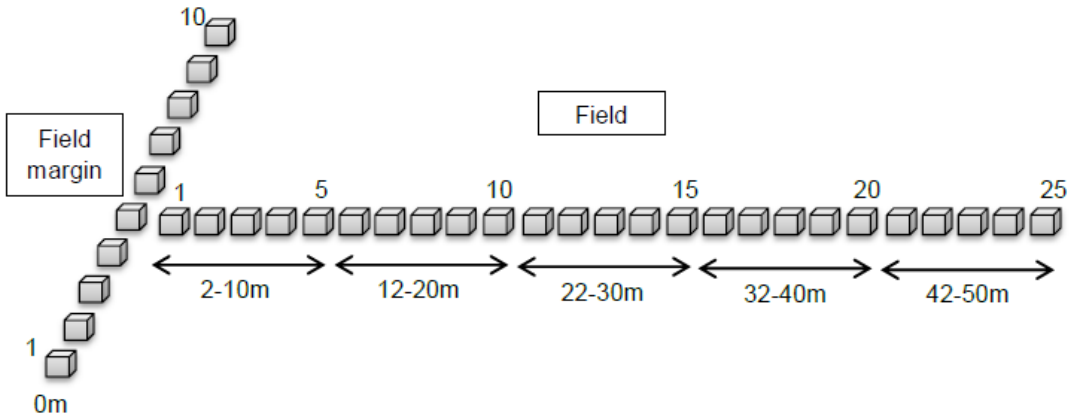

Figure 1. Sampling unit ("T"-shaped trap matrix) consisting of 35 traps: 10 traps in the field margin and 25 traps inside the field. In the field, traps have been regrouped into 5 distance to the field margins categories (of 10 m each, traps being 2 meters apart).

Trapped small mammals (*n* = 6053) were identified and we recorded in which individual trap each capture occurred. Most captures were of common voles (49.31%), followed by wood mice *Apodemus sylvaticus* (26.43%), Algerian mice *Mus spretus* (16.41%), greater white-toothed shrews *Crocidura russula* (5.96%), least weasels *Mustela nivalis* (1.17%) and other species (0.71%).

For each sampling unit, we estimated: 1) the overall abundance of common vole as the number captured divided by the number of traps available for capture (the 35 set traps minus those that captured species other than common vole) and multiplied by 100 (hereafter "vole abundance", in number of voles/100 traps/24 h). We similarly estimated: 2) vole abundance in the field margin (using the 10 traps set up in the margin, hereafter "field margin abundance") and 3) vole abundance inside the field (using the 25 traps set up inside the field; hereafter "field abundance").

In order to describe more precisely how vole abundance varied from the field margin towards the inside of fields, we also estimated within each sampling unit vole abundance for the following 6 distance categories (hereafter "distance to the field margin"): "0" = within the field margin (n = 10 traps); "10" = traps located 2–10 m from the margin (n = 5); "20" = the traps located 12–20 m from the margin (n = 5); "30" = the traps located 22-30 m from the margin (n = 5); "40" = the traps located 32–40 m from the margin (n = 5); and finally, "50" = the traps located 42–50 m from the margin (n = 5) (Fig. 1). The variable "distance to the field margin" was subsequently used as a regressor, and trap groups ("0" to "50") used as distance categories further improved convergence of capture probability models.

2.3. Vegetation characteristics of sampled fields and margins

We characterized the vegetation of the field and margins for 531 sampling units surveyed (due to field work constrains, not all vegetation characteristics were collected for all the sampled fields). We characterised: (1) the type of field margin, according to its topography (three categories: ditches, n = 290, flat margins, n = 100, and margins with slope > 45° (i.e., ridges), n = 141); and (2) the margin width (in meters). Field margin topography may affect vole abundance in several ways. For instance, ditches may better retain water and tend to have denser and greener vegetation, whereas ridges could act as refuges when adjacent fields are flooded after heavy rainfall. We also characterized the following vegetation characteristics: (3) margin vegetation height (average height of the herbaceous vegetation and shrubs, in centimetres); (4) margin vegetation cover (percentage of ground covered by vegetation); (5) margin green vegetation cover (% of ground covered by green vegetation); (6) field vegetation height (average of height of the crop/fallow, in centimetres); (7) field vegetation cover (percentage of ground covered by crop/fallow) and (8) field green vegetation cover (% of ground covered by green crop/fallow). Vegetation variables were obtained by visual estimation and were indicative of the surface occupied by the line of traps (inside fields, a bandwidth of 1 m at both sides of the trapping line was considered to evaluate vegetation variables). The vegetation height is an estimated average between the tallest and the shortest herbaceous vegetation or shrubs.

2.4. Statistical analyses

We used R v3.1.3 for all statistical analyses (R Core Team, 2015). We used Generalized Linear Mixed Models (GLMMs) and Tukey tests for post-hoc pairwise comparisons to test whether vegetation characteristics (height, cover, green cover) differed between habitats (considering four habitat types: field margin, cereal, alfalfa and fallow) by season (using separate models for spring, summer and autumn). The GLMMs included the variable “area-year” (unique combinations of study area- 3 levels- and years, 2009–2014) as random factor to account for the non-independence of abundance data collected in a given study area and year. We used GLMMs to test for differences in field margin characteristics according to season and crop type (cereal, alfalfa, fallow), including season, crop type and their interaction as explanatory variables. The variable “area-year” was used as random factor in all GLMMs performed in this study.

Differences in common vole abundance between habitats were tested by season using GLMMs and a post-hoc pairwise comparison. We also evaluated the temporal variations in vole abundance between habitats using a GLMM.

We modelled vole abundance (captures/100 traps/24 h) according to 6 distance categories to the field margin; (Fig. 1) included as a regressor using GLMMs that included the variables "sample unit" and "area-year" as random effects (to account for the non-independence of data from the same sampled unit and differences in abundance between study areas and years). The dependent variable was a two-vector response variable (number of traps that captured voles/number of traps that did not capture, for a given distance to the margin) fitted to models using a binomial error distribution and a logit link function (using the lme4 package in R; Bates et al., 2014). We analysed each crop type separately and compared three different models: (i) a null model (without the variable "distance to the margin") that included only the explanatory variable Season; (ii) a model with a linear distance effect (abundance = a × Distance + b) that included the explanatory variables Distance (continuous), Season and the interaction Distance × Season; and (iii) a model with an exponential decay distance effect (abundance = a × exp[-Distance] + b) that included the explanatory variables exp[-Distance], Season and the interaction exp[-Distance] × Season. The best model(s) describing abundance variation was (were) chosen by the lowest value of Akaike's Information Criterion (AIC). The strength of the "edge effect" (how and by how much the abundance decreases towards the inside of fields) is described by the type of model supported (from no effect - null model- to moderate effect −linear model- or strong effect −exponential decay model-) and, for a given type of model, by the values of the slope parameter estimates "a" (for a given crop type and season).

We investigated variation in the proportional abundance of voles in the field margin relative to the overall abundance in a given sampled unit using Generalized Linear Models (GLMs). The dependent variable was a two-vector response variable (abundance in the margin/overall abundance) and was fitted to GLMs using a quasi-binomial error distribution and a logit link function. For these analyses, we considered only sampled units with voles (i.e. overall abundance >0; $n = 275$). Explanatory variables included vole abundance (voles/100 traps/24 h; Log-transformed), season (spring, summer autumn), crop type (alfalfa, cereal, fallow) and all the interactions between these variables. For these analyses, we were interested in identifying which variables best explained the relative use of field margins by voles, so we used a stepwise backward model selection approach. Non-significant variables (at $P = 0.05$) were dropped sequentially starting with interactions following a F-test-based backward selection using the drop1 function in R.

We also investigated variation in the proportional abundance of voles in the field margin according to the margin and field characteristics using sampled units with voles (overall abundance >0) and for which we had data on all the margin and field characteristics (margin type and width, and vegetation height, cover and green cover of the margin and field). For these analyses, we considered

only two crop types (alfalfa, n = 98; and cereal, n = 65) affected by agricultural practices and for which the field vegetation characteristics strongly varied between seasons (see results). Margin and field vegetation characteristics were weakly correlated (all $r < 0.4$) and had variance inflation factors (VIF) below 1.5, so there was no issue of collinearity amongst these explanatory variables. Initial models included overall vole abundance, season, the interaction vole abundance × season, and all the variables describing the margin and field characteristics. Non-significant variables were dropped sequentially following a manual F-test-based stepwise backward procedure removing the least significant variable at each step (using the drop1 function in R), with all terms with P (χ^2) < 0.05.

3. Results

3.1. Variations in common vole abundance according to habitat type and season

Seasonal changes in abundance consisted of lower numbers in spring followed by higher numbers in summer-autumn (Figs. 2A; 3). Mean common vole abundance differed between habitats in all seasons (spring: $F_{3,360} = 8.34$, $P < 0.001$; summer: $F_{3,432} = 15.57$, $P < 0.001$; autumn: $F_{3,432} = 24.00$, $P < 0.001$; Fig. 2A) and was consistently higher in field margins than inside fields. Vole abundance was higher in field margins than in cereals in all seasons (45%, 23% and 46% higher in spring, summer and autumn, respectively). Vole abundance was also higher in field margins than in alfalfas during spring (30% higher) and autumn (26% higher), but not during summer (no significant difference). Finally, vole abundance was higher in margins than in fallows in all seasons (29, 13 and 33% higher in spring, summer and autumn, respectively).

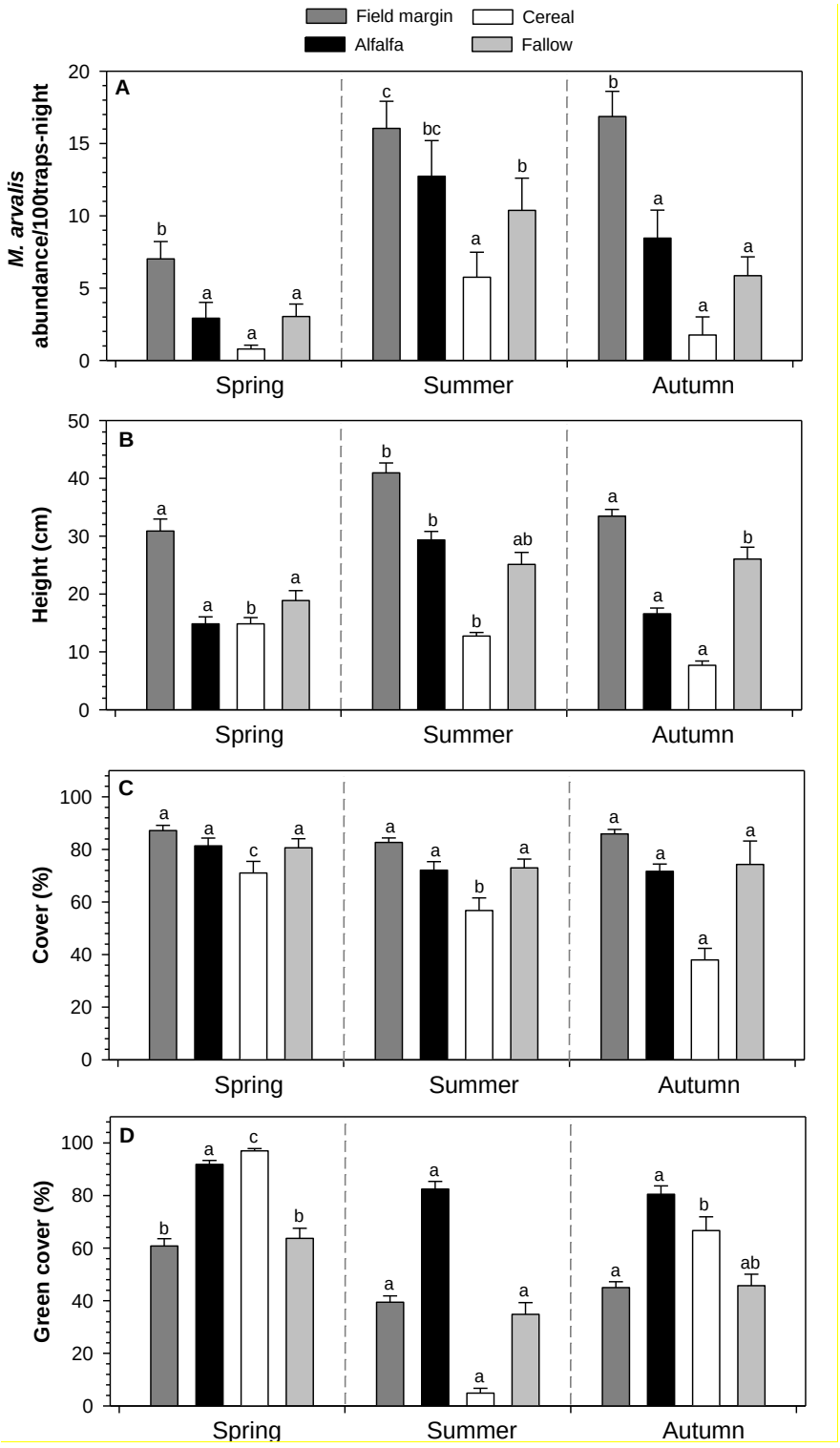

Figure 2. Seasonal variations in vole abundance and vegetation characteristics according to habitat. (A) Common vole abundance (captures/100 traps/24 h), (B) vegetation height (cm), (C) vegetation cover (%), and (D) green vegetation cover (%). *n* = 531 sampled fields. Habitat types: field margin = dark grey, alfalfa = black, cereal = white, and fallow = light grey field) and season. For pairwise comparisons within seasons, different letters indicate significant differences ($P < 0.05$) between habitats (Tukey's tests) in a given season.

3.2. Temporal variations in common vole abundance by habitat type

When considering inter-annual seasonal variations in vole abundance, similar consistent differences among habitats were found (χ^2 = 34.77, d.f. = 3, P < 0.001; Fig. 3). During our study period, vole abundance peaked twice, in November 2011 and again in July 2014. Both peaks were characterized by greater vole abundances in field margins than in fields. This was particularly marked during 2011, when differences in abundance between field margins and other habitats were greatest for cereals (Tukey contrasts: +21.03 ± 3.61; P < 0.01) and intermediate for alfalfas (+13.99 ± 3.61; P < 0.001) and fallows (+12.27 ± 3.61; P < 0.001). By contrast, during the pronounced 2014 outbreak, vole abundance increased in all habitats, including cereal crops. Again, differences were found between margins and other habitats (Tukey contrasts: Cereal crops: +24.87 ± 3.45; P < 0.001. Alfalfa crops: +12.20 ± 3.45; P < 0.01. Fallows: +16.83 ± 3.45; P < 0.001).

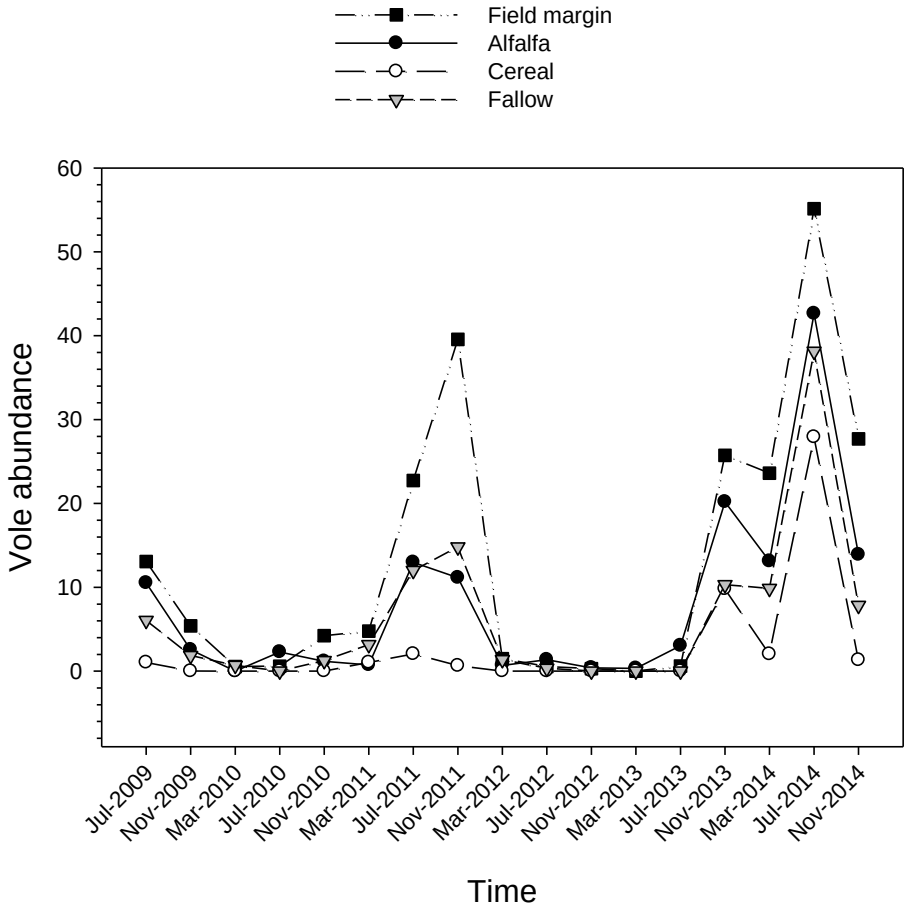

Figure 3. Temporal changes in common vole abundance (captures/100 traps/24 h) according to habitat type (margins = black squares; cereal = white circles; alfalfa = black circles; fallow = grey triangles) during the course of the study (July 2009 to November 2014). Abundance data are averaged for the three study areas and were collected every four months. Note the two population peaks (November 2011 and July 2014) when vole abundances were greater in field margins.

3.3. Vegetation characteristics of fields and margins

In our study areas, the width, vegetation height, cover and green cover of field margins averaged 3.1 ± 0.1 m, 35.7 ± 1.0 cm, 85.2 ± 1.0% and 47.2 ± 1.5%, respectively (*n* = 532). As expected, the seasonal variations in the vegetation characteristics of field margins were independent of the adjacent crop type (all crop type × season interactions were non-significant), given that margins are not cultivated. Vegetation height, cover and green cover of study fields averaged 18.7 ± 0.6 cm, 68.1 ± 1.4% and 61.5 ± 1.7%, respectively (*n* = 532). However, unlike with margins, these field vegetation characteristics showed important seasonal variations, depending on crop type (Fig. 2B–D).

The vegetation characteristics of margins and fallows were overall very similar in all seasons (Fig. 2B–D), except for vegetation height, which was greater in margins than in fallows in all seasons (Fig. 2B). Cereal field characteristics were highly seasonal and characterised by a reduced vegetation height (Fig. 2B) and cover in all seasons (Fig. 2B), high levels of green cover in spring, but a lack of green cover in summer (Fig. 2D).

In terms of green vegetation cover, alfalfa crops had high values all year round, and were greener than other habitats in summer, that is, during the drier months (Fig. 2C), when voles were also abundant in this habitat (Fig. 2A).

3.4. Spill-over: variation in vole abundance from field margins towards the inside of fields

We found that vole abundance declined exponentially with an increasing distance from the field margin towards the inside of fields. Such an edge effect was evident in all crop types (Table 1; Fig. 4) but its magnitude varied depending on crop types and seasons (see below). In all cases, the null models (no edge effect) or the linear models (linear decrease in abundance towards the interior of fields) were the least supported.

Table 1. Results of Generalized Linear Mixed Models (GLMMs) describing how vole abundance varied with increasing distance to the field margin. The null model included Season as the only fixed effect. All other models included as fixed effects Season, Distance and the interaction Season × Distance (see methods). The best models (lowest AICs) are highlighted in bold.

Crop type	Model	d.f.	AIC	ΔAIC
Alfalfa	Null	5	1685.84	62.88
	Linear	8	1643.17	20.21
	Exp. decay	8	1622.96	0.00
Cereal	Null	5	1188.62	319.80
	Linear	8	989.58	120.76
	Exp. decay	8	868.82	0.00
Fallow	Null	5	1440.26	76.26
	Linear	8	1400.76	36.76
	Exp. decay	8	1364.00	0.00

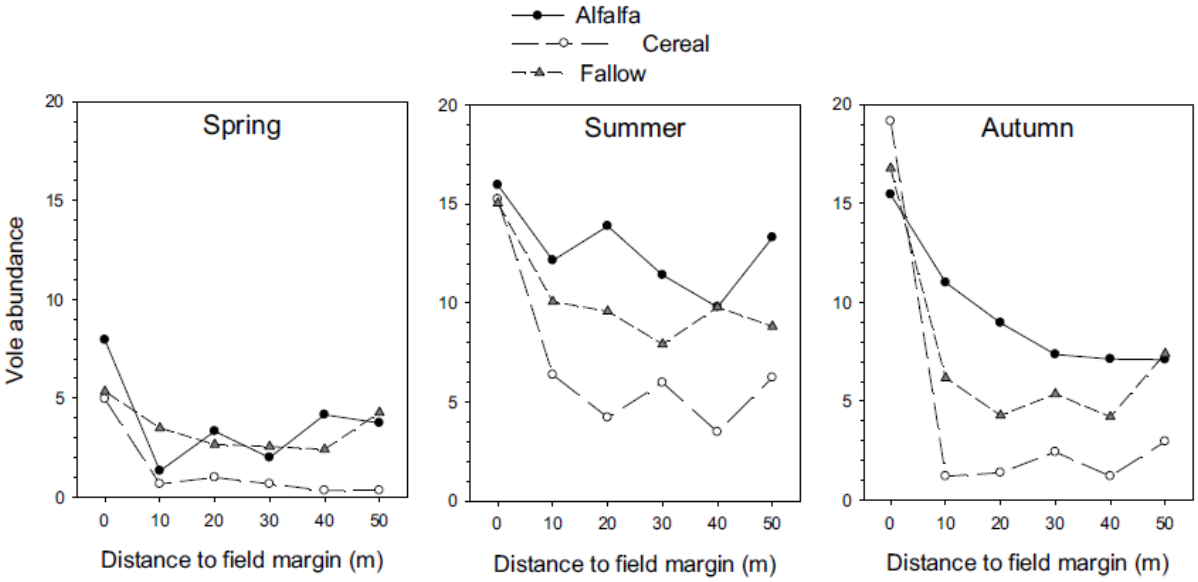

Figure 4. Average common vole capture rate (captures/100 traps/24 h) according to the distance to the field margin (m), season and crop type (alfalfa: black dots-solid line; cereal: white dotted-long dashed line; fallow: grey triangles, short dashed line).

For cereal crops, the best model included the exponential decay, indicating a strong edge effect (Table 1). The Season × exp[-distance] interaction was significant ($\chi^2 = 30.22$, d.f. = 2, $P < 0.001$), and slope parameter estimates comparisons among seasons indicated that the exponential decay in

abundance with increasing distance to the margin was stronger in autumn (slope ± se: 2.161 ± 0.439) and summer (1.830 ± 0.209) than in spring (0.644 ± 0.464).

For fallows, the best model also included the exponential decay (Table 1) and the Season × exp[-distance] interaction was significant (χ^2 = 7.61, d.f. = 2, P < 0.05). The exponential decrease in abundance with increasing distance to the margin was stronger in autumn (slope ± se: 0.635 ± 0.264) and summer (0.814 ± 0.176) than in spring (−0.112 ± 0.319).

For alfalfa, the exponential decay model was also supported, with a significant Season × exp[-distance] interaction (χ^2 = 10.27, d.f. = 2, P < 0.01). The decrease in abundance with increasing distance to the margin was stronger in spring (slope ± se: 0.843 ± 0.301) than summer (0.471 ± 0.158) or autumn (0.575 ± 0.238).

3.5. Proportional vole abundance in field margins according to overall abundance, crop type and season

The proportion of common voles captured in the margin as opposed to within the fields significantly varied with vole abundance depending on crop types and seasons (abundance × crop type × season interaction: χ^2 = 14.16, d.f. = 4, P < 0.001). We further explored these density-dependent patterns of margin use variation by season.

In spring, the proportional abundance of voles in margins depended on abundance and crop type (crop type × abundance interaction: χ^2 = 7.10, d.f. = 2, P < 0.05; Fig. 4), with a positive relationship in cereal (estimate ± se: 4.042 ± 2.113, n = 65), such that nearly all voles where in margin in spring at higher density, but there was no significant density-dependent relationships in alfalfa (0.036 ± 0.448, n = 65) or in fallows (0.320 ± 0.667, n = 65). When spring density increased, an increasing proportion of voles occupied the margins of cereal fields, but not of other crops (Fig. 5).

In summer, the proportion of common voles in field margins depended on vole abundance and crop type (significant interaction: χ^2 = 5.08, d.f. = 2, P < 0.01), with a negative relationship in cereal (estimate ± se: −1.590 ± 0.510, n = 100), but no significant relationship in fallows (-0.499 ± 0.312, n = 100) and a positive trend in alfalfa fields (0.562 ± 0.202, n = 100). When summer vole density increased, voles spilled over from the margins towards the inside of cereal fields, but no such density-dependent change occurred in fallow lands and alfalfa crops (Fig. 5).

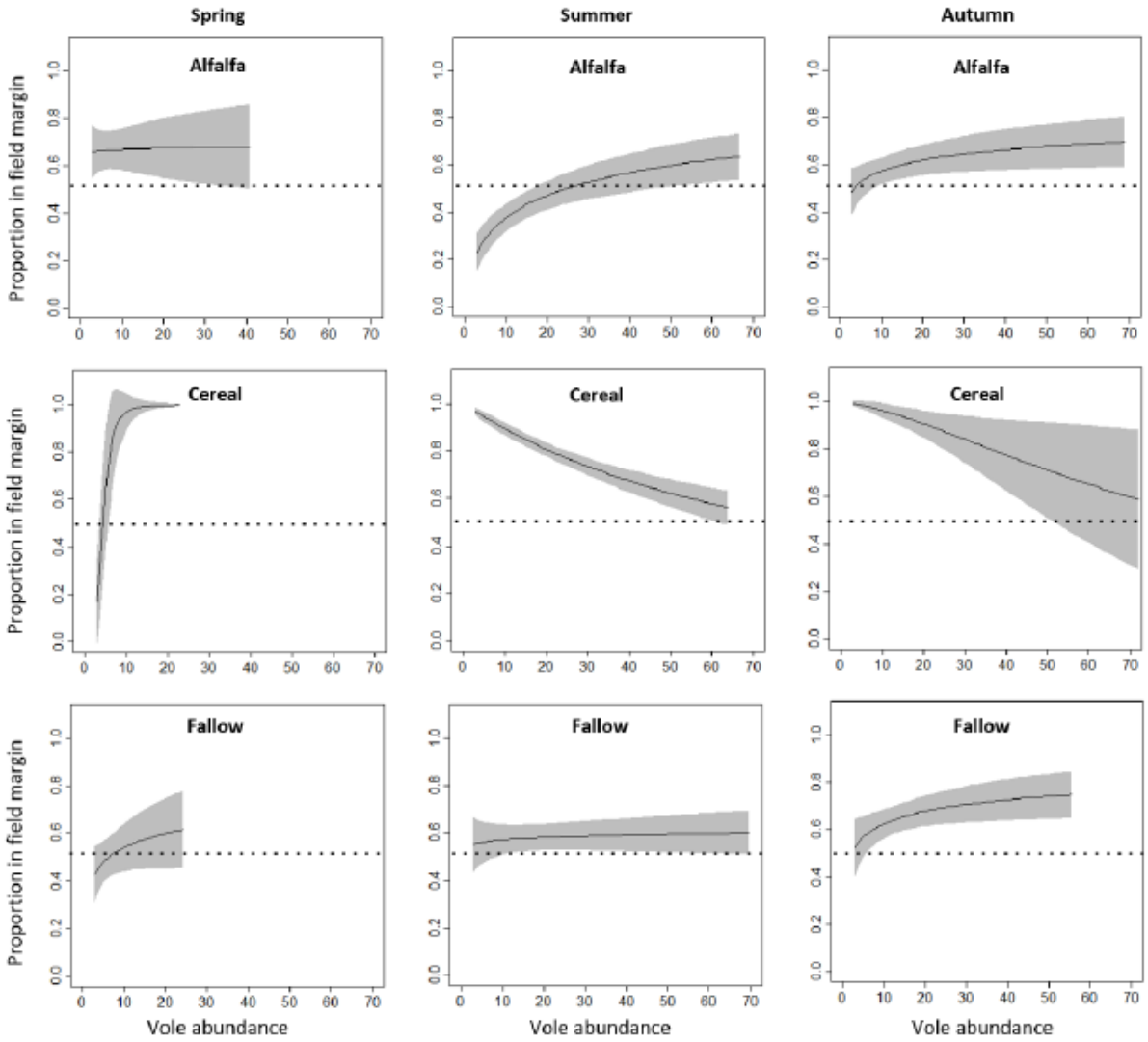

Figure 5. Proportional abundance of common vole in the field margins according to season, crop type and overall vole abundance (captures/100 traps/24 h). Grey shades denote 95% confidence intervals of the predicted curves. The horizontal dotted line indicates a proportion of 0.5 (equal abundance in the margin and in the field).

In autumn, the proportion of common voles in margins also depended on vole abundance and crop type (significant interaction: $\chi^2 = 3.78$, d.f. = 2, $P < 0.05$), with a negative relationship in cereal (estimate ± se: −1.779 ± 0.789, $n = 110$), and no relationship in alfalfa (0.274 ± 0.258, $n = 110$) or in fallows (0.063 ± 0.417, $n = 110$). As during summer, when autumn density increased, a decreasing proportion of voles occupied the margin of cereal fields, but no such density-dependent change occurred in alfalfa crops or fallows (Fig. 5).

3.6. Proportional vole abundance in the field margin according to vegetation characteristics

Using vole sampling occasions for which we measured vegetation characteristics (margin type and width, and vegetation height, cover and green cover of the margin and field), we further investigated whether these influenced patterns of margin use by voles in the two studied crops (alfalfa and cereal).

In alfalfa crops, the proportion of common vole in field margins varied significantly with vole abundance depending on season (abundance × season interaction: $\chi^2 = 71.70$, d.f. = 3, $P < 0.05$) and with crop height ($\chi^2 = 70.90$, d.f. = 1, $P < 0.01$), but not with other vegetation characteristics of the crops or margins. The proportional abundance of voles in the margins increased with decreasing vegetation height in the alfalfa field (estimate ± se: −0.0040 ± 0.0160, $n = 98$; Fig. 6).

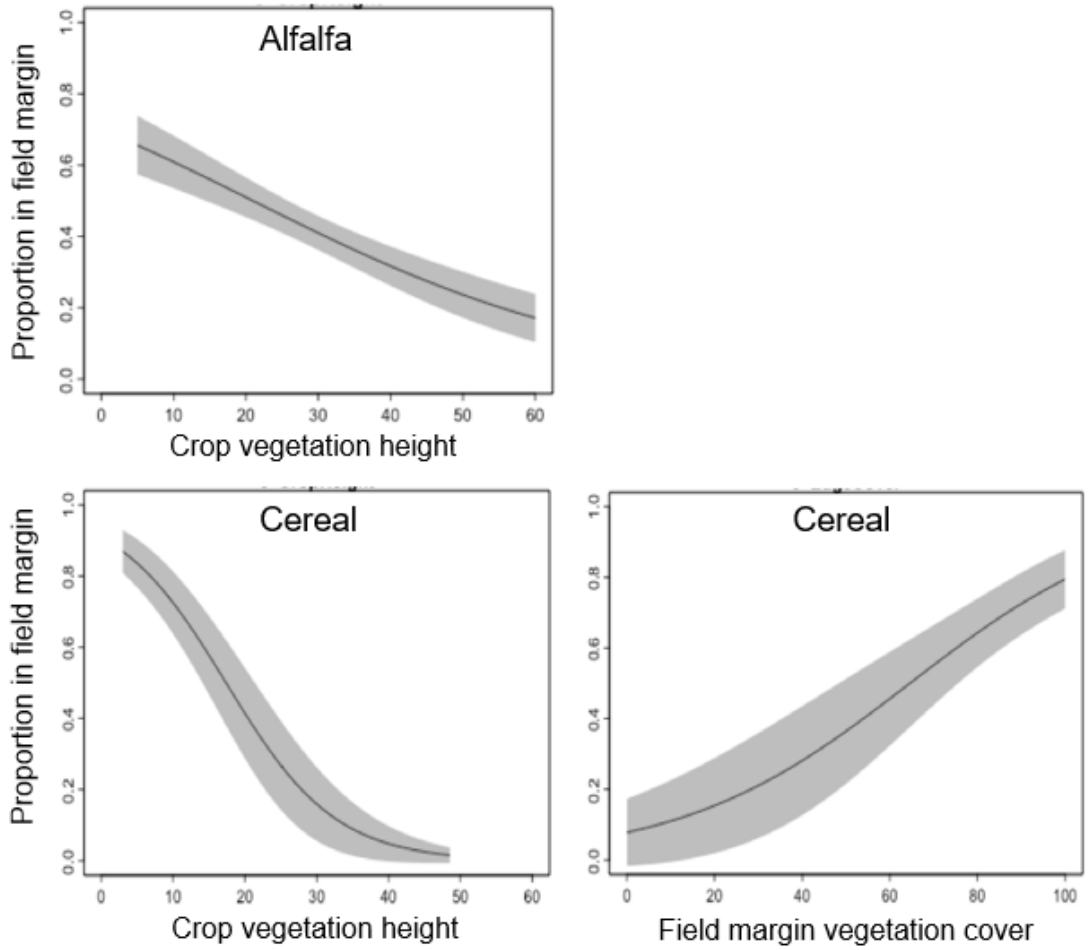

Figure 6. Proportional common vole abundance in the field margins according to the characteristics of the cropped fields (vegetation height, in cm) and of margins (vegetation cover, in %). Grey shades denote 95% confidence intervals of the predicted curves.

In cereal crops, the proportion of voles in field margins depended on vole abundance and season (significant abundance × season interaction: χ^2 = 22.26, d.f. = 3, P < 0.05), and was also explained by crop height (χ^2 = 23.35, d.f. = 1, P < 0.001), margin type (χ^2 = 22.22, d.f. = 2, P < 0.01), margin height (χ^2 = 18.69, d.f. = 1, P < 0.05) and margin cover (χ^2 = 19.12, d.f. = 1, P < 0.05). The proportion of common voles in field margins increased with increasing margin vegetation cover (0.0381 ± 0.0181, n = 65) and with decreasing vegetation height in the margins (−0.0332 ± 0.0176, n = 65) and cropped field (−0.1316 ± 0.0411, n = 65). Regarding differences between margin types, the proportional abundance in the margins was lower in ditches (0.76 ± 0.35, n = 35) than in flat margins (0.89 ± 0.13, n = 9) or in sloped margins (0.94 ± 0.12, n = 21).

4. Discussion

Field margins represent a key habitat for common voles in the Mediterranean agricultural landscapes of southern Europe. This is in agreement with other studies conducted in northern and eastern regions of Europe, where conditions are less arid and semi-natural habitats are also optimal habitats for voles (Briner et al., 2005; Butet et al., 2006; de Redon et al., 2010; Delattre et al., 2009). Our study is the first to highlight and quantify the use of field margins by *Microtus arvalis* in semi-arid Mediterranean farmland, where climatic conditions likely generate seasonal "bottleneck" periods, in particular the summer droughts that represent a major constrain for voles in terms of food availability and vegetation cover.

In intensive Mediterranean agricultural landscapes of NW Spain field margins are a relatively scarce habitat (less than 5% of the total agrarian surface), which nevertheless host disproportionately large abundances of common voles: about 2.3 times higher on average than within fields. The use of margins by common voles is dynamic and varied depending on crop type, season, and vole abundance, as well as according to vegetation characteristics of the margins. Remarkably, vole abundance in the margins of cereal crops was 8–9 times higher than in fields during spring and autumn. Considering our estimated vole abundances in margins vs. fields, and an estimated 5% of the agrarian surface corresponding to field margins (vs. 95% for fields), we could infer that margins host about 11% of the overall vole population in agricultural landscapes, although this varied depending on vole density (9–15%) and crop types (Fig. 3). In the case of the margins of cereals, the dominant crop in the region (48% of the landscape), those estimates would reach 30, 14 and 34% of the overall vole population of cereals in spring, summer and autumn, respectively. By contrast, the margins of alfalfa fields would host 12, 7 and 9% of the overall vole population of alfalfas in spring, summer and autumn, respectively.

Considering inter-annual variations in vole density, we observed that the greatest use of margins was for cereal crops during the (moderate) population peak of 2011, when margins hosted an estimated 53% of the overall cereal vole population. However, during the (large) population peak of July 2014, a much lower proportion of voles occupied the cereal field margins (c. 1%).

Common vole abundance in field margins varied seasonally and was low in spring (7.02 voles/100 traps/24 h), but still twice that found in the other habitats during that season. Abundance indices doubled by the beginning of summer (16.04 voles/100 traps/24 h) and reached the highest values in autumn (16.87 voles/100 traps/24 h). Such differences between margins and fields were observed in all habitats and have been also reported in central Europe (Janova et al., 2011), where vole abundance was always greater in field margins than in the other habitats, irrespective of the season. Field margins are key refuges in spring and, possibly, source habitats in summer and autumn, when voles move inside fields as density increases. This is particularly important for cereal crops that dominate the agrarian landscape and are particularly impacted in terms of crop damage during outbreaks. Seasonal cereal crops were found to be the least suitable habitat for common voles, with lower abundances and greater seasonal and density-dependent variations in abundance than in other habitats. The increase in vole abundance during summer and later in autumn was associated with a decrease in the proportion of common vole in margins. Again, this pattern suggests a source-sink dynamic between temporary and permanent habitats (Butet and Leroux, 2001).

A spill-over of common voles from margins towards the inside of fields was well modelled as an exponential decay in abundance with increasing distance from the margin (edge effect). This confirms a marked edge effect (Fig. 4), despite the possibility of vole movements from the margin to a distance of 50 m inside the field (dispersing voles can move 10–100 m per day; Boyce and Boyce, 1988). Importantly, the strength of this edge effect depended on common vole density, type of adjacent field and season. In general, when maximum population density was reached, common voles spread from the margin to the adjacent field. The edge effect appeared to be weaker in alfalfa crops and fallows as compared with cereal crops, and was strongest in cereal crops in summer and autumn. This likely reflected the impact of cereal harvesting on voles at the end of summer, with the associated drastic reduction of vegetation height and cover within crops; this may also likely be associated with an increased predation risk. In addition, ploughing and sowing in autumn typically destroys vole burrows though it is known that the extent of damages to common vole population depends on the depth of ploughing (Jug et al., 2008). Thus, seasonal agricultural practices required in cereal crops (i.e., tillage) should limit vole populations to field margins (Bonnet et al., 2013). Contrary to previous observations in agricultural landscapes of central Europe, where the highest common vole

abundances typically occur in cereals during spring followed by a decrease after harvesting in summer (Bonnet et al., 2013; Gauffre et al., 2008; Janova et al., 2011), we found that vole abundances were lowest in spring and increased during summer. This may be related to the timing of the intensive ploughing activity that is recorded by the end of autumn, which destroys burrows and can literally eradicate common voles at local scale (Jacob, 2003).

With the exception of field margins, alfalfa crops harboured the highest vole abundances. Alfalfa is a multiannual perennial crop that, in our study area, remains at least five years without being ploughed and are subjected to repeated (up to 4 on average) mowing (cuts) during summer. Consequently, alfalfas provide voles with a stable habitat for underground breeding colonies and enough protective cover against avian predators, and older alfalfa fields typically harbour greater vole densities (Babinska-Werka, 1979; Heroldová et al., 2007, 2004; Jacob and Hempel, 2003). Edge effects are expected to be greater for younger alfalfa fields (more colonization from the margins) than for older ones (with already established colonies inside the field). Unfortunately, we did not know the age of sampled alfalfa fields, so we cannot exclude the possibility that we detected stronger edge effects in spring and autumn because we may have sampled a greater proportion of young alfalfa fields then. Alfalfas also offer higher quality food (high protein content) than fallows or cereal crops (Janova et al., 2008; Lantová and Lanta, 2009), which also contributes to greater vole abundances. Alfalfa crops were the habitat with the highest percentage of green vegetation cover (80–‒90%) from spring to autumn, providing voles with year-round green food. In field margins, fallows and cereal crops green cover ranged from 30% to 64%, with the exception of cereal in summer that had almost no green cover (c. 4%). This would imply that, in this Mediterranean landscape, fresh food availability and soil stability of alfalfa crops are not only important in summer, but also in autumn. During both seasons, common vole abundance increases and, in some occasions, reaches outbreak situations (as in November 2011 and in July 2014 in our study areas). Fluctuations in abundance of common voles in alfalfa crops were greater than in fallows. This result is in accordance with those of Janova et al. (2008) who found that populations of common voles living in alfalfa crops reached higher abundances than populations in grasses or set-aside habitats.

Although fallows and field margins had similar vegetation characteristics, they had different seasonal vole abundances. Most of the studies conducted in European temperate farmlands suggest that the suitability of fallows' vegetation for wildlife is not as high as in margins, which generally hold greater plant biodiversity (Ernoult et al., 2013). In our study, however, we do not have the relevant data to evaluate this assumption. In farmland from central and northern Europe, fallows are considered as a suboptimal habitat for voles (Janova et al., 2008). The relatively lower vole

abundances that fallows harbour in comparison to alfalfa crops could be explained by differences in green vegetation cover, which is higher in alfalfa than in fallows, the preferences of voles for certain plants (annual or biannual plants from fallows versus protein-rich herbaceous perennial plants from alfalfa crops) and also, for some parts of the plant, such as green parts, buds and roots from alfalfa (Heroldová et al., 2005; Lantová and Lanta, 2009). Nevertheless, fallows also represented an attractive habitat for common vole (with abundances comparable to those of alfalfas at different voles densities; Fig. 3), particularly in spring when the vegetation height of the adjacent crops is lower and fallows remain un-mowed. Although fallows occur moderately within our study area (ca. 21% of the agricultural surface), they could act as reservoir habitats from winter to spring, in addition to field margins. Both of them provide variable scenarios of stable vegetation cover (protection against predators and a permanent food supply) and soil stability.

We also found that some vegetation characteristics of crop field affected the use of margins by common voles. The use of margins was greater when the vegetation height of cereal and alfalfa fields was lower, and was greatest in margins of cereal fields with greater vegetation cover. Vegetation cover and height are key determinants of predation risk and these observed patterns suggest that margins can be important refuges to avoid predation (Jacob and Brown, 2000). In our study, margin width averaged 3 m and did not seem to affect vole abundance, contrary to findings by Renwick and Lambin (2011) that pointed thresholds of margins width below which the vole densities quickly decrease. Finally, the topography of field margins influenced their relative use by voles, with proportionally fewer individuals in ditches than in flat or slope margins. This difference may arise because ditches usually become flooded after rainfall, mainly in winter and spring, and thus negatively affecting the survival of common vole colonies.

4.1. Management implications

The management of outbreaking common voles in farming landscapes implies understanding: (1) how their populations are numerically and spatially distributed across the landscape, (2) how such distribution changes seasonally and with density, and (3) how these changes are affected by vegetation characteristics. The integration of empirical knowledge about all these aspects should facilitate the development of more explicit and scientifically-informed vole management strategies in farmland ecosystems. Our results tentatively suggest that, if preventive vole outbreak management actions were to be implemented (e.g. chemical control, vegetation burning or removal by scrapping) in semi-natural habitats, these would be more effective if they targeted only the margins of cereal fields during early spring and autumn, thereby leaving fractions of semi-natural habitats unmanaged so that other important species can persist. Indeed, spring is the time when the relative use of this

habitat by voles is greatest, so targeting margin cereals in advance may contribute to reduce vole spill-overs inside fields later in summer. In our study area, the control of common voles during early outbreaks was based on chemical control campaigns at large scales, primarily using anticoagulant rodenticides. As frequently described in ecological scenarios holding chemical wars against rodents, the region also recorded major adverse toxicological effects on non-target species, including the secondary poisoning of endangered species (Sánchez-Barbudo et al., 2012). Alternative ecologically-based management actions have subsequently been promoted, such as the provision of nest-boxes to increase avian predation pressure on voles (Paz et al., 2013), deep ploughing of fields to destroy burrows, local flooding (whose effectiveness depends on field soil characteristics) or management actions on the field margins such as controlled burning and mechanical removal of soil or vegetation clearing (Caminero Saldaña et al., 2015). Most of the latter traditional management actions totally destroy vegetation (and sometimes soil horizons), affecting not only vole populations, but also numerous non-target species and biological communities (including legally protected and small game species). Field margins play a key functional role in the conservation of biodiversity in agrarian landscapes because their inter-connected webs of semi-natural habitats directly contribute to diversify agricultural mosaic systems, also enhancing the natural control of crop pests (Marshall et al., 2003). So the potential benefits of management actions on field margins (especially those that consider their physical destruction), in terms of reduced vole abundance, must be traded-off against potential adverse and cascading effects on other species also inhabiting these semi-natural habitats (and which may contribute to maintain vole numbers down).

5. Conclusions

The recent occurrence of common vole populations in Mediterranean agricultural landscapes of SW Europe, where severe water deficit periods could seasonally limit the species distribution comparing with northern European latitudes, implies understanding how the species is distributed and its population dynamics. Field margins, reduced to linear patches inter-connected inside agricultural landscapes, are key habitats for common vole distribution acting as source habitats. Their vegetation characteristics remain relatively constant along time, mainly as these habitats are not subjected to farming practises altering soils such as tillage. Consequently, at high vole densities, margins act as source habitats; on the other hand, cereal crops act as sink habitats, which in farming terms is important during summer when cereal crops are totally grown and mature. The role of alfalfas as key crop habitat for common vole populations is also confirmed in Mediterranean agricultural landscapes. Despite of being mowed several times per year alfalfas typically hold well-established vole colonies

over long periods of time, indicating: (1) that voles are not limited by the seasonal mowing of above ground plant parts (i.e., soil stability is putatively most relevant), and (2) that this high-protein fodder crop can effectively act as a source habitat for common vole populations across European farmlands.

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CHAPTER 2

HOST-PATOGHEN INTERACTION

CHAPTER 2.A

FRANCISELLA TULARENSIS

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Tularemia Outbreaks and Common Vole (*Microtus arvalis*) Irruptive Population Dynamics in North-western Spain, 1997-2014

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Running title: Tularemia and Vole Outbreaks in Spain

Abstract

During last decades, large tularemia outbreaks in humans have coincided in time and space with population outbreaks of common voles in North-western Spain, leading us to hypothesize that this rodent species acts as a key spillover agent of *Francisella tularensis* in the region. Here, we evaluate for the first time a potential link between irruptive vole numbers and human tularemia outbreaks in Spain. We compiled vole abundance estimates obtained through live-trapping monitoring studies and official reports of human tularemia cases during the period 1997-2014. We confirm a significant positive association between yearly cases of tularemia infection in humans and vole abundance. High vole densities during outbreaks (up to 1000 voles/ha) may therefore enhance disease transmission and spillover contamination in the environment. If this ecological link is further confirmed, the apparent multi-annual cyclicity of common vole outbreaks might provide a basis for forecasting the risk of tularemia outbreaks in North-western Spain.

Keywords: Tularemia, *Francisella tularensis*, Common vole, *Microtus arvalis*, Outbreaks, Spain

1. Introduction

Tularemia is caused by the etiological agent *Francisella tularensis*, a highly infectious gram-negative zoonotic bacterium that is known to affect more than 250 animal species (Mörner 1992). *F. tularensis* subsp. *holarctica* (Type B) is the only subspecies found in Europe, where lagomorphs and rodents are the main putative mammalian reservoir hosts and haematophagous arthropods play a role as vectors and hosts (Mörner 1992, Gratz 2006). In Europe, *F. tularensis* infections frequently appear as epidemic outbreaks although these are usually not linked to vector transmission (Gratz 2006). Besides the relevance of this pathogen, listed as a Class A biothreat agent (Ellis et al. 2002), its epidemiology remains poorly understood (Gyuranecz et al. 2012).

In Spain, tularemia has been a notifiable disease since 1997. Two large outbreaks have been declared in North-western Spain during 1997-1998 and 2007-2008, accumulating over 1000 confirmed human cases. Exactly the same *F. tularensis* subsp. *holarctica* genotypes caused tularemia in both outbreaks, indicating that reemergence of the disease in Spain resulted from persistence of the pathogen in the environment rather than the reintroduction of exotic strains (Ariza-Miguel et al. 2014). While the 1997-1998 outbreak was mainly associated with handling of hunted hares (*Lepus spp.*), the second one (2007-2008) has been considered to occur in a "*different epidemiological context*", its timing coinciding with a large population outbreak of common voles (*Microtus arvalis*) (Ariza-Miguel et al. 2014). Here, we point out that recently published evidence indicates that both the 1997-1998 and 2007-2008 tularemia outbreaks coincided in time and space with common vole population outbreaks in North-western Spain (Luque-Larena et al. 2013). A further, contemporary increase of human cases of tularemia (2014) also coincides with a major ongoing common vole outbreak in the region. This leads us to hypothesize that there is a causative link between irruptive vole numbers and human tularemia outbreaks in North-western Spain, as also suggested in similar ecological systems of central Europe, such as in Eastern Hungary (Gyuranecz et al. 2012). Here, we use 18 years of data on vole abundance and declared tularemia cases in North-western Spain (1997-2014) to test for such a link between fluctuating common vole abundance and tularemia outbreaks. If *M. arvalis* acts as a spillover agent in agrarian ecosystems of North-western Spain, increases in vole abundance should be temporally associated with increases in human tularemia declared infections.

2. Materials and Methods

To reconstruct annual fluctuations in common vole abundance in North-western Spain (Castilla-y-León) during 1997-2014, we used information from two complementary long-term studies that monitored vole abundance through live-trapping methods: i) published data from one population located in the Segovia province (1997-2007; Fargallo et al. 2009), and ii) data from our own monitoring of three vole populations in "Tierra de Campos" (Palencia, Valladolid and Zamora provinces; 2007-2014). "Tierra de Campos" is an agricultural region in Castilla-y-León where both vole and tularemia outbreaks have been most often recorded (Luque-Larena et al. 2013, Fig. 3 in Ariza-Miguel et al. 2014).

The peak vole abundance years in Segovia were confirmed to be synchronous with vole outbreak years in "Tierra de Campos" (Luque-Larena et al. 2013). Because vole abundance estimates from both studies were obtained with slightly different trapping methods, we standardized both data sets (mean = 0, variance = 1) before combining them to obtain a vole abundance time-series for 1997-2014 that can be compared with annual reports of human tularemia cases.

We used reports from the "National Network of Epidemiological Surveillance" (*Red Nacional de Vigilancia Epidemiológica*) of Spain, managed by the National Center of Epidemiology in Madrid, to compile data on the number of accumulated human cases of tularemia each year during 1997-2014 (updated until the end of November 2014). We selected only cases declared in the Castilla-y-León region. We cross-correlated the vole abundance and tularemia cases time series using the PAST software (http://palaeo-electronica.org/2001_1/past/issue1_01.htm).

3. Results

Annual variations in standardized vole abundance and numbers of declared human tularemia cases in Castilla-y-León during 1997-2014 are shown in Figure 1a. Vole abundance greatly fluctuated, with marked peaks in 1997-98, 2007 and 2014, and lesser peaks in 2004 and 2011. Annual tularemia cases ranged from 0 to 585, and also peaked during vole outbreak years (1997, 2007 and 2014, respectively). The cross-correlation analysis confirmed a significant positive association between yearly numbers of human tularemia cases and vole abundance, with no time lag ($r=0.495$; $n=18$; $P<0.05$; Fig 1b).

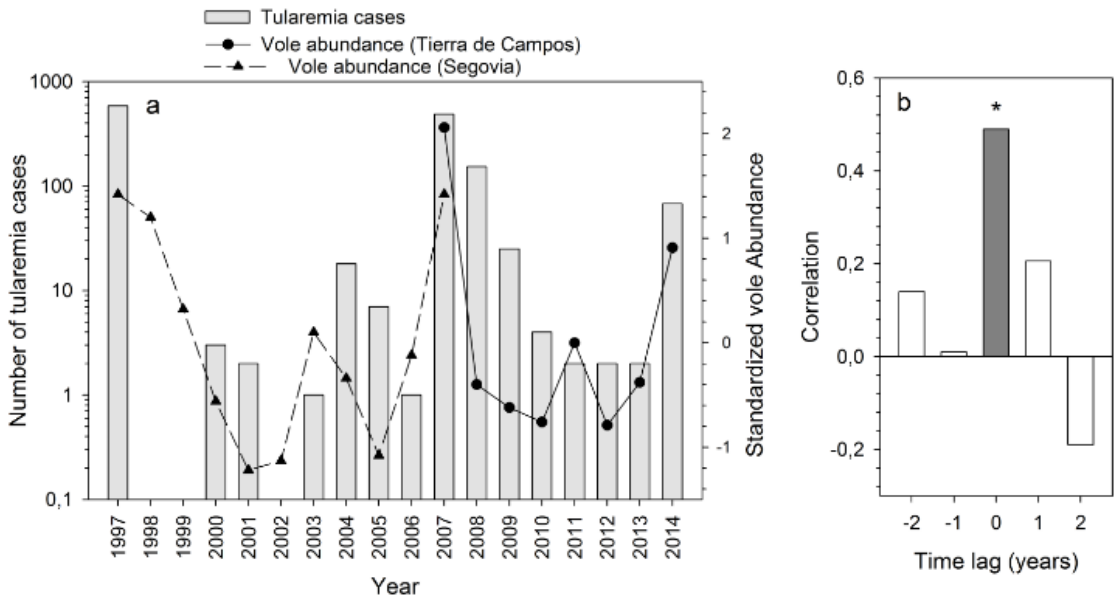

Figure 1. (a) Annual variations in the number of human tularemia cases reported in Castilla-y-León (grey bars; note the log-scale) and standardized vole abundances in Segovia (black triangles, dotted line) and Tierra de Campos (black circles, continuous line; see methods); **(b)** Correlations between number of tularemia cases (log-transformed) and standardized vole abundance during 1997-2014 at different time lags (-2 to +2 years). The asterisk indicates the significant (P<0.05) correlation.

4. Discussion

We showed for the first time a temporal association between multi-annual fluctuations in common vole abundance and human tularemia cases in North-western Spain during the last 18 years, supporting our hypothesis that voles may be acting as a spillover agent during outbreak years. *M. arvalis* is highly susceptible to *F. tularensis*, and disease transmission and spillover contamination of the environment may be enhanced at high vole densities (Gyuranecz et al. 2012). In fact, recent studies have experimentally supported a role for voles as amplification hosts of *F. tularensis* (Rossow et al. 2014). Since the early 1980s, common voles have rapidly and completely colonized agricultural landscapes in North-western Spain, favored by a large increase of irrigated land (Luque-Larena et al. 2013). *F. tularensis* subsp. *holarctica* is reported to have a mainly water-borne cycle with rodent species linked to aquatic habitats in the Northern Hemisphere (Mörner 1992). This fits well with its presence in irrigated crops where common voles are mainly found in North-western Spain (Luque-Larena et al. 2013).

As recently suggested by Ariza-Miguel et al. (2014), the different clinical forms recorded during tularemia outbreaks in 1997-1998 and 2007-2008 (game handling and inhalation, respectively) may have been determined by ecological processes involved in infection. In Europe

F. tularensis is frequently seen in hares (*Lepus spp.*), which can constitute a reservoir for the disease between epizootics (Gyuranecz et al. 2012). Human infection in Spain is commonly associated with handling of hares and crayfish, mainly *Procambarus clarkii* (Anda et al. 2001), that inhabit the same ecosystems as *M. arvalis* and are authorized game and fishing species respectively. Thus, contacts between humans and these species are more likely than with voles. In Eastern Hungary, human cases are highly correlated with *F. tularensis* seroprevalence in hares; interestingly, seroprevalence in hares correlated positively with common vole abundance, but negatively with hare abundance (Gyuranecz et al. 2012). The fact that the same genotype was isolated from hares, voles and humans during tularemia outbreaks in North-western Spain (Ariza-Miguel et al. 2014), further supports a role of voles as a spillover agent in the system.

Iberian hares have dramatically declined in numbers during the last decades (Duarte 2000), and this may explain the scarcity of hare-related human infections in recent outbreaks (i.e., during 2007-2008) (Allue et al. 2008). High prevalence of *F. tularensis* in *M. arvalis* was documented during the 2007-2008 tularemia outbreak in those same areas (Vidal et al. 2009). During the 2007-2008 outbreak crop harvest occurred when there were many dead voles on the ground (Vidal et al. 2009), which may have facilitated the airborne transmission of the bacteria (consistent with inhalation being then the main form of human infection) (Ariza-Miguel et al. 2014).

We conclude that, due to their irruptive dynamics and high densities (up to 1000 voles/ha) during outbreaks, common voles are the likely key spillover agent in the ecosystems of North-western Spain (Luque-Larena et al. 2013). Preventing future tularemia outbreaks should focus on unraveling and managing the ecological cycle of *F. tularensi*s, and this includes understanding the relative role that keystone organisms with irruptive population dynamics, such as common voles, play within ecosystems. If this ecological link is further confirmed, the apparent multi-annual cyclicity of vole outbreaks might provide a basis for forecasting the risk of tularemia outbreaks in North-western Spain.

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CHAPTER 2.B

FRANCISELLA TULARENSIS

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Density-dependent prevalence of *Francisella tularensis* in fluctuating vole populations in NW Spain

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Abstract

Occurrence of tularemia in humans in NW Spain is linked to vole outbreaks. Prevalence of *F. tularensis* in common voles increased with vole abundance during a population fluctuation, reaching 33% at peak. This confirms voles as spill-over agents during outbreaks and the need to consider ecological interactions for tularemia prevention.

Emerging infectious diseases of zoonotic nature are rising worldwide and most zoonoses are linked to wildlife (1-2). Quantifying disease prevalence in potential wildlife hosts is thus critical to understanding the outbreak dynamics of zoonoses (3).

Tularemia, caused by *Francisella tularensis*, is a problematic zoonotic disease worldwide but its ecology remains poorly understood. This pathogen is classified by the CDC as a Class A bio-threat agent (*F. tularensis* subs. *holarctica* in Europe) since only a few bacteria are needed to induce tularemia in humans or susceptible animal species (>250 hosts described) (4). Yet, the relative epidemiologic roles (i.e., reservoir, spill over and amplification agents) of different hosts are uncertain. An important hotspot for tularemia in Europe occurs in NW Spain (Castilla-y-León region), where the largest recent outbreaks of the disease have been recorded (>1,000 officially confirmed human cases in 1997-98 and 2007-08) (5). In intensive European farmlands, rodents and lagomorphs are the main putative mammalian hosts (5-6), but most studies addressing the epidemiological roles of these species have been correlative or used opportunistic sampling. Recent work suggests that common voles (*Microtus arvalis*) are a key agent in NW Spain, based on a spatial and temporal coincidence between human tularemia cases and the occurrence of vole outbreaks (5). Voles periodically fluctuate in density and can reach high abundances during outbreak years in farming landscapes (5). Dead voles infected with *F. tularensis* subs. *holartica* have been reported in the region during the collapse phase of population outbreaks (7). If, as previously hypothesized, common voles are a key amplifying and spill over agent of the disease in intensive-farming landscapes of NW Spain (5), we should expect an increased tularemia prevalence in voles as their numbers rise. It is thus crucial to empirically evaluate whether such density-dependent pattern occurs in natural populations.

Here, we use samples from live voles periodically collected throughout a complete population outbreak (2013-2015) in NW Spain to determine how prevalence of *F. tularensis* in common voles varies with population density.

The Study

Between 2013 and 2015, a common vole fluctuation, peaking in 2014, was recorded in agricultural landscapes of Castilla-y-León (8). This vole outbreak was moderate (in terms of peak density) compared with previous outbreaks when tularemia outbreaks among humans were also officially declared (1997-98 and 2007-08) by the National Network of Epidemiological Surveillance of Spain (5, 8). In 2014, no outbreak of tularemia was declared, but a higher-than-average number of identified cases of tularemia among humans (n = 95) occurred in the area

(the regional average cases/year is 3 (range 0-11), excluding outbreak years) (5). To monitor vole abundance during the complete population fluctuation, we sampled 80-km^2 of farmland located in Palencia province (42°01´N, 4°42´W), where human tularemia cases have previously occurred (5, 8). We live-trapped voles seasonally (every 4 months) from March 2013 to March 2015. Our vole trapping effort was constant (840 traps set for 24h per seasonal sampling), and our sampling design was spatially stratified (we randomly sampled 8 alfalfas, 8 cereals and 8 fallows at each seasonal sampling). Vole abundance was estimated as the number of captures/100 traps/24h in each season. Trapping was extractive and animals were brought alive to the lab in rodent cages provided with food, water and bedding immediately after their capture. At the lab, voles were euthanized with medical CO_2; subsequently carcasses were individually frozen at -30° C. DNA was extracted from a homogenized mix of liver and spleen (ca. 25 mg). DNA extraction was conducted using standard procedures (QIAamp® DNA Mini Kit, Qiagen). A phylogenetically informative region of lpnA (231 bp) was amplified by conventional PCR and further hybridization with specific probes by reverse line blotting (RLB) as previously described (9). Positive samples were further tested using a real-time multi-target TaqMan PCR, using tul4 and ISFtu2 assays (10). A negative PCR control as well as a negative control for DNA extraction were included in each group of samples processed. We used R 3.2.4 for statistical analyses.

We tested 243 live voles and found an average prevalence of *F. tularensis* of 20.16%. Prevalence greatly varied between samplings (range 0-33%), and was strongly related to vole abundance (Binomial GLM, Chi^2=21.64; df=1, p<0.001), with a direct and positive density-dependent association (Figure 1a, b). The predicted odds of tularemia infection increased by 1.037 (95% confidence interval: 1.021-1.056) when vole density increased by +1 captured vole/100 traps/24h (range during the study 1 - 60 voles/100 traps/24h). During the vole population peak in July 2014, 33% of sampled live voles (n = 102) were infected with *F. tularensis*.

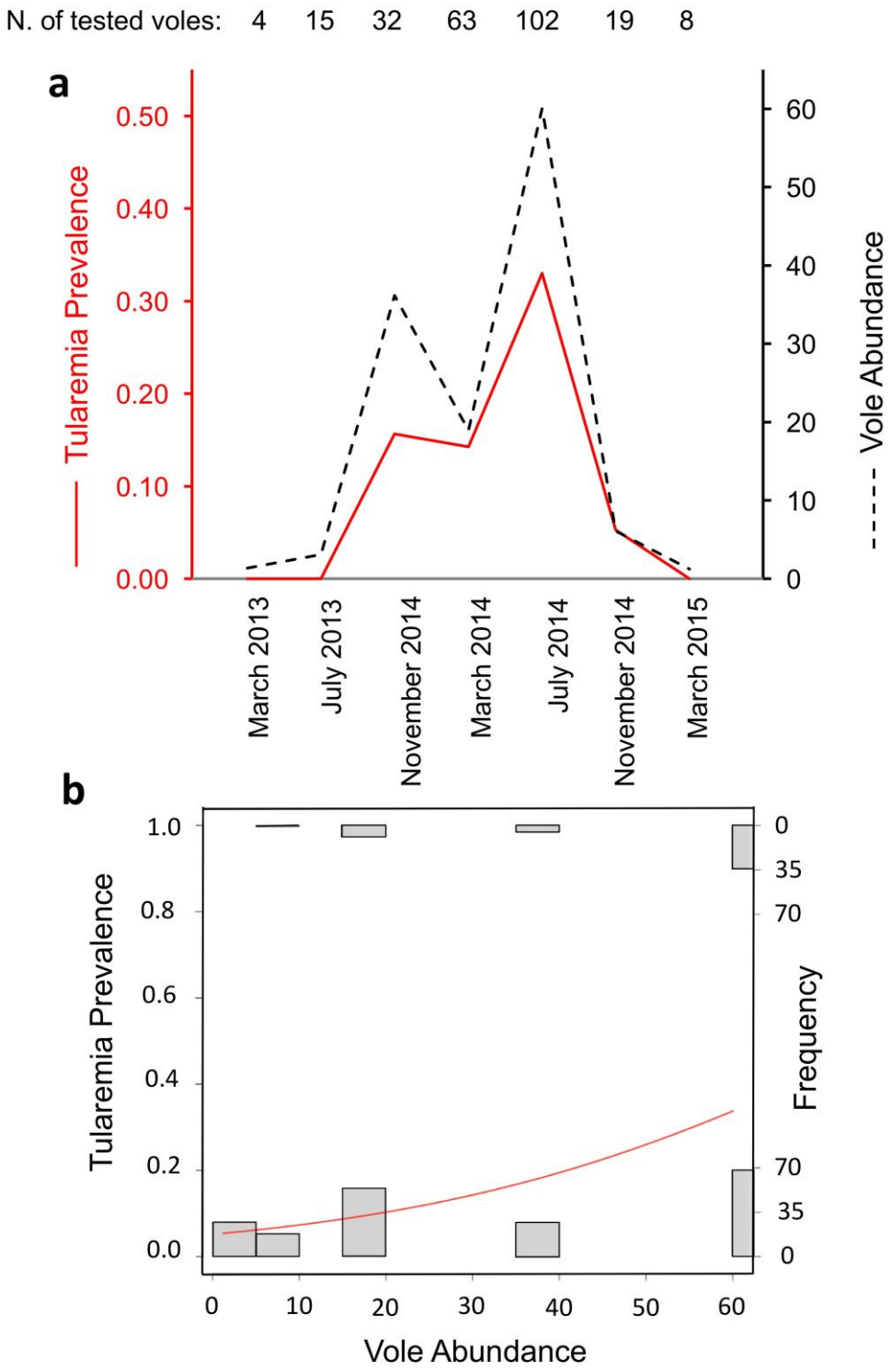

Figure 1. (a) Temporal variations in vole abundance (number of captures/100 traps in 24h; dashed black line) and in tularemia prevalence in voles (red line); **(b)** Relationship between tularemia prevalence and vole abundance. The histograms show the number of positive (top) or negative (bottom) voles sampled at each level of vole density. The red line shows the model result.

Conclusions

We report a direct and positive density-dependent association between prevalence of *F. tularensis* in common voles and their abundance in agricultural landscapes. This is consistent with vole-to-vole transmission and amplification of the bacterium as vole density increases. Voles experimentally infected with *F. tularensis* die within a few days following a rapid acute infection, and generally with very high bacterial loads in organs (11). Transmission between voles may thus involve direct contact, cannibalism or contamination of the environment. In our study all the tested voles were alive and free of obvious signs of disease when captured, implying that prevalence could be higher than estimated here if moribund voles were less trappable, and under-represented in the trapping-based samples. The role that exogenous sources might play (i.e. other animals, environmental sources) in modulating infection prevalence among vole populations still needs to be clarified. Notwithstanding and irrespective of the precise mechanism(s) of transmission, our results support the hypothesis that the exponential growth of common vole populations is crucial for the amplification of the disease transmission in intensive farmlands, and that vole outbreaks are linked with the periodic emergence of human cases of tularemia in Spain (5). Vole density can reach >1,000 voles/ha (i.e. >300 tularemia-infected voles/ha) during outbreaks, potentially leading to the contamination of the environment and other wildlife including harvestable species such as crayfish and hares which have higher contact rates with humans than voles (5).

Tularemia is probably not completely enzootic in vole populations, as we did not detect *F. tularensis* at very low vole densities, suggesting the involvement of animal or environmental reservoirs. Indeed, a key unknown facet of the ecological cycle of *F. tularensis* is where does it persist between epizootic periods (5-6). There is no evidence of *F. tularensis* replication in arthropods, although ticks may represent a true reservoir of this pathogen because they remain infected lifelong after they have been infected. In any case, mammal populations are probably needed to amplify the disease in the environment (11). The characteristic spatial and social behaviours of voles during outbreaks, including elevated contact rates and increased aggression and wounding, readily account for disease amplification transmission rates and spread across the landscape (5, 12). While reservoir and vector hosts of *F. tularensis* occurring at variable densities can play distinct contributions to the ecological cycle of tularemia in different ecosystems, there appears to be a common link between tularemia outbreaks and rodent fluctuations across Europe (5-6, 11, 13).

Common voles are important targets for the surveillance of this wildlife disease in the region, and strategic prevention should incorporate their temporal fluctuations in planned preventive actions. Since vole numbers seem to modulate the risk of disease exposure to humans, monitoring their population dynamics can help anticipate and increase awareness of the risk of tularemia in rural areas of Spain.

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CHAPTER 2.C

FRANCISELLA TULARENSIS

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Irruptive mammal host populations shape tularemia epidemiology

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1. Host population dynamics are the key of wildlife zoonotic risk

Infectious diseases affecting humans and involving rodents are rising and ubiquitous. One of every ten rodent species is a zoonotic host of up to 244 zoonotic pathogens, including bacteria, viruses, helminths and protozoa [1]. Muroid rodents (rats, mice, voles, gerbils, hamsters) account for 25 % of extant mammal species and their high reproductive output and rapid population turnover make them highly permissible amplification agents of zoonotic pathogens [1]. Many muroid populations show strong rates of increase and high amplitude multi-annual fluctuations in abundance ("population outbreaks"), spanning 2-3 orders of magnitude. The prevalence of zoonotic pathogens is claimed to be higher in populations that experience outbreaks [1]. Where zoonotic host populations fluctuate in size, considering how such fluctuations contribute to variation in zoonotic disease risk is paramount [2].

Variation in transmission efficiency underpins the dynamics of pathogens [3]. Zoonotic pathogens are often harboured by multiple vector and reservoir species. A precise knowledge of the life cycle and zoonotic transmission routes, and of their variation with host abundance, is therefore essential to understand the dynamics of zoonotic diseases. Yet, surveying the temporal changes in abundance of a few species may suffice to predict zoonotic risk changes. For instance, consideration of changes in the numbers of key hosts and vectors is integral to prevention strategies for zoonotic cholera, dengue, west Nile virus, Hantavirus or Lyme disease [4]. Rapid population growth in such key species translates into a subsequent increased infection risk to humans. It is thus a research priority to acquire basic epidemiological information about how temporal changes in host abundance modulate zoonotic risk for those wildlife-derived zoonoses that show episodic outbreaks in humans [5].

One infectious disease with highly variable incidence in Europe is tularemia, caused by the etiological agent *Francisella tularensis* subs. *holarctica*, a facultative intracellular gram-negative bacterium of extremely high-infectivity and listed as a Class A bio-threat agent by the CDC. More than 15,000 human cases have been reported during 1997-2013 [6], most of which during discrete outbreak episodes separated by inter-epizootic periods. Up to 250 different animal species are susceptible to infection by *F. tularensis* [7] but empirical evidence about transmission routes remains limited. Novel insights from southern Europe may however shed light on the dynamics of this highly infectious zoonotic pathogen.

2. Aquatic and terrestrial agents of tularemia coexist in nature

It has recently been suggested that tularemia has both a terrestrial and a distinct aquatic life cycle in Europe, owing to terrestrial and aquatic organisms having been implicated as vectors of transmission to humans [6]. The former involves primarily lagomorphs (rabbits, hares), terrestrial rodents and ticks, whereas the aquatic cycle involves mosquitoes and their larvae, as well as semi-aquatic rodents [6]. Recent evidence from Spain [8, 9] where both hypothetical life cycles are said to occur [6] is however compatible with a single, more unified life cycle including coexisting zoonotic hosts and either terrestrial or aquatic amplification (Fig 1).

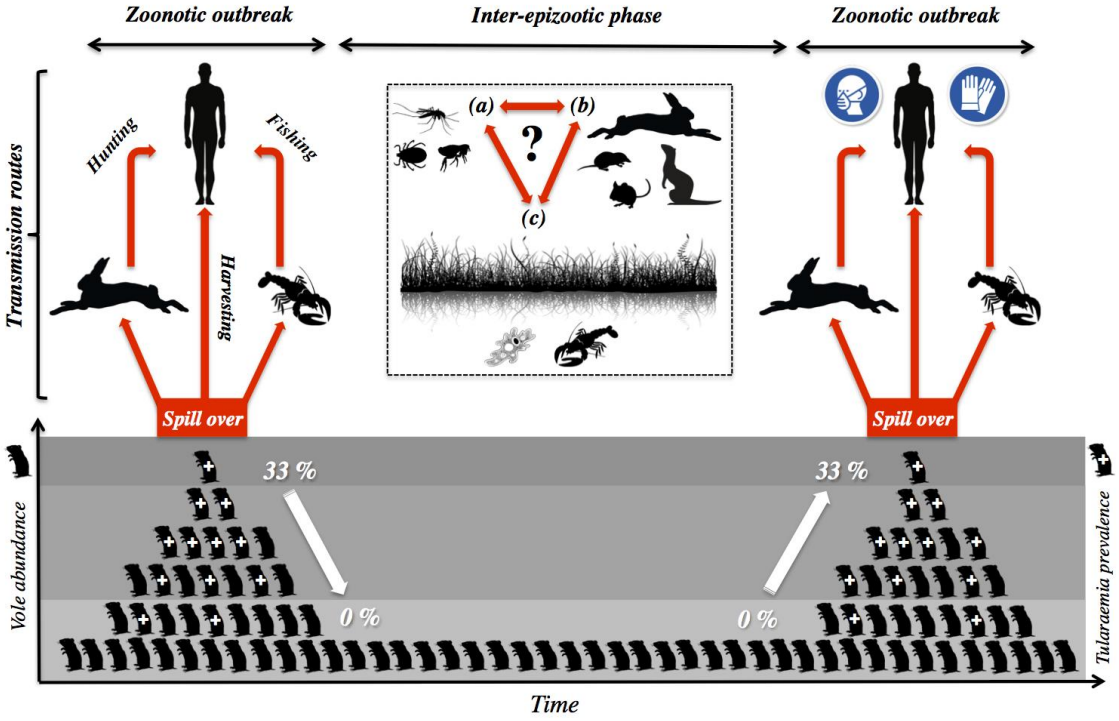

Figure 1. Dynamics of tularemia outbreaks in NW Spain.

Common voles (*Microtus arvalis*) are key agents for this disease in NW Spain (Castilla-y-León region), where outbreaks of tularemia among humans are endemic in farming landscapes since 1997 (>1,300 cases between 1997-2016). Voles have been identified as a main spill over and amplification agent of tularemia because epizootic and epidemic episodes coincide in time and space with vole outbreaks. When the rodents reach peak densities (>1,000 voles/ha), up to 33% of them are infected with tularemia. Therefore, as vole numbers increase so does the bacterium in the environment. Transmission routes of tularemia to humans during zoonotic outbreaks include: (i) direct contact with wildlife species such as hares or crayfish, which coexist with voles in the same habitats, and (ii) trough inhalation during the harvesting of vole-infested crop fields. At low vole densities the bacterium is not found among the rodents, indicating that, between vole outbreaks, populations of *Francisella tularensis* subs. *holarctica* may remain at lower numbers associated with some yet-unknown reservoirs. Enzootic cycles in other local wildlife than voles, including hematophagous arthropods (a) and other small and medium-sized mammals (b), may also contribute to sustain the bacteria in the environment during inter-epizootic periods. Water is a main habitat for reservoir candidates (c), as it is a well-known favourable habitat for tularemia (most especially in these semi-arid landscapes of NW Spain).

Human tularemia is endemic in Spain, with 1,386 clinical cases described between 1997 and 2016 by the National Network of Epidemiologic Surveillance of Spain (Red Nacional de Vigilancia Epidemiológica – RENAVE, Instituto de Salud Carlos III, Madrid). Virtually all cases (> 1,300) have been described in the region of Castilla-y-León, NW Spain. Additionally, an isolated outbreak (19 cases) of human ulceroglandular tularemia was reported in central Spain in 1998 [10]. The latter was associated with manipulation of non-native crayfish (*Procambarus clarkii*) in a water reservoir, consistent with a role for aquatic zoonotic vectors. Most instances of human-acquired tularemia in Castilla-y-León occurred during two larger outbreaks recorded in 1997-1998 (585 human cases) and 2007-2008 (639 human cases) (RENAVE), and were associated with: (i) contact with Iberian hares (*Lepus granatensis*) or common voles (*Microtus arvalis*) (ulceroglandular and glandular forms) (71 % of cases in 1997-1998), and (ii) inhalation during harvesting of crops invaded by common voles (pneumonic and typhoidal forms) (65 % of cases in 2007-2008) [11]. In 2014, 95 human cases of tularemia were also confirmed in Castilla-y-León coinciding with a regional increase of vole numbers [8]. Terrestrial vectors such as voles and hares evidently transmit this zoonosis, but a human clinical case involving aquatic crayfish handling was also described in the same region in 2001 during an inter-epizootic period [12], implying that the bacterium is also present in water. Therefore, both aquatic and terrestrial agents of tularemia coexist in nature in NW Spain.

3. Irrigation has provided aquatic reservoirs and a grass-loving amplification agent for tularemia in Spain

The climate of Castilla-y-León features hot and dry summers and a hostile environment for the survival of *F. tularensis* on land [13]. Mesic habitats also restrict the abundance of terrestrial reservoirs such as ticks. *F. tularensis* can however survive in water [13], including through the parasitism of protozoans that act as reservoir hosts [14]. Indeed, proximity to water is associated with higher incidence rates of tularemia in northern Europe [15]. Crucially, the bacterium is not amplified in water and its life cycle requires mammalian hosts for amplification [13, 16]. The surface area of irrigated crops doubled in the agro-ecosystem of Castilla-y-León between the 1970s and 1990s [17], prior to the local emergence of tularemia [8, 11]. The extensive network of irrigation canals and ditches not only provides suitable conditions for an aquatic persistence of tularemia, but the presence of irrigated crops has also triggered the colonization of millions of hectares of hitherto unoccupied habitats by common voles [17]. Common voles have been identified as a main spillover and amplification agent of tularemia because: (i) epizootic and

epidemic episodes coincide in time with vole outbreaks [8], and (ii) at peak density (>1,000 voles/ha), up to 33% of live voles are infected with tularemia [9]. What was an inhospitable semi-arid landscape has become a suitable environment for the spread and maintenance of tularemia as an endemic disease in Castilla-y-León.

4. Fluctuating mammalian populations shape tularemia epidemiology

It has long been accepted that fluctuations in the abundance of wild herbivorous mammals (hares, voles) play a key role in tularemia epidemiology in European countries accumulating the largest numbers of clinical cases (i.e., Sweden, Finland, Czech Republic, Hungary, Spain) [6, 8, 14, 16, 18]. Irrespective of whether human infection is vectored by ticks or mosquitoes, contact with harvested fish and game or contaminated water air or food, epidemics coincide temporally with increases in the abundance of a *F. tularensis*-mammalian host. In Sweden, peaks in vole and hare populations and outbreaks of tularemia in humans were simultaneously recorded during the 1960s and 1970s [18]. In the Novosibirsk region (Russia) the number of human cases of tularemia was also correlated with the density of the water vole population between 1956 and 2000 [19]. The high amplitude multi-annual fluctuations in the abundance of muroid rodents and hares are wholly consistent with irruptive increases of tularemia prevalence among these vector hosts leading to rapid amplification of the bacterium and contamination of the environment as hares and voles succumb to tularemia.

There is also evidence that the contribution of lagomorphs and rodents may change over time according to their abundance. In Saskatchewan (Canada), contact with lagomorphs was the common route for human infection before the 1950s, while rodents became of greater importance afterwards [20]. Extensive serological surveys among human populations in Castilla-y-León showed practically no evidence of *F. tularensis* (prevalence of antibodies < 0,19%) until 1997 [21], coinciding with the final stage of the colonization of the agro-ecosystem by common voles [17], but the early human outbreak (1997-1998) was associated with handling of shot hares and an episode of massive hare mortality, which led to enduring low hare abundance [11]. Subsequent human tularemia outbreaks (2007-2008, 2014) have been associated with periods of super abundance of common voles [8], which attain much higher abundance and biomass than hares and rabbits. The empirical evidence suggests that pulses of abundance of hosts that amplify the bacterium within host populations and ultimately contaminate the terrestrial and

aquatic environments may be of greater epidemiological significance than host taxonomy, probably associated to the low competent-host specificity of tularemia.

5. Tularemia surveillance must target unstable mammalian host populations

The "One-Health" concept advocates a broad view of medicine for the successful development of policies and practices that reduce the impact of zoonoses through targeted surveillance and strategic prevention [5]. Monitoring of populations of key epidemiological agents such as voles and hares should be central to prevention strategies. Vole surveillance programs are already implemented in the Castilla-y-León region, showing a degree of predictability to vole populations fluctuating with region-wide outbreaks every 5 years [22]. Extending vole monitoring to include tularemia, particularly during increasing and outbreak population phases, would provide crucial data to parameterize spatial-temporal models of disease risk and help predict when people engaging in non-optional (e.g. crop harvesting) and optional (e.g. hare hunting, crayfish fishing) risky activities should adopt appropriate risk minimising techniques (e.g. farmers using breathing masks during summer harvests in vole outbreaks, hunters and fisherman using gloves during hare butchering or crayfish cleaning) (Fig 1).

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CHAPTER 2.D

OTHER PATHOGENS

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Zoonotic pathogens in fluctuating common vole (*Microtus arvalis*) populations: occurrence and dynamics.

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Running title: Pathogens, fleas and common vole.

Abstract

Diseases and host dynamics are linked, but their associations may vary in strength, be time-lagged, or depend on environmental influences. Where a vector is involved in disease transmission, its dynamics are an additional influence, and we often lack a general understanding on how diseases, hosts and vectors interact. We report on the occurrence of six zoonotic arthropod-borne pathogens (*Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella*, *Francisella* and *Rickettsia*) in common voles (*Microtus arvalis*) throughout a population fluctuation and how their prevalence vary according to host density, seasonality, and vector prevalence. *Rikettsia* spp., *A. phagocytophilum*, *Borrelia* spp., and *C. burnetii* were not detected in voles. We detected four species of *Bartonella*. *B. taylorii* and *B. grahamii* prevalence increased and decreased with current host (vole and mouse) density, respectively, and increased with flea prevalence. *B. doshiae* prevalence decreased with mouse density. These three *Bartonella* species were also more prevalent during winter. *B. rochalimae* prevalence varied with current and previous vole density (delayed-density dependence), but not with season. Coinfection with *F. tularensis* and *Bartonella* occurred as expected from the respective prevalence of each disease in voles. Our results highlight that simultaneously considering pathogen, vector and host dynamics provides a better understanding of the epidemiological dynamics of zoonoses in farmland rodents.

Key words: rodent- and arthropod-borne pathogens; mixed infections; population outbreaks; *Microtus arvalis*; fleas; zoonotic diseases dynamics; *Bartonella*; *Francisella tularensis*.

Key findings:

_ Common voles are reservoirs for *Francisella tularensis* and several *Bartonella* species.

_ *Bartonella* spp. prevalence depended on host density, season and flea infestation.

_ *B. taylorii*, *B. rochalimae* and *B. grahamii* were the most prevalent pathogens in voles.

_ *Bartonella* spp. prevalence was greater than *F. tularenis* prevalence.

_ Coinfection of *Francisella* and *Bartonella* occurred as expected from respective prevalence.

1. Introduction

Rodents are frequently exposed to ectoparasites that transmit pathogens (Gratz, 1994). Many pathogens are transmitted by arthropods to rodents and from rodents to humans, livestock and domestic animals. Among arthropods, ticks, mosquitoes and fleas are the main vectors of pathogens that constitute a burden to public health. The re-emergence of zoonotic diseases of risk to humans heightens the necessity to understand how infections are maintained and transmitted in ecosystems (Morner *et al.* 2002). In particular, vector-borne pathogens offer the opportunity to determine how vector and pathogen dynamics are linked to host dynamics in order to identify reservoirs and transmission pathways. For instance, the dynamics of *Trypanosoma microti*, a flea-borne protozoan, were strongly influenced by flea dynamics in cyclic populations of field voles (*Microtus agrestis*) (Smith *et al.* 2005), whereas vole host density was more influential than flea abundance in explaining the dynamics of a flea-borne bacterium, *Bartonella* spp. (Telfer *et al.* 2007*a*). These findings were attributed to fleas exploiting, and being affected by, several host species in the ecosystem.

Coinfections also occur when a host is infected by different parasites, at the same time or sequentially. Parasite interactions can result in a co-occurrence or in a competition between parasites for a shared resource, such as food or habitat, thus affecting host population and resulting in direct interactions. Alternatively, the immune response of the host to one parasite may affect the host's ability to control a second parasite species, i.e., indirect interactions (Telfer *et al*. 2010). In this case, the presence of a parasite can increase the host susceptibility to be infected with a second parasite or, on the contrary, decrease the infection probability of other parasite due to an immune response (Cox, 2001). Coinfections not only result from the interactions among parasites, but also from shared risk factors such as environmental and climatic conditions, vectors or groups of vectors, host density or host physiological conditions. Many studies have shown that rodents can be simultaneously infected by more than one pathogen (Meerburg *et al.* 2009; Buffet *et al.* 2012; Kallio *et al.* 2014; Razzauti *et al.* 2015; Koskela *et al.* 2017). However, the existence and types of interactions between parasites in natural systems, which may be essential to predict disease dynamics and control parasites, remains poorly known (but see Telfer *et al.* 2010).

The common vole (*Microtus arvalis*) is one of the most abundant and widespread mammals in continental Europe (Jacob and Tkadlec, 2010). Throughout its range, common vole populations typically exhibit regular fluctuations in abundance or irruptive outbreaks (Tkadlec and Stenseth, 2001; Lambin *et al.* 2006). The species recently colonized ca. 5 million ha of

farmland in Northwest Spain, coinciding with an increase in the surface area of irrigated herbaceous crops, in particular alfalfa (Luque-Larena *et al.* 2013; Jareño *et al.* 2015). Since the colonization, vole population outbreaks have regularly occurred in the region, reaching high abundances during peak phases (>1,000 individuals/ha). These outbreaks have caused unprecedented public health risks because voles carry and amplify tularemia, a highly infectious disease caused by the bacterium *Francisella tularensis* (Rossow *et al.* 2015; Luque-Larena *et al.* 2017). *F. tularensis* infection in voles was direct-density dependent (Rodríguez-Pastor *et al.* 2017) and infections in voles and humans coincided in space and time (Luque-Larena *et al.* 2015). As it has been reported in other rodents, common voles from Northwest Spain could be simultaneously infected by other vector-borne pathogens, but the occurrence, dynamics and coinfection patterns of several pathogens remain empirically unknown for these populations, as well as their interactions with vectors. Ticks and fleas can be found on voles elsewhere, and both vectors can transmit *F. tularensis* as well as other pathogens. Therefore, to obtain a complete understanding of the pathogens dynamics it is necessary to take into account not only the dynamics of the hosts, but also the dynamics of vectors as well as pathogen interactions (mixed coinfections) and their consequences in the environment.

Here, we investigated the occurrence and dynamics of six vector-borne pathogens of zoonotic risk to humans in fluctuating populations of common voles in Northwest Spain. Specifically, we screened for the occurrence of three tick-borne bacteria (*Anaplasma phagocytophilum*, *Borrelia* spp., and *Coxiella burnetii*), and three flea- and tick-borne bacteria (*Bartonella* spp., *Rikettsia* spp. and *F. tularensis*) that are often reported in vole populations. We also investigated whether the prevalence of these pathogens detected in voles varied with vole population density and the density of other coexisting potential hosts (the wood mouse *Apodemus sylvaticus*, and the Algerian mouse *Mus spretus*). Common voles typically occur at much greater abundances that coexisting mice (Lambin *et al.* 2006; Rodríguez-Pastor *et al.* 2016), so we expected pathogen prevalence to be more heavily influenced by vole density (positive density-dependence). We also looked for associations between pathogen and vector (flea) to assess whether vectors participated in the transmission of some pathogens. Finally, we investigated coinfection patterns and tested whether the infection probability by a given pathogen varied depending on the presence of a second pathogen.

2. Material and methods

We held all the necessary licenses and permits for conducting this work: JJLL, FM and RRP held official animal experimentation licenses of level B for Spain, and capture permits were provided by the Dirección General del Medio Natural, Junta de Castilla y León.

2.1. Study area

The study was conducted in an 80-km^2 area of farmland located in Palencia province, Castilla-y-León autonomous region, Northwestern Spain (42°01´N, 4°42´W), which is recurrently affected by common vole outbreaks (Luque-Larena *et al.* 2013). We sampled voles between March 2013 and March 2015, when vole abundance increased region-wide, peaked to outbreak densities in July 2014, and thereafter declined (Luque-Larena *et al.* 2015; Rodríguez-Pastor *et al.* 2017). Pre-outbreak vole abundance data (2009-2013) were also available, allowing us to investigate delayed-density dependent patterns.

2.2. Common vole sampling

Common vole abundance, as well as pathogen and vector prevalence were monitored every 4 months: March, July and November. Voles were live trapped using LFAHD Sherman© traps (8 cm × 9 cm × 23 cm) baited with carrots. At each seasonal sampling, trap lines were set in 24 randomly selected fields and their adjacent margins. Thirty-five traps per trap line spaced by 2 m between each other were operated, with 10 traps set along a margin and 25 traps set perpendicularly inside the field (see Rodríguez-Pastor *et al.* 2016 for more details on the trapping scheme). Traps were opened in the morning and checked the following morning with a constant vole trapping effort (840 traps set for 24h per seasonal sampling, making up a total sampling effort of 5,880 trap night). Common voles live in sympatry with other rodent species in the area, but the majority of captures were voles (76.1%; 929/1221), followed by *A. sylvaticus* (18.5%; 226/1221) and *M. spretus* (5.4%; 66/1221). From a total of 929 voles captured between March 2013 and March 2015, a subset of 240 randomly-selected voles (105 males and 135 females) were used for pathogen and vector screening. The random selection was based on a representative sample of captured voles that arrived alive to the laboratory and was stratified by seasonal sampling event and vole gender.

2.3. Laboratory procedure

Each vole was sexed, weighed and euthanatized through medical CO_2 inhalation, following a protocol approved by our institution ethics committee (CEEBA, Universidad de Valladolid;

authorisation code: 4801646). Immediately after death, each individual was examined for ectoparasites (fleas and ticks) through careful visual inspection and by gently blowing the vole's fur while holding the animal over a white plastic tray (520 × 420 × 95 mm) filled with water. Collected ectoparasites were counted and preserved at room temperature in individually labelled tubes filled with 70% ethanol. Fleas, but not ticks, were identified to species level. Three flea species were identified (*Ctenophthalmus apertus*, *Nosopsyllus fasciatus* and *Leptopsylla taschenbergi*) under a binocular microscope (x10 and x40 magnification; Nikon Optiphot-2) based on morphological traits following Gómez *et al*. (2004). Vole carcasses were kept frozen at -23˚C until dissection, which followed standard protocols. The spleen and liver were kept separately in labelled tubes and stored at -23˚C for molecular detection of pathogen.

2.4. DNA extraction and multiplex PCR-Reverse Line Blot

DNA was extracted from a homogenized mix of liver and spleen (ca. 25 mg) using commercial kits (QIAamp® DNA Mini Kit, Qiagen, Hilden, Germany) according to the standard procedures of the manufacturer. A multiplex Polymerase Chain Reaction (PCR) was set up for the simultaneous detection of six vector-borne pathogens (*A. phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *C. burnetii*, *F. tularensis* and *Rickettsia* spp.) combined with a reverse line blotting (RLB), as previously described (Anda *et al.* 2012). All positive samples to any given pathogen were further tested separately using specific-probes with an individual PCR and subsequent RLB.

2.5. Detection of F. tularensis

We used a phylogenetically informative region of *lpnA* (231 bp) that was amplified by conventional PCR and further hybridization with specific probes by RLB as previous described in Escudero *et al*. (2008). Positive samples were tested using a real-time multitarget TaqMan PCR, using tul4 and ISFtu2 assays (Versage *et al.* 2003). A negative PCR control as well as a negative control for DNA extraction was included in each group of samples tested. For real-time PCR using tul4, ISFtu2, a type A positive control was used, as type A strains are restricted to North America. Rodríguez-Pastor *et al*. (2017) previously screened 243 common voles for a single pathogen (*F. tularensis*); here, we screened 240 (99%) of these voles for 6 pathogens (including *F. tularensis*) using the multiplex PCR (Escudero *et al.* 2008).

2.6. Identification of Bartonella *species infecting voles*

Bartonella positive samples were further analysed using a multiplex PCR targeting the 16S rRNA and the intergenic transcribed spacer (ITS) 16S-23S rRNA. Subsequently, amplicons were

analysed with a RLB that included 36 probes for the identification of the different genotypes and species of *Bartonella* (Garcia-Esteban *et al.* 2008; Gil *et al.* 2010).

2.7. Statistical analyses

We used R v3.4.1 (R Development Core Team, 2017) for all analyses. In order to evaluate hypotheses on pathogen prevalence, we calculated time-varying host population-level covariates and individual-level vole host covariates. The former included mean vole abundance, mean mouse abundance (wood and Algerian mice pooled) per seasonal sampling (mean vole and mouse abundances were estimated as the average number of captures per 100 traps per 24h for a given seasonal sampling period), and mean prevalence of *F. tularensis* and *Bartonella* spp. for each seasonal sampling (hereafter, *Bartonella* spp. refers to all species of *Bartonella*). From individual data, we calculated seasonal sampling specific pathogen prevalence as the number of voles positive for a particular pathogen, over the total number of voles analysed. Individual level covariates included vole sex; *F. tularensis* PCR result (0/1); *Bartonella* spp. PCR result (0/1); overall flea prevalence (0/1) and flea burden (number per host); species-specific flea prevalence and flea burden (i.e., *C. apertus, N. fasciatus* and *L. taschenbergi* separately); tick prevalence and tick burden. Burdens of ectoparasites were estimated as the number of fleas, or ticks, collected per individual vole.

To investigate whether host density influenced *Bartonella* spp. prevalence, we used Generalized Linear Models (GLM) with a binomial error structure and logit link. Similar analyses were conducted for each *Bartonella* species. The dependent variables were the probability of a vole being infected (categorical variable: "0" vs. "1", as dependent variable) at time $_t$ according to vole abundance (at time $_t$), previous vole abundance (4 months before, times $_{t-4}$) and mouse abundance (wood and Algerian mouse abundance at time $_t$). As host abundance changed seasonally and by sex, the categorical variables season (spring/March, summer/July and winter/November) and sex (male and female) were also included in the models. In order to address collinearity issues and improve model fitting to the data, vole abundances were log-transformed when included as explanatory variables. A series of models including the different explanatory variables were built. Model selection was performed using the Aikake Information Criterion for small sample size (Δ-AICc) with the "AICcmodavg" package in R and compared (model selection procedure explained below).

We examined whether flea infestation influenced *Bartonella* spp. prevalence in voles, using binomial GLMs. We considered flea prevalence (whether or not a vole had fleas) and vole sex as explanatory variables. These models were also fitted for each *Bartonella* species in turn

to examine species-specific relationships. We further tested which flea species better explained the prevalence of *Bartonella* spp., as well as of each *Bartonella* species separately, using binomial GLMs including the prevalence of each flea species and vole sex as explanatory variables.

Finally, we tested whether *Bartonella* spp. infection in common voles was associated with *F. tularensis* infection. For this, we used binomial GLMs considering *F. tularensis* prevalence, vole abundance and sex, and the 2-way interaction between *F. tularensis* prevalence and vole abundance as explanatory variables. We similarly tested for associations between *F. tularensis* and each *Bartonella* species separately.

3. Results

3.1. Pathogens prevalence in common vole

Among the six pathogens screened, only *F. tularensis* and *Bartonella* spp. were detected using PCRs. *Bartonella* spp. prevalence averaged 46.7% (112/240), with marked differences between seasonal samplings: prevalence was maximum during the summer peak in vole density (July 2014), when 69.3% (70/101) of voles were infected (Fig. 1). For *F. tularensis*, we also confirmed that 20.4% (49/240) of voles were infected on average, and that in July 2014, prevalence peaked at 33.7% (34/101; Fig. 1).

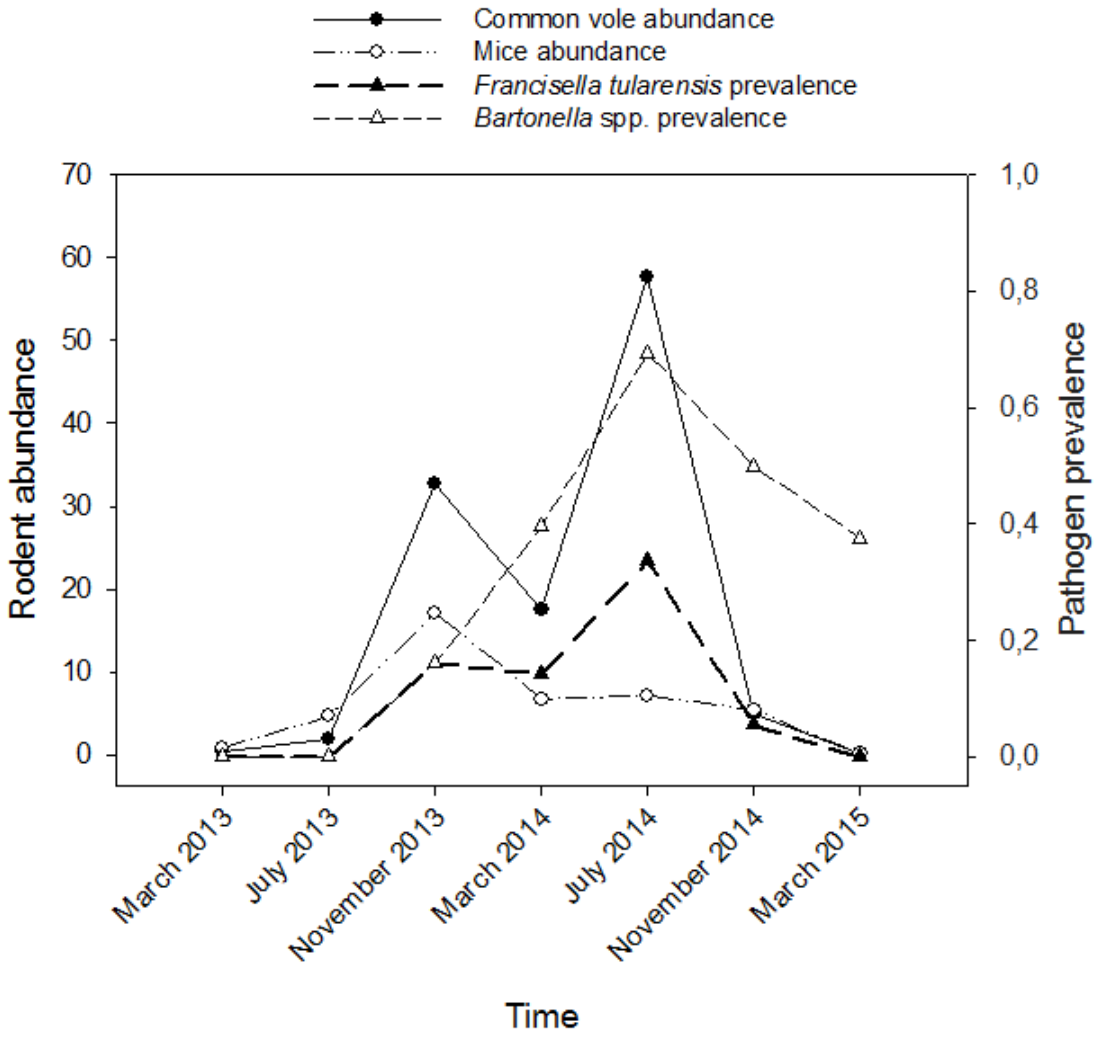

Figure 1. Temporal changes in rodent abundance and in pathogen prevalence in common vole during the course of the study (March 2013 to March 2015). Common vole abundance (captures/100 traps/24 h) = black solid line and black circles; mouse abundance (wood mouse and Algerian mouse; captures /100 traps/24 h) = black dashed line and white circles; *F. tularensis* prevalence = thick black dashed line and black triangles; *Bartonella* spp. prevalence = black dashed line and white triangles.

3.2. Bartonella *spp. infecting voles*

Five *Bartonella* species were identified among infected voles (Table 1): *B. taylorii*, *B. grahamii*, *B. rochalimae*, *B. doshiae*, and *B. clarridgeiae*. The most frequent species was *B. taylorii*, which was detected in 64.8% (72/111) of all the *Bartonella*-positive voles. Mixed infections with different *Bartonella* species were detected in 58.6% (65/111) of the positive voles (Table 1). Moreover, a mix of three different *Bartonella* species was found in 8.1% (9/111) of the positive voles. One of the samples reacted with the 16S rRNA probe, but not with any of the other 36

Bartonella species-specific ITS probes (Table 1). Attempts to sequence the ITS amplicon were unsuccessful and the sample was classified as belonging to an unknown *Bartonella* species.

Table 1. Species-specific occurrence of *Bartonella* species in infected common voles (n=111) according to infection type: single *Bartonella* species infection, or mixed-*Bartonella* species infection.

Bartonella species	N (%)
B. taylorii	19 (17.1)
with *B. grahamii*	27 (24.3)
with *B. rochalimae*	17 (15.3)
with *B. rochalimae* and *B. grahamii*	4 (3.6)
with *B. rochalimae* and *B. doshiae*	3 (2.7)
with *B. doshiae* and *B. grahamii*	2 (1.8)
B. rochalimae	14 (12.6)
with *B. doshiae*	4 (3.6)
with *B. grahamii*	3 (2.7)
with *B. clarridgeae*	1 (0.9)
B. grahamii	11 (9.9)
with *B. doshiae*	4 (3.6)
B. doshiae	1 (0.9)
Bartonella spp.	1 (0.9)
Total	111 (100)

3.3. Density-dependence: host-pathogen interactions

The models that best explained variation in *Bartonella* ssp. prevalence in voles included vole abundance (direct, positive density-dependence), mouse abundance (direct, negative density-dependence) and season (see model selection in Table 2 and Fig. 2). Both mouse and vole abundance were statistically significant: vole abundance influenced prevalence positively (slope ± standard error: +3.45 ± 0.80), but mouse abundance influenced prevalence negatively (-0.39 ± 0.09; Fig. 2). In addition, pathogen prevalence in voles was relatively higher in winter than in summer or spring (Fig. 2).

Table 2. Results of Generalized Linear Models (GLMs) describing how host density, sex and season influenced *Bartonella* spp. prevalence in common vole population. The best models (lowest AICs) are highlighted in bold. Vole abundances were log-transformed. Vole Ab: contemporary vole abundance (at time $_t$); Vole Ab4: previous vole abundance (4 months before, time $_{t-4}$); Mouse Ab: contemporary mouse abundance (wood mouse and Algerian mouse, at time $_t$); Sex: female vs. male common vole; Season: spring (from March to July), summer (from July to November) and winter (from November to March).

	k	AIC	AICc	Δ-AICc	Pseudo-R^2
***Bartonella* spp. ~ Season + Mouse Ab + Log Vole Ab**	**5**	**288.33**	**288.59**	**0.00**	**0.266**
***Bartonella* spp. ~ Season + Mouse Ab + Log Vole Ab + Sex**	**6**	**289.59**	**289.95**	**1.36**	**0.269**
Bartonella spp. ~ Season + Mouse Ab + Log Vole Ab + Log VoleAb4 + Sex	7	291.26	291.74	3.15	0.271
***B. doshiae* ~ Season + Mouse Ab**	**4**	**104.08**	**104.25**	**0.00**	**0.121**
***B. doshiae* ~ Season + Mouse Ab + Log Vole Ab**	**5**	**104.77**	**105.02**	**0.77**	**0.135**
B. doshiae ~ Season + Mouse Ab + Log Vole Ab + Sex	6	105.48	105.84	1.59	0.150
B. doshiae ~ Season + Mouse Ab + Log Vole Ab + Log Vole Ab4 + Sex	7	106.38	106.87	2.62	0.162
***B. grahamii* ~ Season + Mouse Ab + Log Vole Ab**	**5**	**215.89**	**216.15**	**0.00**	**0.264**
B. grahamii ~ Season + Mouse Ab + Log Vole Ab + Sex	6	217.06	217.42	1.27	0.268
B. grahamii ~ Season + Mouse Ab + Log Vole Ab + Log Vole Ab4 + Sex	7	218.73	219.22	3.07	0.270
***B. rochalimae* ~ Mouse Ab + Log Vole Ab + Log Vole Ab4**	**4**	**228.50**	**228.67**	**0.00**	**0.091**
***B. rochalimae* ~ Log Vole Ab + Log Vole Ab4**	**3**	**228.83**	**228.93**	**0.26**	**0.076**
B. rochalimae ~ Mouse Ab + Log Vole Ab4 + Log Vole Ab + Sex	5	230.08	230.34	1.66	0.094
B. rochalimae ~ Season + Mouse Ab + Log Vole Ab + Log Vole Ab4 + Sex	7	234.02	234.50	5.83	0.094
***B. taylorii* ~ Season + Mouse Ab + Log Vole Ab**	**5**	**264.90**	**265.15**	**0.00**	**0.217**
B. taylorii ~ Season + Mouse Ab + Log Vole Ab + Sex	6	266.77	267.13	1.98	0.218
B. taylorii ~ Season + Mouse Ab + Log Vole Ab + Log Vole Ab4 + Sex	7	268.76	269.25	4.09	0.218

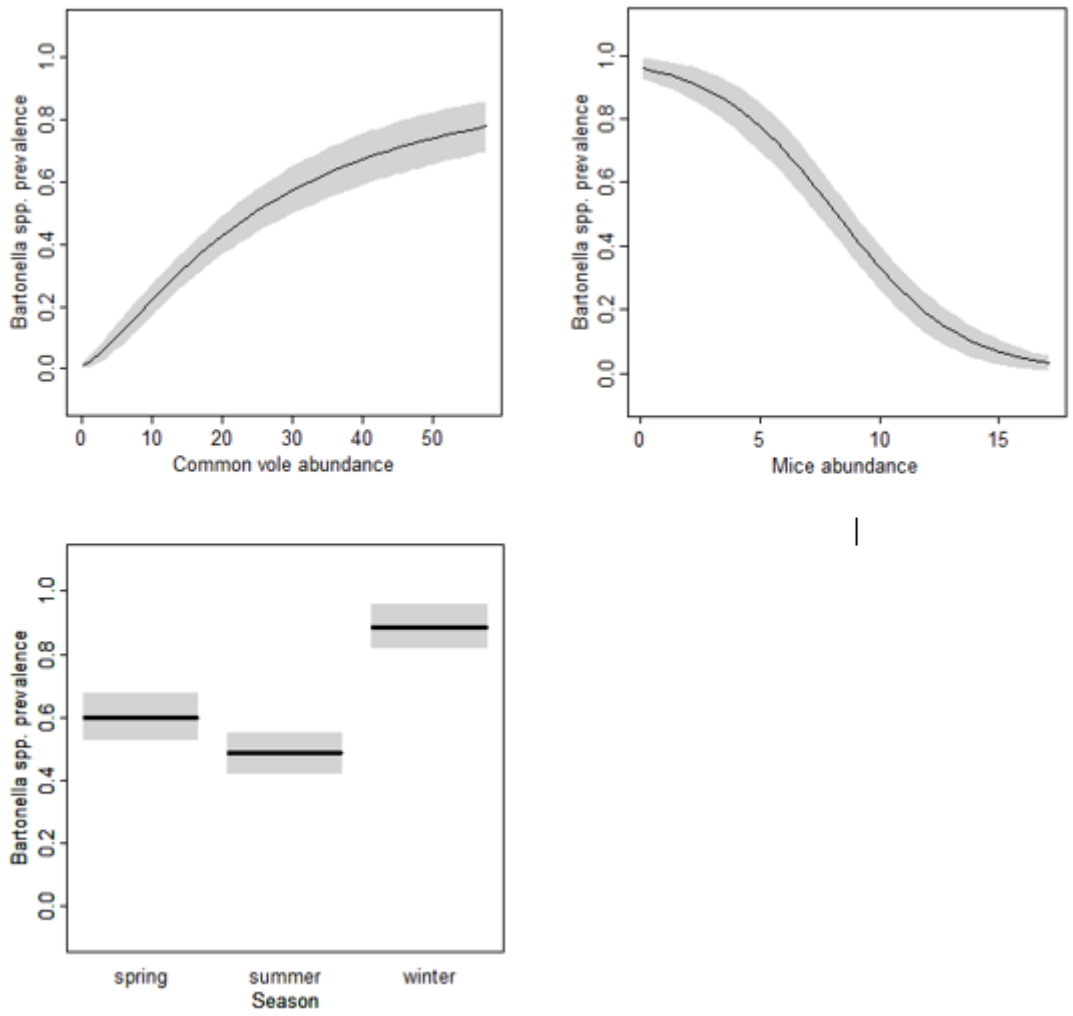

Figure 2. *Bartonella* spp. prevalence in common vole populations according to current common vole abundance (at time $_t$); current mouse abundance (wood mouse abundance and Algerian mouse abundance, at time $_t$); and season. The graphs show model outputs (Table 2), with grey shades denoting 95% confidence intervals of the predicted curves.

Two models explained *B. doshiae* prevalence in voles equally well, and included season and mouse abundance, or these variables plus vole abundance (Δ-AICc < 2; Table 2). Prevalence decreased with increasing mouse abundance (slope ± se: -0.19 ± 0.09), was higher in winter (estimate ± se: +3.12 ± 1.18) and summer (+2.02 ± 1.10) than in spring (-3.35 ± 1.09) and increased with vole abundance.

For *B. rochalimae,* two models also explained equally well prevalence variation in voles (Δ-AICc < 2; Table 2). One model included contemporary and previous vole densities, while the other model also included mouse abundance. However, mouse density was marginally significant, and the omission of this variable improved the significance of vole densities (Table 2). *B. rochalimae* prevalence increased with current vole density (slope ± se = +0.88 ± 0.48) and

with vole density 4 months before (slope ± se = +1.27 ± 0.55). This was the only species of *Bartonella* that showed a positive delayed density-dependence and its prevalence did not differed between seasons.

B. grahamii and *B. taylorii* prevalence varied like *Bartonella* spp. prevalence. In both species, prevalence in voles increased with vole density (slope ± se: +3.20 ± 1.26, for *B. grahamii*; and +3.61 ± 1.11, for *B. taylorii*) and decreased with mouse density (-0.40 ± 0.16, for *B. grahamii*; and -0.50 ± 0.15, for *B. taylorii*) (Table 2). *B. grahamii* prevalence was higher in winter (estimate ± se: +1.88 ± 0.88) than in summer (+0.53 ± 0.77) and lowest in spring (-3.72 ± 1.09). *B. taylorii* prevalence in voles was lower in spring (estimate ± se = -2.27 ± 0.79) than in winter (+1.54 ± 0.69) and there was a null effect in summer (coefficient not significant).

3.4. Flea-pathogen interactions

Almost all (94%; 225/240) of the voles that were used in this study arrived alive to the laboratory. Among them, 55% (125/225) were females and 45% (100/225) were males. A total of 153 (68%) voles were infested with fleas, with 643 fleas collected from 70 male voles and 83 female voles. By contrast, only 5 (2.22%) voles were infested with ticks, considering both larvae and nymphs (29 ticks collected from 4 females and 1 male). The community of fleas was dominated by *C. apertus* (62.52%), followed by *N. fasciatus* (36.70%), and with *L. taschenbergi* (0.78%) occurring in a minor proportion. Details about flea prevalence and tick prevalence on voles at each sampling period are shown in Table 3.

Table 3. Prevalence of fleas, ticks, *F. tularensis*, *Bartonella* spp. and co-infections (with both *F. tularensis* and *Bartonella* spp.) in common voles at each sampling time. Note that sample sizes differ for ectoparasite and pathogen prevalence because only those common voles that did not die in traps were considered for ectoparasite prevalence.

Time	Voles sampled for ectoparasites	% infested by fleas	% infested by tick	Voles sampled for pathogens	% infected with *F. tularensis*	% infected with *Bartonella* spp.	% with co-infection (*F. tularensis* + *Bartonella* spp.)	% with expected co-infection (*F. tularensis* + *Bartonella* spp.)	% infected with *B. doshiae*	% infected with *B. grahamii*	% infected with *B. rochalimae*	% infected with *B. taylorii*
March 2013	2	50.00	0.00	4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
July 2013	14	71.43	7.14	15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
November 2013	31	35.48	0.00	31	16.13	16.13	6.45	2.60	3.23	3.23	12.90	3.23
March 2014	58	43.10	0.00	63	14.29	39.68	6.35	5.67	0.00	7.94	23.81	25.40
July 2014	101	87.13	2.97	101	33.63	69.31	23.76	23.33	7.92	41.58	22.77	47.52
November 2014	12	91.67	0.00	18	5.55	50.00	5.55	2.78	22.22	13.67	22.22	33.33
March 2015	7	100.00	0.00	8	0.00	37.50	0.00	0.00	12.50	12.50	0.00	25.00
Total	225	68.00	1.78	240	20.42	46.67	12.92	95.30	5.83	21.67	19.17	30.42

Bartonella spp. prevalence was positively correlated with flea prevalence (estimate ± se = +0.60 ± 0.29). Considering species-specific prevalence, *B. doshiae* and *B. rochalimae* prevalence were not related to flea prevalence, while *B. grahamii* and with *B. taylorii* both increased when voles had fleas (*B. grahamii*: estimate ± se = +1.49 ± 0.46; *B. taylorii*: +0.79 ± 0.34).

At flea species level, *Bartonella* spp. prevalence in voles increased with *N. fasciatus* prevalence (estimate ± se = +0.61 ± 0.27) but not with the prevalence of other flea species. This positive association between *Bartonella* prevalence and *N. fasciatus* was found in *B. grahamii* (estimate ± se = +0.75 ± 0.33) and in *B. taylorii*, but marginally significant, (estimate ± se = +0.51 ± 0.29; p = 0.07). There was a positive correlation between *B. doshiae* prevalence and *C. apertus*, but marginally significant (estimate ± se = +1.14 ± 0.64, p = 0.07).

3.5. Pathogen-pathogen interactions

The presence of both *F. tularensis* and *Bartonella* spp. was detected in 12.9% (31/240) of the screened voles (Table 3). Coinfection rate (*F. tularensis* and *Bartonella* spp) reached a maximum of 23.8% (24/101 voles) in July 2014 when voles reached their maximum density (Table 4). Overall, the probability of a vole being infected by both pathogens was not different from that predicted from the prevalence of each pathogen alone at a given sampling time (Table 3). Coinfection rate was 14.8% (20/135) in female voles and 10.5% (11/105) in male voles (Table 4). We observed that the probability of being infected by both pathogens was not different from the predicted prevalence of each pathogen in voles (χ^2_1= 6.81, p < 0.05; Table 3). When vole abundance and sex were considered in the model, the probability of infection with *Bartonella* spp. did not depend on *F. tularensis* prevalence but only depended on vole density (slope ± se = +0.03 ± 0.01). This positive association with vole density was found for *B. grahamii* (slope ± se = +0.05 ± 0.01) and *B. taylorii* (+0.03 ± 0.01).

Table 4. Occurrences of co-infections with both *F. tularensis* and *Bartonella* spp. in studied common voles (n=240). "Positive" = voles with the pathogen(s); "Negative" = voles without the pathogen(s). Percentages are indicated in parentheses.

			F. tularensis		Total
			Negative	Positive	
Bartonella spp.	Female	Negative	59 (24.58)	14 (5.83)	73
		Positive	42 (17.50)	20 (8.33)	62
	Male	Negative	51 (21.25)	4 (1.67)	55
		Positive	39 (16.25)	11 (4.58)	50
	Total		191	49	240

4. Discussion

Prevalence of *F. tularensis* and *Bartonella* spp. has been studied in some small mammals (*M. spretus*, brown rat *Rattus norvegicus*, *A. sylvaticus*, white-toothed shrews *Crocidura russula* and *M. arvalis*) from Mediterranean areas (Márquez *et al.* 2008; Gil *et al.* 2010; Cevidanes *et al.* 2017; Rodríguez-Pastor *et al.* 2017), although the relationship between the dynamics of hosts, pathogens and vectors, as well as the interactions between pathogens, have not been studied previously. Our study shown a significant correlation between host and pathogen dynamics, and that the probability of infection with *Bartonella* spp. increased with flea prevalence, which is consistent with *Bartonella* spp. being a flea-borne pathogen. We also provided evidence that the occurrence of one zoonotic pathogen (*Bartonella* spp.) was not dependent on the occurrence of the other (*F. tularensis*) in vole populations.

Bartonella spp. was the most prevalent bacteria in voles, infecting almost half (46.7%) of all the voles analysed, while just a fifth (20.42%) of all the voles were infected with *F. tularensis*. This *Bartonella* spp. proportion falls within the range (between 11 and 72 %) of prevalence previously reported in rodents from other European countries (Gutiérrez *et al.* 2015).

B. taylorii, *B. rochalimae* and *B. grahamii* were the most prevalent pathogens in voles, and there was a high percentage of mixed infections (58.6%), with the highest dual infections among *B. taylorii* and *B. grahamii*. This relatively high percentage may be reflecting a host specificity of these species. However, to assert this, it will be necessary to screen the prevalence of the species of *Bartonella* in other rodents that cohabit with voles.

An effect of host density on *Bartonella* spp. prevalence has been demonstrated in several rodent species. For instance, in a study of a Mediterranean peri-urban environment and in absence of voles, *Bartonella* spp. occurrence was positively correlated with wood mouse abundance, the most abundant species in such small mammal community, but not with Algerian mouse abundance, despite prevalence being higher in autumn than in spring for both rodent species (Cevidanes *et al.* 2017). In that case, density-dependence was tested considering a pool of various species of *Bartonella*, so the density-dependent pattern found may have been masked by the most prevalent species of *Bartonella*. In another study using long-term data in a highly Atlantic moist climate from another vole species (i.e., field voles), and which populations also experience abundance outbreaks and are infested by fleas, (Telfer *et al.* 2007*a*) found that different species of *Bartonella* exhibited contrasting dynamics in two alternative hosts: field voles and wood mice. The probability of infection with *B. doshiae* and *B. taylorii* increased with field vole density, while *B. doshiae* and *B. grahamii* did so with density of wood mice. In another study with different rodent hosts (bank voles and wood mice), *B. taylorii* and *B. doshiae* were more prevalent in wood mice, while *B. birtlesii* was more prevalent in bank voles (Telfer *et al.* 2007*b*). This suggests that the distribution and abundance of each *Bartonella* species do not follow common patterns and that their response to host density depends on the most abundant preferred host. These findings highlight that each species of *Bartonella* exhibits different patterns in its distribution and abundance, has different host specificity, seasonality and response to host density. Therefore, to study the relationship between pathogen and host dynamics requires considering each species of *Bartonella* separately (Telfer *et al.* 2007*b*). In agreement with previous findings by Telfer et al. (2007*a*, *b*), we provided evidence for a density-dependence response that differed among *Bartonella* species and rodent hosts: i.e., *B. taylorii* and *B. grahamii* responded to both vole and mouse densities, while *B. doshiae* responded to mouse density (direct response), and *B. rochalimae* to vole density (direct and delayed responses). The positive direct density-dependence to vole density suggests that the pathogen spreads quickly between individuals, and that voles may have low resistance to pathogen infection. Moreover, the negative relationship with mouse density suggests that voles may influence infection prevalence in mice.

Factors such as seasonality can also determine variation of pathogen prevalence in reservoir hosts. *Bartonella* spp. prevalence in small mammals follows a seasonal pattern, although with contrasting results among studies: *Bartonella* spp. prevalence can peak in summer (Paziewska *et al.* 2012), but also in autumn (Cevidanes *et al.* 2017). However, these seasonal patterns are based on a pool of *Bartonella* spp., not on the prevalence at species level (but see Telfer *et al.* 2007*b*). Overall, we found that *Bartonella* spp. prevalence in voles was highest during winter (Fig. 2) when taking into account host densities. Altogether, more fleas were collected in spring and summer than during winter. An increase in the infection probability with *Bartonella* spp. in winter could be the result of an increase in the occurrence of infected alternative hosts, increasing the infection probability in voles. However, we need to know the *Bartonella* spp. prevalence of the alternative rodent hosts (mice) as well as in the main vector (fleas) in order to better understand these interactions. At the species level, the infection probability with *B*. *grahamii*, *B*. *taylorii* and *B*. *doshiae* in voles followed a marked seasonal variation, i.e., increased in winter and decreased in spring. *B*. *rochalimae* was the only species whose prevalence did not vary seasonally, but was also the one with the lowest prevalence in voles. A seasonal pattern for *B. grahamii* has been also found in other vole species, but not for *B*. *taylorii* and *B*. *doshiae* (Telfer *et al.* 2007*a*). Such seasonal differences may be due to the dynamic and phenology of the fleas that transmit *Bartonella* spp.

Pathogen prevalence also varies with vector dynamics. *Bartonella* spp. prevalence has been previously shown to be higher in mice carrying greater flea burdens (Cevidanes *et al.* 2017). In our studied common vole population, *B. taylorii* and *B. grahamii* were the most prevalent species and the infection probability increased when voles were infested by fleas, independently of the flea burden. This positive relationship between flea and pathogen was found between *N. fasciatus* and both species of *Bartonella*, providing evidence for vector specificity: these bacteria were likely transmitted by *N. fasci*atus. Indeed, both *B. taylorii* and *B. grahamii* have been previously detected in *N. fasciatus* collected from rodents (Silaghi *et al.* 2016). However, we need to confirm the role of fleas in the transmission process, because when host density and flea prevalence were simultaneously considered, variation in pathogen infection was explained by host dynamics rather than flea prevalence. A lack of effect of flea prevalence on *Bartonella* dynamics has been previously shown in voles (Telfer *et al.* 2007*a*). Therefore, our findings should be considered with caution because we do not know which proportion of fleas become infected, what species of *Bartonella* occur in fleas, and whether there are other vectors or modes of transmission. Some species of *Bartonella* are transmitted by ticks, and others can be transmitted vertically between mother and offspring (Kosoy *et al.* 1998; Chang *et al.* 2001). A relatively

weaker role of fleas, in contrast to vole density in modulating *Bartonella* prevalence over time, could also be explained by a delayed-density dependence response of flea burden to common vole density that we observed in our study system (a lag of 8 months; unpublished data), but more work is needed to test this hypothesis.

Coinfection with more than one pathogen seems to be common in wildlife. We found coinfection between *Bartonella* spp., a flea-borne bacterium, and *F. tularensis*, a facultative flea-borne bacterium. In the absence of tick-borne infection, the pairwise combination was limited, and the pattern of infection was consistent with concurrent exposure rather than variation in susceptibility. Around 13% of all the common voles screened here were simultaneously infected with *F. tularensis* and *Bartonella* spp., and this percentage of coinfection reached 23.80% during the population peak in July 2014 (see Table 3). The high percentage of infected individuals with two pathogens suggested that there could be some type of interaction modulated by characteristics of the host and the environment. Coinfections by both bacteria may occur non-randomly and thus, the infection with *F. tularensis* may increase the probability of infection with *Bartonella* spp. or vice-versa. *F. tularensis* is expected to cause an acute and lethal infection in voles (Rossow *et al.* 2014), and *Bartonella* spp. a more chronic but non-lethal infection (Harms and Dehio, 2012). However, we do not know the average length of infection in common voles when infected by both bacteria. Voles could be initially infected with *Bartonella* spp. and later with *F. tularenis*, killing the animal. However, the initial association among the two bacteria disappeared when we considered host density. The lack of correlation between both pathogens reflected the similarity of percentages of coinfection to those expected by adding the percentage of infected individuals by each pathogen independently (see Table 3), so we have no evidence of pathogen interactions. This preliminary result about coinfection should be considered with caution and would need to be confirmed by experimental studies focusing on interactions between *Francisella* and *Bartonella,* and some measures of infection duration in common voles.

The lack of detection of *Rikettsia* spp., *A. phagocytophilum*, *Borrelia* spp., and *C. burnetii* in the studied voles could be due to the climatic conditions (seasonally semi-arid Mediterranean climate) and the habitat type (agricultural landscape) of the study area, as well as the absence of other more suitable vectors, such as ticks (that infected less than 5% of sampled voles). In contrast to our study, and in a region with an Atlantic climate (mild temperature and significant precipitations) in areas surrounding farms, forested and recreational areas, Barandika *et al.* (2007) were able to study the prevalence and diversity of *Borrelia* spp., *A. phagocytophilum*, *C. burnetii*, and the spotted fever group rickettsiae infecting several species of small mammals: the

wood mouse, the yellow-necked field mouse (*A. flavicollis*), the bank vole, the crowned shrew (*Sorex coronatus*), the white-toothed shrew, the house mouse (*M. domesticus*) and the European mole (*Talpa europaea*). They found that infection rates with *Borrelia*, *Anaplasma* and *Coxiella* differed between small mammal species, although like in our study, *Ricketssia* spp. was not detected. However, small mammals were heavily infested by ticks.

All the results shown in this study were conducted in one site, so they should be interpreted with caution and not generalized to all common vole populations. Notwithstanding, we found that voles were infected with four species of *Bartonella*, which had different dynamics according to host density (vole and mouse), season and flea infestation. Moreover, voles were infected with *Bartonella* spp. and *F. tularensis*, but we did not find a clear pattern of association among pathogens. Ongoing studies could focus on identifying other suitable reservoirs as well as the effect of these pathogens in voles at individual level and how the infective process happens.

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Competing interests

The authors declare that they have no competing interests.

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CHAPTER 3

PARASITE-PATHOGEN INTERACTION

Zoonotic bacteria in fleas parasitizing common voles in northwest Spain

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Capsule

Francisella tularensis and *Bartonella* spp. were detected in fleas parasitizing common voles with a mean prevalence of 6.1% and 51%, respectively, indicating that these ectoparasites may play a role in the transmission cycles of these pathogens in nature.

Abstract

Tularemia is endemic to Northwest Spain where human case numbers increase during common vole (*Microtus arvalis*) outbreaks in agricultural landscapes. There, common voles are frequently infected by *Francisella tularensis*, the etiological agent of tularemia, and also by *Bartonella* spp., particularly at high vole densities. Both zoonotic pathogens can be transmitted by arthropods, in particular fleas, which are the main ectoparasite of common voles in this region. In this study, we screened the DNA extracted from 191 fleas of two species (*Ctenophthalmus apertus* and *Nosopsyllus fasciatus*) collected from 90 live common voles from farmland in Northwest Spain. These flea-hosting voles were first checked for prevalence of *F. tularensis* (27 *F. tularensis*-positive and 63 *F. tularensis*-negative voles) and subsequently for other zoonotic bacteria of risk to humans, among which only *Bartonella* spp. was found to infect this population. Through molecular analyses we looked for prevalence of *F. tularensis* and other zoonotic bacteria among fleas, including: *Anaplasma*, *Bartonella*, *Borrelia*, *Coxiella* and *Rickettsia*. We only detected *F. tularensis* and *Bartonella* spp. in fleas. A total of 3.3% of voles carried *F. tularensis*-positive fleas, all these voles being *F. tularensis*-positive. Additionally, 31% of voles carried *Bartonella*-positive fleas, but just half of these hosts were *Bartonella*-positive voles. Both bacteria were detected in *C. apertus* and *N. fasciatus*, so both flea species could potentially act as vectors for tularemia and *Bartonella* infections. Our results suggest that fleas should be taken into account for understanding the transmission cycle of these zoonotic bacteria in the region. Further molecular surveys should be conducted to quantify transmission pathways in this host-vector-pathogen system, particularly in ecosystems where humans are in close contact with wildlife and thus exposed to such endemic diseases.

1. Introduction

Arthropod-borne pathogens are commonly surveyed due to their important medical relevance (e.g., Keesing and Ostfeld, 2018). Changes in climate and landscape have increased the risk of infection by zoonotic pathogens for humans, livestock and pets (Altizer et al., 2013; Jones et al., 2013; Mills et al., 2010). Among wildlife, rodents are a relevant target group for disease surveillance as they are abundant, widely distributed and are typically infected by many zoonotic pathogens, frequently playing an important role in their transmission cycles (Han et al., 2015). Additionally, rodents are also commonly infested by a wide range of ectoparasites (e.g., ticks, fleas, mites …), which transmit pathogens among rodents and from rodents to humans and other animals. For instance, fleas are well-known vectors that can transmit infectious pathogens to humans such as plague, rickettsioses, and *Bartonella* infections (Bitam et al., 2010). Therefore, the detection of such pathogens in fleas is a first step to determine human risks for flea-borne diseases and can help diagnosis and treatment. Studies about the simultaneous occurrence of zoonotic pathogens in rodent hosts and their fleas are also necessary to elucidate realistic transmission routes in nature (e.g., Gutiérrez et al., 2015; Hornok et al., 2015; Silaghi et al., 2016; Stevenson et al., 2003).

Some rodent populations widely fluctuate in numbers, and they offer an excellent opportunity to study the effect of varying rodent densities in the transmission dynamics and spill over patterns of pathogens in the environment. This is the case of the common vole (*Microtus arvalis*), one of the most abundant small mammals in Europe and an important vertebrate pest species in many agricultural regions (Jacob and Tkadlec, 2010). In temperate Central Europe, some studies have pointed out that common voles can be infected by different zoonotic pathogens of risk to humans, such as *Bartonella* spp., *Babesia microti*, *Trypanosoma* sp., *Francisella tularensis*, *Borrelia* spp., *Leptospira* spp., and *Rickettsia* spp. (Elashvili et al., 2015; Pawelczyk et al., 2004; Schmidt et al., 2014; Welc-Faleciak et al., 2010). In semi-arid Mediterranean environments of southern Europe, such as the agricultural landscapes of Northwest Spain, it has been recently shown that common voles are as well infected by some zoonotic pathogens of risk to humans: *F. tularensis* and *Bartonella* spp., and that the prevalence of these pathogens is positively correlated with vole density (Rodríguez-Pastor et al., 2017; Rodríguez-Pastor et al., submitted – Chapter 2.d).

F. tularensis, the etiological agent of tularemia, is a highly infective gram-negative bacterium widely distributed in Northern hemisphere. The bacterium can be transmitted via different routes including water, air and arthropod vectors (Rossow et al., 2014), and is

associated with a wide range of hosts, with lagomorphs and rodents acting as key hosts. Ectoparasites likely play an important role in the transmission and maintenance of *F. tularensis* within host populations (Petersen et al., 2009). Transmission of tularemia by ticks and diptera has been documented empirically (Hopla, 1974; Olsufiev et al., 1943; Philip et al., 1932), but evidence of flea-borne transmission of tularemia under natural conditions is still very scarce (but see Bibikova, 1977). Despite that, fleas are not considered an important vector of *F. tularensis* to humans, they may play a greater role than is currently recognized in the transmission of tularemia between rodents (Hopla, 1974; Hopla and Hopla, 1994). Indeed, the squirrel flea (*Ceratophyllus acutus*) was the first ectoparasite found infected with *F. tularensis* (McCoy, 1911). In Northwest Spain, a region where tularemia is now endemic, infection outbreaks in humans have been associated with high vole densities (Luque-Larena et al., 2017, 2015). Moreover, the seasonally-dry climatic conditions of this region results in common voles being parasitized by fleas much more frequently than by ticks (Rodríguez-Pastor et al., submitted – Chapter 2.d), but their role in the epidemiology of tularemia is still unknown.

The occurrence of tularemia in common vole populations from Northwest Spain urges to evaluate whether fleas parasitizing voles also harbour *F. tularensis*, as well as other zoonotic pathogens of risk to humans further detected in this rodent host, like *Bartonella* spp. (Rodríguez-Pastor et al., submitted – Chapter 2.d). During recent years, the genus *Bartonella* has risen medical attention as increasing reported clinical illnesses have been associated with *Bartonella* infections (Eremeeva et al., 2007; Kosoy et al., 2010; Vayssier-Taussat et al., 2016). This bacterium occurs in a variety of mammalian species and their ectoparasites all over the world (Gundi et al., 2012; Malania et al., 2016; Tsai et al., 2010). Some species of *Bartonella*, isolated from rodents, are causative agents of human diseases (Angelakis and Raoult, 2014). In many rodent species, *Bartonella* spp. prevalence is extremely high (Buffet et al., 2013; Razzauti et al., 2015) and the same rodent can be co-infected with more than one species of *Bartonella* (Morick et al., 2011; Rodríguez-Pastor et al., submitted – Chapter 2.d). Different ectoparasites of small mammals are involved in the transmission of *Bartonella* spp., but fleas harbour the greatest biodiversity of *Bartonella* spp., and are considered their main vector (Chomel et al., 2009).

The main aim of the present study is to investigate the prevalence of tularemia in fleas harboured by common voles from farming landscapes in Northwest Spain. For this, we compared the prevalence of *F. tularensis* in fleas from voles that were previously tested for tularemia (i.e., *F. tularensis*-positive vs. *F. tularensis*-negative voles). Under this pseudo-experimental setting, and if there is a real transmission pathway between fleas and voles, we expect to find greater tularemia prevalence in vectors from those hosts known to be infected by

the pathogen. By using a multiplex PCR method, in addition to tularemia we simultaneously screened several vector-borne zoonotic pathogens of risk to humans in the DNA extracted from the studied fleas, including *Anaplasma phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, *F. tularensis* and *Rickettsia* spp. As we had previously detected *F. tularensis* and *Bartonella* spp. in common voles, we expected to find both pathogens in their fleas. The results will contribute to the understanding of the role of fleas as vectors of zoonotic pathogens in natural environments.

2. Material and methods

2.1. Sample collecting

The study was conducted in Palencia province, Castilla-y-León region, northwest Spain (42°01´N, 4°42´W). Common voles were live trapped in an agricultural area using LFAHD Sherman© traps (8 cm × 9 cm × 23 cm) between March 2013 and March 2015 (see more details about the study area and trapping design in Rodríguez-Pastor et al., 2016). Captured voles were taken to the lab alive, where they were euthanatized through medical CO_2 inhalation, following a protocol approved by our institution ethics committee (CEEBA, Universidad de Valladolid; authorisation code: 4801646). Immediately after death, the fur of voles was inspected carefully for fleas. We collected and identified flea species collected from 225 individual voles. Fleas collected from each individual vole were counted and preserved in labelled tubes with 70% ethanol kept at room temperature until investigation. Fleas were later identified using a binocular microscope based on morphological criteria following Gómez et al. (2004). For this study, we selected flea pools that were collected from 90 different voles, which were analysed at molecular level in pools (191 fleas in total, 90 pools). A given pool consisted of fleas belonging to the same species, i.e., we considered those voles whose flea pools belonged to the same flea species: either *Ctenophthalmus apertus* (78 fleas in 39 pools) or *Nosopsyllus fasciatus* (113 fleas in 51 pools). Thus, the number of flea pools and the number of voles is the same, i.e., n = 90. We did not analyse pools containing a mix of different flea species. Pools were selected based on an *a priori* knowledge of *F. tularensis* prevalence in the voles that hosted them (Rodríguez-Pastor et al., 2017), but irrespective of the prevalence of other bacteria, like *Bartonella* spp. The selected flea pools came from 27 *F. tularensis* positive voles and 63 *F. tularensis* negative voles. Since we used a multiplex PCR method to analyse the DNA of the common voles that hosted the fleas studied here (see Rodríguez-Pastor et al., submitted – Chapter 2.d; and *PCR methods* sub-section below),

we also obtained information on the prevalence of other zoonotic pathogens in their rodent host, including *A. phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *C. burnetii*, and *Rickettsia* spp.

2.2. DNA extraction

DNA from each flea pool was extracted using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the standard procedures of the manufacturer.

2.3. PCR methods

Pathogen detection in the DNA extracted from fleas was carried out using a multiplex Polymerase Chain Reaction (PCR), that simultaneously detected six vector-borne pathogens (*A. phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *C. burnetii*, *F. tularensis* and *Rickettsia* spp.), combined with a reverse line blotting (RLB), as previously described (Anda et al., 2012). The same methodology was used to detect these same pathogens in common voles (see Rodríguez-Pastor et al., submitted – Chapter 2.d), including those hosting the fleas analysed here. All positive samples to any given pathogen were further tested separately using specific-probes with an individual PCR and subsequent RLB.

For detection of *F. tularensis* DNA in a flea pool, a phylogenetically informative region of lpnA (231 bp) was amplified by conventional PCR and further hybridization with specific probes by RLB, as previous described in Escudero et al. (2008). Positive samples were tested for confirmation of the results using a real-time multitarget TaqMan PCR, targeting tul4 and ISFtu2 assays (Versage et al., 2003). A negative PCR control as well as a negative control for DNA extraction was included in each group of samples tested.

2.4. Statistical analyses

As the number of fleas per pool ranged from 1 to 9 fleas, and all the fleas in each pool were screened together, we estimated an average pathogen prevalence per pool as the mean prevalence between the minimum and maximum prevalence. We thus assumed that either only one of the fleas was positive (minimum prevalence estimate), or that all the fleas from the pool were positive (maximum prevalence estimate). Average pathogen prevalence was estimated for all the fleas and for each flea species separately.

We used an analysis of variance (ANOVA) to test whether the pathogen prevalence in voles had an effect on the average pathogen prevalence in fleas. We also tested whether the

average prevalence of on pathogen in fleas was related with the average prevalence of other pathogens in fleas. A p-value < 0.05 was considered significant.

3. Results

From a total of 191 fleas (90 monospecific flea pools) we collected, 78 were *C. apertus* fleas (40.8%) and 113 *N. fasciatus* fleas (59.2%). The average number of fleas per analysed pool was 2.12 (range: 1-9). Most pools (> 70%) contained one (51.1%) or two fleas (22.2%) (Table 1).

While we detected DNA from *F. tularensis* and *Bartonella* spp. among analysed fleas, DNA from *A. phagocytophilum*, *Borrelia* spp., *C. burnetii* and *Rickettsia* spp. was not found by our analyses.

F. tularensis DNA was detected in 3.3% of flea pools (3 out of 90), which represented a mean prevalence of 6.1% (Table 1). Three *F. tularensis* positive voles hosted all the positive flea pools. The bacterium was detected in both flea species: 1 positive pool of 3 *N. fasciatus* (mean *F. tularensis* prevalence = 6.9%) and, 2 positive pools of 1 and 4 *C. apertus* (mean *F. tularensis* prevalence = 5.1%). There was significant difference between the average *F. tularensis* prevalence in fleas according to prevalence in voles (ANOVA, $R^2 = 0.072$, $F_{0.05,\ 1,\ 88} = 6.81$, $p = 0.011$). Tularemia prevalence in fleas was estimated at 6% overall, reaching 20% in fleas parasitizing *F. tularensis* positive voles (Table 1).

Bartonella DNA was detected in 31.1% of flea pools (28 out of 90, see Table 1) and was detected in pools of both flea species (37% of *N. fasciatus* pools and 23% of *C. apertus* pools). *Bartonella* spp. was detected in fleas collected from *Bartonella* positive and *Bartonella* negative voles in equal proportions (50% of pools). Estimated average *Bartonella* spp. prevalence was higher in *N. fasciatus* (65%) than in *C. apertus* (33%). There were no significant differences between the average *Bartonella* spp. prevalence in fleas and the prevalence in voles (ANOVA, $R^2 = 0.006$, $F_{0.05,\ 1,\ 88} = 0.53$, $p = 0.467$).

A simultaneous detection of *F. tularenis* and *Bartonella* spp. in the analysed flea pools never occurred, providing no evidence of coinfection in fleas. Indeed, there were no significant association between the prevalence of both pathogens in flea pools (ANOVA, $R^2 = 0.011$, $F_{0.05,\ 1,\ 88} = 0.97$, $p = 0.328$).

Table 1. Detection of *Francisella tularensis* (FRA) and *Bartonella* spp. (BART) in two species of fleas (CA: *Ctenophthalmus apertus*; and NF: *Nosopsyllus fasciatus*) collected from live common voles.

	Flea species	% FRA positive pools	% BART positive pools	Number of pools	FRA prevalence in fleas [min-max]	BART prevalence in fleas [min-max]	Number of fleas
All voles	**All fleas**	**3.3%**	**31.1%**	**90**	**6.1% [3.3-8.8]**	**51.1% [31.1-71.1]**	**191**
	NF	2.6%	37.3%	51	6.9% [3.9-9.8]	64.7% [37.3-92.2]	113
	CA	3.9%	23.1%	39	5.1% [2.6-7.7]	33.3% [23.1-43.6]	78
FRA negative voles	**All fleas**			**63**	**0**		**127**
	NF			32	0		71
	CA			31	0		56
FRA positive voles	**All fleas**			**27**	**20.4% [11.1-29.6]**		**64**
	NF			19	18.4% [10.5-26.3]		42
	CA			8	25.0% [12.5-37.5]		22
BART negative voles	**All fleas**			**45**		**44.4% [26.7-62.2]**	**93**
	NF			21		71.4% [38.1-100]	53
	CA			24		20.8% [16.7-25.0]	40
BART positive voles	**All fleas**			**45**		**51.1% [31.1-71.1]**	**98**
	NF			30		60% [36.7-83.3]	60
	CA			15		53.3% [33.3-73.3]	38

4. Discussion

The prevalence of zoonotic pathogens of risk to humans including tularemia have been previously studied in common vole populations from intensive farmland in Northwest Spain (see Rodríguez-Pastor et al., 2017 and Rodríguez-Pastor et al., submitted – Chapter 2.d). Since ectoparasites vectors may play an important role in the transmission cycle of such pathogens in nature, and as fleas are the main ectoparasites of common voles from this region, we studied their prevalence in this blood-sucking insect group. We found that *F. tularensis* and *Bartonella* spp. infected fleas carried by common voles. These results are in concordance with our previous findings, in which *F. tularensis* and *Bartonella* spp. were detected in the voles hosting the analysed fleas. Fleas were not infected with *A. phagocytophilum*, *Borrelia* spp., *C. burnetii* and *Rickettsia* spp, all which were neither found in their rodent hosts (see Rodríguez-Pastor et al., 2017 and Rodríguez-Pastor et al., submitted – Chapter 2.d).

Fleas collected from infested voles harboured *F. tularensis* DNA. However, pathogen prevalence in fleas was overall low (3.3% of pools, overall prevalence estimated at 6%). As it was expected, all the *F. tularensis* positive fleas came from *F. tularensis* positive voles (three individuals). The three voles infected with tularemia and with *F. tularensis*-positive fleas were captured in July 2014, when common vole populations reached their highest densities (Rodríguez-Pastor et al., 2017). This result supported the idea that high vole densities favour the spread of the bacteria in the environment (Luque-Larena et al., 2017) and that infected fleas might act as vector increasing the infection risk between voles and perhaps other alternative coexisting hosts (e.g., mice). The detection of *F. tularensis* in fleas thus indicates a likely role in the epidemiological cycle of the bacteria. Yet, the low prevalence of tularemia detected in fleas does not elucidate the quantitative significance of the role of fleas in the transmission and circulation of tularemia. By now, we are not able to know how the interaction between fleas, voles and tularemia occurs. The detection of a pathogen in a vector could mean that the host must have been infected; however, if the vector tests negative for the pathogen, the host might still have been infected but did not transmit the pathogen to the vector (Ostfeld and Mills, 2007). Due to the scarce knowledge about the role of fleas in the transmission of tularemia, further studies are required to monitor tularemia, not only in voles, but also in other hosts that cohabit with voles. For instance, in our study area, common voles share habitat and fleas with other small mammals, such as the wood mouse (*Apodemus sylvaticus*), the Algerian mouse (*Mus spretus*), and the white-toothed shrew (*Crocidura russula*). The social behaviour of common voles, i.e., they form colonies inhabiting underground burrows (Frank, 1957), favours their exposition to fleas harboured by other small mammals and, consequently, to be infested by

different flea species. Indeed, fleas are most abundant and diverse ectoparasite on small burrowing mammals and they can alternatively occur on the body of their host and in its burrow or nest (Krasnov et al., 2002). We have observed a flea-host specificity in the small mammal community with two flea species (*C. apertus* and *N. fasciatus*) shared by voles and mice, and one flea species (*Leptopsylla taschenbergi*) that is mainly specific to mice, although it occurs in a minor proportion in voles (Rodríguez-Pastor et al. unpublished data – Chapter 4). Pathogen-flea specificity has been also reported in previous studies (Castle et al., 2004). *F. tularensis* has been detected in at least 20 flea species of eight genera (*Amphipsylla*, *Cediopsylla*, *Ceratophyllus*, *Ctenophthalmus*, *Malaracus*, *Megabothris*, *Neopsylla*, and *Pulex*), but their specific role in the spread of this bacterium is still unclear (Olsufiev and Dunayeva, 1970). In the common voles studied here, *N. fasciatus* was the predominant flea species (59.2% of the fleas collected). This flea species infests a wide range of hosts, mainly rodent hosts with underground behaviour, such is the case of the studied species, and is considered to be implicated in the maintenance and transmission of several pathogens, such as *Yersinia pestis*, *Salmonella enteriditis*, *F. tularensis* and *Trypanosoma lewisi* (Bitam et al., 2010). However, from the three flea pools positive to *F. tularensis*, the pathogen was detected in 2 pools of *N. fasciatus* and in 1 pool of *C. apertus*, which does not provide strong evidence for pathogen-flea specificity by now.

We also detected *Bartonella* spp. in the analysed fleas, which supported the fact that fleas are a well-known vector of *Bartonella* in wild rodents (Morick et al., 2011). *Bartonella* spp. DNA was detected more frequently in fleas than *F. tularensis*, with an average prevalence estimated at 51%. Again, most of the *Bartonella* spp. positive flea pools were detected during the vole population peak in July 2014 (17 out of 28 positive pools). Thus, fleas parasitizing common voles were more frequently infected with *Bartonella* spp. than with *F. tularensis* at high common vole densities. The differences between prevalence of *Bartonella* spp. and *F. tularensis* in fleas may be related with the highest prevalence of *Bartonella* spp. detected in the hosting voles (i.e., 69.3% of voles infected by *Bartonella* spp. vs. 33.7% of voles infected by *F. tularensis* at population peak in July 2014) (see Rodríguez-Pastor et al., submitted – Chapter 2.d). Several species of *Bartonella* have been previously detected in *N. fasciatus* collected from other wild rodents, such as *Apodemus flavicollis*, *A. agrarius* and *Myodes glareolus* (Silaghi et al., 2016). In common voles, our estimated *Bartonella* spp. prevalence was higher (37%) in *N. fasciatus* pools (19 out of 51) than in *C. apertus* pools (23%, 9 out of 39).

Interestingly, no coinfection with *F. tularensis* and *Bartonella* spp. was found in the flea pools, although the simultaneous presence of both pathogens was previously detected in around 13% of voles. This coinfection in voles occurred when population density peaked (in July

2014) (see Rodríguez-Pastor et al., submitted – Chapter 2.d). This result highlights that coinfection does not follow a general pattern and is depended on the capability of pathogens to evolve and adapt to host and vector characteristics. Following this line, more studies should be conducted in order to understand how host-vector-pathogens interaction occur in wildlife.

Our results confirm that *F. tularensis* and *Bartonella* spp. occur in both wild common voles and in their fleas, which is an essential first step to establish a link between vectors and zoonotic pathogens of risk to humans circulating in nature. However, the detection of a pathogen does not necessarily imply that the hosting voles acquire the bacteria from fleas. Future studies and experiments are now necessary to confirm the functional role of fleas in the transmission of these pathogens, in particular of *F. tularensis,* amongst voles and other coexisting alternative small mammal hosts. A more realistic scenario including several flea species and rodent hosts with dynamic densities throughout time represent a challenging study target in order to ascertain transmission routes and maintenance of risky zoonotic pathogens in natural environments.

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CHAPTER 4

HOST-PARASITE INTERACTION

Dynamics and species-specific interactions between fleas and *boom-bust* rodent hosts

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Abstract

Parasitism has been suggested as one main extrinsic process influencing the population dynamics of hosts. Parasites induce reduction in host fitness, which enhances selection for host resistance mechanisms, in turn these novel host defences increase selection pressures on the parasite (i.e., parasite-host evolutionary arms race process). Experiments have shown that ectoparasites such as fleas can reduce condition, growth, life span, litter size or juvenile survival of rodent hosts. These effects might be due to a direct effect of fleas withdrawing resources from parasitized hosts, and/or to indirect effects of pathogens transmitted by fleas. The effects at the individual host level are expected to project at, and influence, host population level, but studies linking flea parasitism to host dynamics in natural populations are currently lacking. Here, we report on variations in flea burden on free-living common vole (*Microtus arvalis*), a rodent characterised by periodic fluctuations in abundance. Common voles cohabit with wood mice (*Apodemus sylvaticus*) and Algerian mice (*Mus spretus*), and fleas have the ability to switch between all these rodent hosts. We studied the whole flea community (3 species: *Ctenophthalmus apertus*, *Nosopsyllus fasciatus*, *Leptopsylla taschenbergi*) occurring in the studied rodent host assemblage, and considered each flea species-specific patterns of variation. We first evaluated how flea burden in common voles varied with vole abundance through time, and whether this variation showed direct or delayed density dependent patterns, also considering the abundance of alternative rodent hosts such as mice. We tested for negative associations between the body condition (mass relative to size) or reproductive performance (number of embryos per female) of voles and individual flea burdens. Finally, we tested for negative associations between seasonal vole population growth rates (PGR) and average species-specific flea burdens. We found that: (1) an increase in flea burdens occurred 8-months after common vole increases in abundance; (2) at the individual level, a greater flea burden was associated with a relatively poorer body condition of voles and with a reduced fertility among females (lower litter sizes); and (3) at population level, greater flea burdens were associated with a reduced vole PGR in summer and winter. These effects were most found between voles and one of the three flea species, *C. apertus*, indicating that flea-vole interactions were species-specific. Altogether the results support the hypothesis that fleas can contribute to shape the dynamics of unstable common vole populations, and more specifically that a delayed increase of flea burdens when vole abundance is reduced can play a role in maintaining the rodent population at a low-density phase, preventing common vole to quickly bounce back after a population peak. Future research should however test apart the direct versus indirect (pathogens vectored by fleas) influence of fleas on vole fitness and natural population dynamics.

Keywords: *Cenophthalmus apertus*; condition; delayed density-dependence; ecto-parasite; *Microtus arvalis*; population growth rate; reproductive output.

1. Introduction

Some rodent populations exhibit multiannual fluctuations in the form of *boom-bust* dynamics (Strayer et al., 2017) and understanding the causes and consequences of these unstable population dynamics is one of the major questions in ecology (Krebs, 2013). Density-dependent extrinsic and extrinsic processes that act with a time-delay have the potential to destabilize populations, and their influence on population growth rate (PGR) has been extensively studied in order to understand rodent dynamics (Krebs, 2002). Natural enemies, such as predators and parasites, are one of the extrinsic biotic mechanisms principally thought to be influential in generating oscillatory behaviour in rodent populations (Korpimäki and Norrdahl, 1991). In this study, we focused on the role that parasitism might play in influencing the abundance fluctuations of their hosts.

Parasites cause harm to hosts, but this harm is not always easy to demonstrate or characterize. Many examples have shown that parasitism can directly affect birth and death rates of hosts (e.g. Begon et al., 2006), but parasites often do not act in isolation and affect hosts through interacting with other factors. For example, infection or infestation may increase the vulnerability of hosts to predation and/or competition (Begon, 2009). The potential role of parasites and pathogens in driving host dynamics by reducing host abundance has been tested under controlled experimental conditions (Pedersen and Fenton, 2015). However, there is a lack of empirical evidence for natural wildlife populations. Theoretical knowledge about parasites and hosts postulates that parasites have the potential to regulate and destabilize host populations under certain conditions. This body of work has focused on endoparasites, mainly helminths, and hosts with unstable dynamics (Anderson and May, 1978; May and Anderson, 1978; Redpath et al., 2006; Tompkins and Begon, 1999). The Anderson and May model (Anderson and May, 1978; May and Anderson, 1978) predicts three destabilizing features: (i) the existence of a time delays in endoparasite abundance in relation to host population size, (ii) a low aggregation level of endoparasites during phases of high host abundance, and (iii) endoparasites should induce reduction in host survival and/or fecundity. Theoretical models predict a positive relationship between parasite abundance and host density, this generalization is complicated to test in natural systems and should be specific to each parasite taxon. For example, a main difference between endoparasites and ectoparasites is that, for the later, hosts are intermittent habitats (not ultimate habitats). Indeed, a host represents an ultimate habitat for an endoparasite, providing it with a place for living, foraging, and mating. Thus, hosts may be considered as habitat patches for most ectoparasites (Krasnov et al., 2002). When an infected host dies, its endoparasites typically die with it, but its ectoparasites may survive and find an alternative host. The time that ectoparasites spend in the hosts may not reflect the real aggregation pattern in a host population. In fact, ectoparasites can

constantly occur in hosts (e.g. Anoplura), sporadically attack the host during some stages of their life cycle (e.g. Ixodidae and Phlebotomidae), or alternate some periods on the hosts and others in their nests or burrows (e.g Siphonaptera) (Krasnov et al., 2002).

Therefore, a direct or delayed density-dependent response of an ectoparasite to changes in host density does not have the same implications than a similar response by endoparasites. A delayed response of ectoparasite burdens to host abundance (i.e., an increase in parasite numbers per host) may be a true numerical response (a change in ectoparasite population size) or may be due to a dilution effect (i.e., a reduced burden when host abundance and diversity are greater, or an increased concentration of ectoparasites when host abundance is scarce or declining, also switching to other alternative host species). In addition, ectoparasites are common vectors of pathogens (viruses and bacteria), which in turn can directly or indirectly affect the fitness and/or body condition of the host. The peculiarities of ectoparasites (i.e., capacity to change their distribution between "host habitat patches") demand to study the role they play in the dynamics of fluctuating host populations.

Fleas are the most abundant and diverse group of ectoparasites on small burrowing mammals and they can alternatively occur on the body of their host and in its burrows or nests (Krasnov et al., 2002). The sole effect of their presence on rodent hosts (i.e., not carrying or carrying fleas and how many) can have profound biological consequences. For instance, the effects of flea (*Nosopsyllus fasciatus*) parasitism on common voles (*Microtus arvalis*) have been experimentally tested at individual level and under captive conditions, showing that fleas impair host development (growth, energy consumption and immune response) and reduce survival probability and reproductive success (Devevey et al., 2008; Devevey and Christe, 2009). Moreover, parasitism on juvenile common voles has long-term effects that do not protect them from the detrimental effects of parasitism when they are adults, resulting in higher metabolic rate (Devevey et al., 2010). These experimental findings highlight that the effects of flea parasitism should be evaluated in studies of host population dynamics in nature. Understanding the consequences of fleas in wild vole populations requires novel and detailed empirical investigations in the field, but also a theoretical framework to propose mechanistic hypotheses and make predictions.

The common vole is one of the most abundant small mammal species in Europe and is an excellent model species to study the influence of direct vs. delayed density-dependence processes in shaping their dynamics (Lambin et al., 2006; Turchin, 2003). Common vole populations are characterised by *boom-bust* cyclic dynamics in many parts of Europe that, as a result, cause populations to periodically reach peak densities of up to 1,000 individuals per hectare (Jacob and Tkadlec, 2010). These high amplitude fluctuations, and a prevailing cycle period of 3 years, have long

been documented in agricultural areas from the west to the east of Europe (Lambin et al., 2006; Tkadlec and Stenseth, 2001), although the tendency of vole cycles vary geographically and their amplitudes are dampening in Europe (Cornulier et al., 2013). A key aspect of delayed density dependent effects is overcompensation, which occurs when a population grows well above its carrying capacity and population cycles occur because processes limiting population growth rate have a delayed effect on population size (Lester and Burns, 2008). Populations that exhibit overcompensation often undergo stable periodical cycles (i.e., showing fixed interval and amplitude), although higher levels of overcompensation can lead to chaotic fluctuations in densities with varying intervals or amplitudes (Lester and Burns, 2008). Common vole population dynamics in farming areas from western France were recently shown to fit to an overcompensating density-dependence pattern, which allows for carrying capacity to overshoot (Barraquand et al., 2014). Although several studies have separately investigated which intrinsic or extrinsic processes affect common vole population dynamics, the combined effects of these are still poorly understood (Krebs, 2013). Parasites or food rather than predators have been proposed as factors driving population crashes of common voles in France (Barraquand et al., 2014).

In this study, we explored the ecological role of fleas on shaping common vole population dynamics in an agricultural landscape of northwest Spain, where common vole populations apparently peak every 3 years. We also considered the densities of alternative rodent hosts, specifically the wood mouse (*Apodemus sylvaticus*) and the Algerian mouse (*Mus spretus*), which are the most abundant rodent sharing common vole habitats (Chapter 1; Rodríguez-Pastor et al., 2016). Voles and mice share the same species of fleas (unpublished data), which suggests that the response of flea burden could vary with both the abundance of the focal hosts (vole) and that of alternative hosts (mice). Our study system thus entails the study of dynamic interactions between two complete guild assemblages from different trophic levels (host assemblage 3 spp. – parasite assemblage 3 spp.). We first tested whether flea burden variation was density-dependent in relation to the three rodent hosts, and investigated if it was direct or delayed. Secondly, we tested whether the delayed density-dependence was a “true” numerical response (increase in flea numbers) or it could arise through host switching and dilution effects, within or between hosts (re-distribution of similar flea numbers). For that, we estimated the total flea population size on the rodent hosts and investigated density-dependence patterns comparing rodent numbers with flea burden. However, we did not know the flea population size outside rodent hosts, i.e., located in the burrows, which may be a limitation. Our null hypothesis was that flea burdens change only because of dilution effect. Yet, large fluctuations in the flea population size on voles and mice (i.e., what we can measure), may reflect a fluctuation in vole abundance. So it was consistent with both an increase in the total flea population size with vole abundance and a

dilution on fewer hosts. At individual level, we studied whether there is any evidence that fleas affect vole condition, reproduction and at population level, we investigated whether seasonal vole PGRs were negatively associated with flea burdens in the natural vole populations.

2. Materials and methods

2.1. Study area and trapping design

The study was carried out in the intensive farmlands of northwest Spain, Castilla-y-León region, in 6 study areas of 40 km^2 each (hereafter referred as to "populations") located in the provinces of Palencia, Valladolid and Zamora (see Jareño et al., 2014 for maps of the study areas). The agricultural landscape is dominated by monocultures of cereal (mainly wheat and barley; ca. 48% of the agricultural surface), irrigated and non-irrigated alfalfa crops (ca. 10%), fallows, pastures or meadows (ca. 21%) and a network of field margins (ca. less than 5%).

Rodents were trapped every 4-months (in March, July and November) over 6 years (July 2009 to July 2015). For each seasonal sampling in each of the six populations, we randomly selected 12 fields (4 planted with cereals, 4 alfalfas and 4 fallows, meadows or grasslands). Inside each field, 35 live-traps (8 cm × 9 cm × 23 cm; LFAHD Sherman©) were laid out with a 2 m spacing in 2 perpendicular lines forming a "T" with 10 traps located in the field margin and 25 traps inside the field; as in Rodríguez-Pastor et al. (2016). Traps were set-up in the morning baited with slices of carrots and checked and retrieved after 24h (morning of the following day). The overall trapping effort was of 420 trap-days per population and season.

2.2. Study hosts and their fleas

Three rodent species were captured in all study areas: the common vole, the wood mouse and the Algerian mouse. Most captures (62.5%, n = 1381) were of common vole, followed by wood mouse (23.6%, n = 522) and Algerian mouse (13.7%, n = 304). *M. arvalis* is a strict vegetarian species that inhabits grasslands from European areas with continental climate with cold winters. However, the species recently colonized the more seasonally-arid agricultural areas of northwest Spain, coinciding with an increase in irrigation and alfalfa crops (Jareño et al., 2015; Luque-Larena et al., 2013). In our study area, the common vole is considered a *boom-bust* pest species that undergoes recurrent outbreaks every 3-5 years. The wood mouse and the Algerian mouse are both omnivores, more reliant on seeds and well adapted to the characteristic seasonally-limiting natural conditions in the region

(summer droughts). Both mouse species show inter-annual fluctuations in abundance without a clear periodicity (Fig. S1).

Three flea species, previously described in the Iberian Peninsula, infect the three rodent species in the study areas: *Ctenophthalmus apertus gilcolladoi* (hereafter *Cteno*), which is endemic of the Iberian Peninsula throughout arid Spain central plateau (Gómez et al., 2003) and a generalist parasite of *Microtus* spp., *A. sylvaticus* and *M. spretus* (Gómez et al., 2003); *Nosopsyllus fasciatus* (hereafter *Nosop*), which has a Palearctic distribution and is known to parasitize common voles elsewhere (Devevey and Christe, 2009; Devevey et al., 2008, 2010); and *Leptopsylla taschenbergi amitina* (hereafter *Lepto*), which mostly parasitizes *A. sylvaticus*, and shows a Mediterranean distribution (southwestern Europe and North Africa) (Beaucournu et al., 1997).

2.3. *Data recorded from trapped animals*

In order to avoid under-estimating burdens, only trapped rodent hosts that arrived alive to our lab were considered for quantifying flea burdens in their populations (individual fleas often abandon carcasses of hosts that die in traps or during transport). Immediately after euthanasia through CO_2 inhalation, rodents were carefully examined for ectoparasites by firmly blowing the animal´s fur while holding the animal over a white plastic tray (520 × 420 × 95 mm) filled with water. In this way fleas typically abandon the freshly sacrificed host and jump dropping into the water tray. Collected fleas were counted and preserved in individually labelled tubes filled with 70% ethanol, and later examined under a binocular microscope (x10 and x40 magnification; Nikon Optiphot-2) to identify them at the species level based on morphological characteristics (Gómez et al. 2004; Gullan and Cranston, 2005). Since all the studied voles were dissected later on for other planned research, any possibly but most infrequently missing flea not recorded immediately post-euthanasia was updated.

Each captured common vole was sexed and weighed to the nearest 0.01 g using an electronic balance, and body length (from the tip of the nose to the base of the tail, i.e., extended body length, excluding tail length) was measured to the nearest ±1 mm using a ruler. We calculated an index of body condition (weight corrected for size) using the residuals from the relationship between Log-body weight and Log-body length (r^2 = 0.84, n = 1356, p < 0.001) (Labocha et al., 2014). Since obesity is not an issue among wildlife populations, this body mass index is a quick and easy intuitive measure of how fit/healthy an individual is physically. The condition index was estimated for both sexes, although we are aware that pregnant female voles could bias the value of the index condition. A sub-sample of females (n = 340) was dissected and those with visible embryos in the uterus under a binocular microscope were characterized as pregnant. When any trapped female gave birth in the trap during

transport from the field or in the lab, they were also considered as pregnant. We recorded the total number of embryos (i.e., litter size) of each pregnant female, as an indicator of its reproductive performance.

2.4. Statistical analysis

At the population level, we calculated the geometric mean of flea burdens (the back-transformed average of the Log-transformed number of fleas (+1) per individual) as a measure of average flea burden on a given host in a given population and season (Redpath et al., 2006). We considered overall flea burden (all flea species pooled) as well as species-specific flea burdens. Rodent species-specific capture rates were used as indicators of host density in a given population and season (number of individuals captured per 100 traps during 24h (see Jareño et al., 2014; Rodríguez-Pastor et al., 2016). We also calculated an index of overall population size of fleas on common voles and on the whole rodent community (voles and mice) in a given population and season by multiplying the host density by the average flea burden on the host(s).

We used Generalized Linear Mixed Model (GLMM) to explain flea burden variation with current and previous host densities, including the sampled populations (6 levels) as a random effect. The dependent variable (flea burden, geometric mean) was fitted to models using the package ´lme4´ (Bates et al., 2015)) and the following independent variables: the logarithm of current host density (at time $_{t}$) and the logarithm of previous host densities (4 and 8 months before, times $_{t-4}$ and $_{t-8}$, respectively). The inclusion of current and previous host densities allowed us to test for direct or delayed density-dependence responses of flea burden to host density. Correlations between the explanatory variables was examined and considered in the model selection. A series of models including the different explanatory variables were built. The full model included current and previous densities of common vole (focal host), as well as those of the wood mouse and the Algerian mouse (alternative hosts). We similarly tested the direct or delayed density-dependence of species-specific flea burdens, considered separately the burdens of *Cteno*, *Nosop* or *Lepto* instead of the overall flea burden. The final model was selected following a stepwise elimination of non-significant interactions and variables using the drop1 function in R.

The relationship between vole body condition index and flea burden was tested at the individual level using GLMMs with a normal distribution that included population (6 levels) and year (7 levels) as random effects. We fitted separate models for male and for female common voles (n = 645 and 711, respectively). The initial models included weight as dependent variable and, body length (to correct weight for size), flea burden, season and the interaction between flea burden and season

as explanatory variables. We also considered which flea species had a major effect on body condition of females and males, considering burdens of each flea species. In all initial models, the variables weight, length and flea burden Log (n+1) were log-transformed. Final model selection was conducted with a stepwise elimination of non-significant interactions and variables using the drop1 function.

We evaluated whether litter size decreased with increasing flea burden using a subset of necropsied female common voles (n = 340). We first analysed the probability of pregnancy (i.e., the probability of having embryos or not) using a GLMM with binomial distribution that included population and year as random factors. Three independent variables were included in the analyses: logarithm of flea burden, season and their interaction (flea burden × season). We first analysed the effect of overall flea burden, and then that of each flea species. We similarly investigated whether litter size (the number of embryos) varied with flea burden. We used a GLMM with a Poisson distribution that included population and year as random factors. The data set included all females captured alive and pregnant, i.e., those females with embryos. As independent variables, we included flea burden (log-transformed), season and their interaction (flea burden × season). We also tested the effect of each flea species. Model selection was by stepwise elimination of non-significant interactions and variables using the drop1 function.

Finally, we investigated the associations between seasonal vole population growth rates (PGRs) and flea burdens. Voles were sampled three times a year every four months (in March, July and November), so we calculated seasonal PGRs for the focal species at each time interval between consecutive samplings events, i.e., spring (March-July), summer (July-November) and winter (November-March) PGRs. In a given season and population, we calculated $PGR_t = Log(Ab_{t+4}) - Log(Ab_t)$; where Ab_t was the common vole abundance in month t and Ab_{t+4} was the vole abundance 4 months later. First, we evaluated whether seasonal PGRs varied with overall flea burden including current common vole density (log-transformed) and the geometric mean of flea burden as fixed factors, and population as a random factor (6 levels). We similarly tested for associations using flea species-specific burdens (geometric mean of *Cteno, Nosop* and *Lepto)* as fixed factors, and population as random effect. Model selections were based on a stepwise elimination of non-significant variables using the Akaike Information Criterion (AIC) and with drop1 function. All analyses were conducted in R v3.3.1 (R Development Core Team, 2016).

3. Results

3.1. Descriptive summary

We measured flea burdens for a total of 1381 common voles (51.85% females and 48.15% males). In addition, for alternative rodent hosts we measured 522 flea burdens for wood mice and 304 for Algerian mice. A total of 4213 individual fleas were collected and identified from these hosts (3446, 686 and 81 fleas originated from common vole, wood mouse and Algerian mouse, respectively). Two flea species were commonly found: *Nosop* (n = 1893, 45%) and *Cteno* (n = 1860, 44%), but a third, *Lepto*, was much less common (n = 460, 11%). Two flea species (*Cteno* and *Nosop*) were shared by both voles and mice; while the less abundant flea species (*Lepto*) seemed mainly link to mice (Table S1). In spring, the mean flea burden was 1.5 times higher in vole males (6.12) than in females (4.04) compared with summer (2.44 in males; 2.81 in females) and winter (2.08 in males; 1.96 in females). In spring, the mean *Cteno* burden was 1.5 times higher in vole males (4.17) than in females (2.84) compared with summer (0.80 in males; 0.93 in females) and winter (1.36 in males; 1.25 in females). The mean *Nosop* burden was higher in vole males than in females in spring (1.83 in males; 1.02 in females) and in winter (0.72 in males; 0.68 in females), and equal in summer (1.31 in males; 1.31 in females).

3.2. Flea burden variation according common vole and alternative host densities

Flea burden in common voles ranged from 1 to 28.95 with a mean of 3.88 and an aggregation coefficient ($k = \bar{X}^2 / (S^2 - \bar{X})$) of 0.90. Overall flea burden on common voles varied with both current (t) and previous (t-8 months) common vole densities (Table 1; Figs. 1a-b). The association of flea burden with contemporary vole density was negative, suggesting a dilution (fewer fleas per vole when vole abundance increase in the environment and reaches its top at outbreak peaks), but it was positive with previous vole density (delayed density-dependence response with a time lag of 8 months), which suggests a lagged numerical response (i.e., more fleas concentrated per vole when abundance of this host has dropped (bust phase) and is very low).

Table 1. Results of the GLMMs testing for associations between flea burden (average number of fleas per vole) and contemporary or previous densities of common vole or alternative host. All initial models included "population" as a random effect (6 levels) and the following explanatory variables: MA-D_t (contemporary *M. arvalis* density); MA-D_{t-4} (*M. arvalis* density 4 months before); MA-D_{t-8} (*M. arvalis* density 8 months before); AS-D_t , AS-D_{t-4} and AS-D_{t-8} (contemporary and previous -4 and 8 months before- *A. sylvaticus* densities); MS-D_t , MS-D_{t-4} and MS-D_{t-8} (contemporary and previous -4 and 8 months before- *M. spretus* densities). Separate models were used for overall flea burden (Fleas/MA) and species-specific flea burdens (*Cteno=Ctenophthalmus apertus gilcollidai*; *Nosop=Nosopsyllus fasciatus*; *Lepto=Leptopsylla taschenbergi amitina*). The asterisk denotes those log-transformed variables.

Dependent variables	Explanatory variables	χ^2	Df, error	p	Estimate ± SE
Fleas/MA_t	Intercept	29.19	1, 74	< 0.001	4.828 ± 0.894
	MA-D_t *	29.04	1, 74	< 0.001	-1.577 ± 0.293
	MA-D_{t-8} *	33.14	1, 74	< 0.001	1.484 ± 0.258
Cteno/MA_t	Intercept	18.23	1, 72	< 0.001	4.279 ± 0.717
	MA-D_t *	34.62	1, 72	< 0.001	-1.188 ± 0.214
	MA-D_{t-8} *	25.24	1, 72	< 0.001	1.377 ± 0.273
	AS-D_{t-8} *	3.94	1, 72	< 0.05	-0.986± 0.428
Nosop/MA_t	Intercept	28.19	1, 73	< 0.001	1.648 ± 0.310
	MA-D_t *	20.29	1, 73	< 0.001	-0.404 ± 0.090
	AS-D_{t-8} *	8.88	1, 73	< 0.01	0.504 ± 0.169
	MS-D_{t-8} *	6.97	1, 73	< 0.01	0.409 ± 0.155

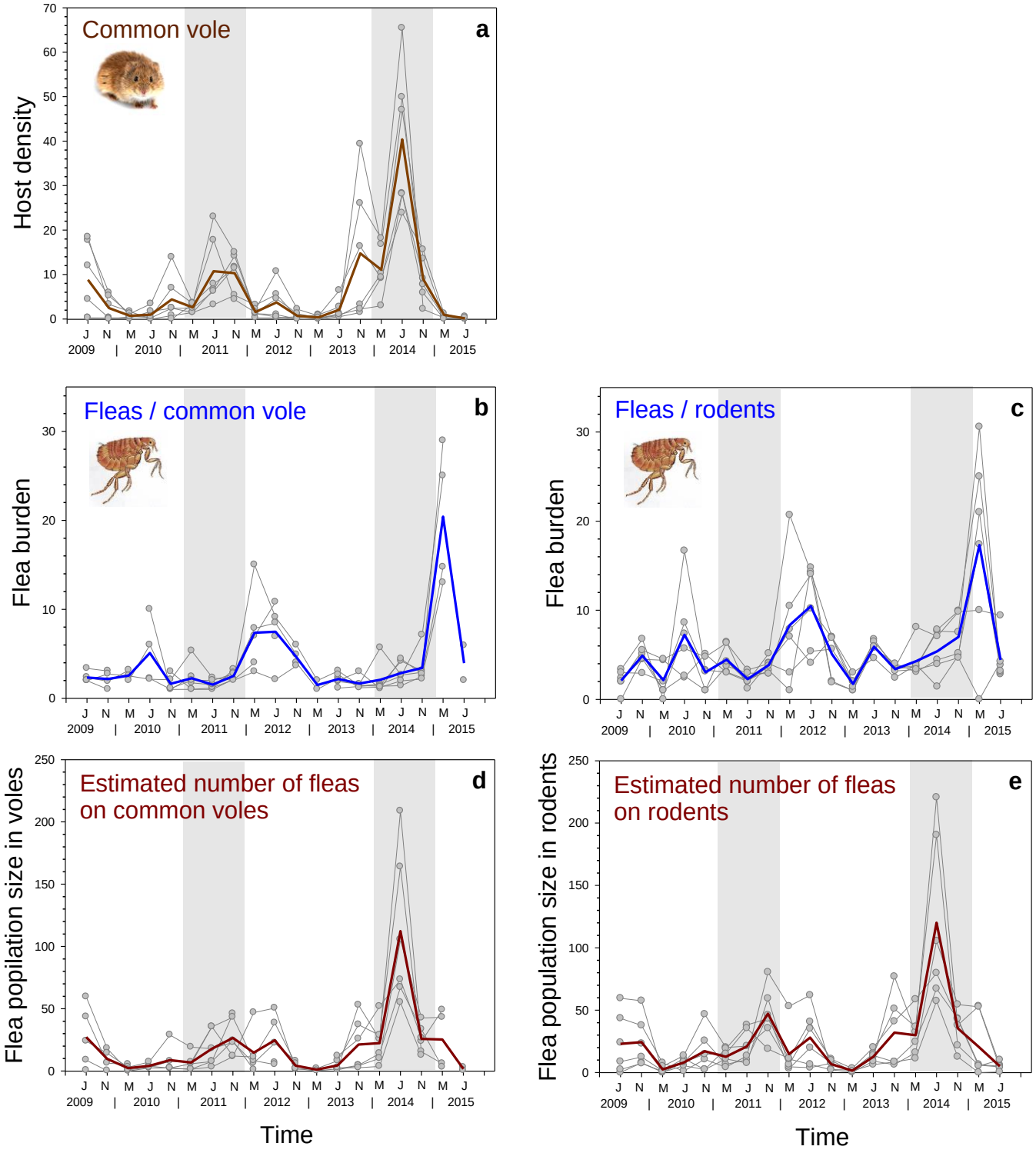

Figure 1. Temporal variations during a 6-year period (July 2009 to July 2015) in: common vole (*Microtus arvalis*) density **(a)**, flea burden on voles (fleas per vole; geometric mean) **(b)**, flea burdens on rodents (fleas per rodent, considering voles and mice; geometric mean) **(c)**, estimated flea population size on common voles **(d)**, estimated flea population size on rodents **(e)**. Graphs show the population dynamic in each of the 6 studied populations separately (grey dots-solid lines) and in the overall population (bold solid line). Grey areas indicate the population outbreaks (*boom-bust* shape variation in abundance) of common voles.

At a flea species level, *Cteno* burden in common vole ranged from 1 to 24.15 (mean = 2.53; *k* = 0.72). It was related to both contemporary (t) and previous (t-8 months) common vole densities (Table 1; Fig. 2a). Again, the association with contemporary vole density was negative, but positive with previous vole densities (delayed density-dependence with a time lag of 8 months; Table 1). *Nosop* burden in voles ranged from 1 to 10 (mean = 2.13), the aggregation coefficient (*k* = 8.58) indicated that the distribution of *Nosop* was random. *Nosop* burden varied with previous wood mouse and Algerian mouse (8 months before), and with contemporary but previous vole densities, suggesting that burdens of this flea species on voles were more dependent on previous mice densities, and decreased with current vole density (Table 1; Fig. 2b). Finally, *Lepto* burden in common vole ranged from 1 to 2 (mean = 0.05; *k* = 0.24), and did not vary significantly with hosts densities (Table 1; Fig. 2c).

Total flea abundance on the three main rodent host (flea burden multiplied by rodent abundance), and which includes voles and mice, fuctuated seasonally and temporally synchronized with rodent host abundance (Figs. 1c, d and e). Flea numbers on rodent hosts were not constant in time, suggesting that the flea population fluctuates in an order of magnitude similar to vole abundance (Fig. 1e).

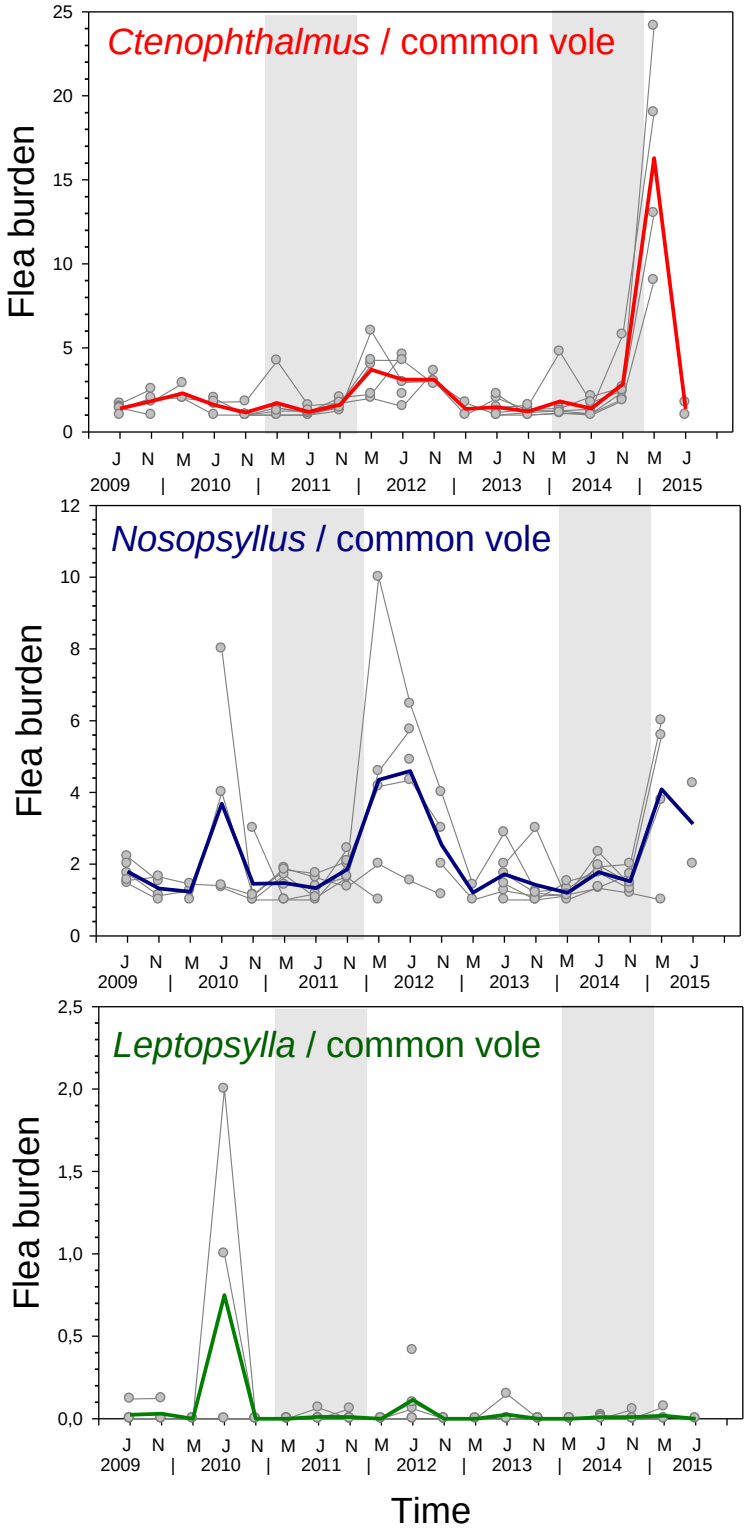

Figure 2. Species-specific temporal variation in flea burdens (geometric means) on their common vole host **(a)** *Ctenophthalmus apertus gilcollidai* **(b)**, *Nosopsyllus fasciatus* **(c)** and, *Leptopsylla taschenbergi amitina*. Burdens on common vole population were studied during a period of 6-years, from July 2009 to July 2015. Graphs show the dynamic in each of the six studied populations separately (grey dots-solid lines) and in overall populations (bold solid line). Grey areas indicate the population peaks of common vole.

3.3. Common vole condition index and reproduction according to flea burden

Body condition in male voles (n = 645) was negatively associated with overall flea burden in spring and winter. At a flea species level, this pattern was found when flea burdens were of *Nosop*. A negative correlation between male condition and *Cteno* burden was also found but in all seasons (Table 2). Female vole condition varied between seasons, but was unrelated to overall flea burden, which could be the result of moving less and be more social than males. When considering species-specific flea burdens, we found a negative association between female vole condition and *Nosop* burden in all seasons (Table 2).

Common vole females with an open vagina and with embryos and/or placental scars were considered reproductively active, whereas females with a closed vagina and without embryos and placental scars and/or embryos were considered as reproductively inactive. The number of reproductively active females was higher in summer (n = 206) than in spring (n = 48) and winter (n = 54), thus we can suggest The probability of pregnancy varied between seasons ($\chi^2_{2, 324}$ = 9.30, Df = 2, $p < 0.01$) and tended to decrease with increasing flea burden ($\chi^2_{1, 324}$ = 3.47, Df = 1; p = 0.063), but was not explained by the burden of any given particular flea species. Pregnancy rate was higher in March (mean ± se: 0.46 ± 0.07) and July (0.28 ± 0.03) than in November (0.18 ± 0.05). In pregnant females, the number of embryos per female also varied significantly between seasons being greater in March (mean = 5.34; CI (95%) = 4.65 - 6.04) and July (5.02; 4.49 - 5.54) than in November (3.57; 2.49 - 4.65). More significantly, the number of embryos was negatively correlated with the overall flea burden, and more specifically with *Cteno* burden (Table 3). The interaction between flea burden and season was non-significant. Noteworthy, an increase in flea burden from 1 to 10 fleas per female vole was associated with a two-embryo reduction in litter size.

Table 2. Results of GLMMs testing for associations between body weight (corrected for body length, included as a covariate) and flea burden according to host gender and season. Separate models investigated the effects of overall flea burden (number of fleas per host) or species-specific flea burdens (number of *Cteno*, *Nosop* or *Lepto* per host). Models included "Population" and "Year" as random factors. The asterisk denotes those log-transformed variables.

Dependent variable	Explanatory variables retained	χ^2	Df	p	Estimates ± SE
Male weight*	Intercept	2385.11	1, 627	< 0.001	-9.764±0.200
	Body length*	4556.75	1, 627	< 0.001	2.814 ± 0.042
	Fleas*	9.67	1, 627	< 0.01	-0.039 ± 0.012
	Season	23.33	2, 627	< 0.001	Summer: -0.089 ±0.021 Winter: -0.119 ± 0.026
	Fleas* x Season	20.24	2, 627	< 0.001	Summer: 0.058 ± 0.014 Winter: 0.013 ± 0.019
Male weight* [1]	Intercept	2418.74	1, 626	< 0.001	-9.819 ± 0.200
	Body length*	4542.10	1, 626	< 0.001	2.823 ± 0.042
	*Cteno**	5.12	1, 626	< 0.05	-0.017 ± 0.007
	*Nosop**	2.96	1, 626	0.085	-0.029 ± 0.017
	Season	25.27	2, 626	< 0.001	Summer : -0.062 ± 0.018 Winter: -0.109 ± 0.022
	*Nosop** x Season	8.27	2, 626	< 0.05	Summer: 0.048 ± 0.019 Winter: 0.011 ± 0.026
Female weight* [2]	Intercept	1508.78	1, 692	< 0.001	-8.808 ± 0.227
	Body length*	2907.51	1, 692	< 0.001	2.601 ± 0.048
	Season	17.80	2, 692	< 0.001	Summer: -0.040 ± 0.017 Winter: -0.090 ± 0.022
Female weight* [3]	Intercept	1519.69	1, 695	< 0.001	-8.815 ± 0.226
	Body length*	2921.70	1, 695	< 0.001	2.603 ± 0.048
	*Nosop**	5.02	1, 695	< 0.05	-0.019 ± 0.008
	Season	16.33	2, 695	< 0.001	Summer: -0.032 ± 0.017 Winter: -0.084 ± 0.021

1.- The variables *Lepto**, *Cteno** x Season and *Lepto** x Season were dropped from the final model.

2.- The variables Fleas* and Fleas* x Season were dropped from the final model.

3.- The variables *Lepto**, *Cteno**, *Lepto** x Season, *Nosop** x Season and *Cteno** x Season were dropped from the final model.

Table 3. Results of GLMMs testing for associations between the number of embryos per pregnant female vole and flea burden according to season. Models included "Population" and "Year" as random effects. The dependent variable was fitted to models using a Poisson error distribution. The asterisk denotes those log-transformed variables.

Dependent variable	Explanatory variables retained	χ^2	Df	p	Estimate ± SE
Number of embryos [1]	Intercept	405.99	1, 83	< 0.001	1.752 ± 0.087
	Fleas*	5.59	1, 83	< 0.05	-0.132 ± 0.056
	Season	6.68	2, 83	< 0.05	Summer: 0.029 ± 0.106
					Winter: -0.365 ± 0.163
Number of embryos [2]	Intercept	385.84	1, 83	< 0.001	1.759 ± 0.089
	*Cteno**	6.76	1, 83	< 0.01	-0.184 ± 0.071
	Season	5.31	2, 83	0.07	Summer: -0.070 ± 0.098
					Winter: -0.376 ± 0.164

1.- The variable Fleas* x Season was dropped from the final model.

2.- The variables *Nosop**, *Lepto**, *Cteno** x Season, *Lepto** x Season and *Nosop** x Season were dropped from the final model.

3.4. Common vole population growth rate (PGR) and flea burden

The spring population growth rate of common voles (spring PGR, from March to July) was positively correlated to current vole density (positive direct density-dependence), but negatively to overall flea burden (all species combined; Table 4). When considering the species-specific flea burdens, spring PGR of voles appeared negatively related to both *Cteno* and *Lepto* burdens. Summer PGR (July to November) was negatively related to both current vole density (negative direct density-dependence) and overall flea burden (Table 4). Summer PGR decreased with increasing burdens of *Cteno* but not with those of other flea species. Similarly, winter PGR (November to March) was negatively related to current vole abundance (negative direct density-dependence) and with overall flea burden, more specifically with *Cteno* burden. Thus, after accounting for direct-density dependence, vole PGR was negatively related to flea burden in all seasons, and was consistently negatively associated with burdens of one flea species, *Cteno* (Fig. 3).

Table 4. Results of GLMMs testing for associations between seasonal Population Growth Rates (PGR) of common voles and flea burden. All models included the variable "Population" as a random effect. We included as explanatory variables the contemporary vole density (MA-D) to test for direct density-dependence, and either overall flea burden (all species combined; Fleas/MA) or species-specific flea burdens (*Cteno*/MA; *Nosop*/MA; *Lepto*/MA). We present the results of the final models (after a stepwise backward selection implemented with the drop1 function in R). The asterisk denotes those log-transformed variables.

Dependent variable	Period	Explanatory variables retained	Chi^2	Df	p	Estimate ± SE
Spring PGR	March-July	Intercept	2.54	1, 18	0.125	0.462 ± 0.290
		$MA\text{-}D_{March}$*	4.29	1, 18	< 0.05	0.351 ± 0.170
		Fleas/MA	11.00	1, 18	< 0.001	-0.060 ± 0.018
Spring PGR [1]	March-July	Intercept	2.13	1, 17	0.119	0.433 ± 0.297
		$MA\text{-}D_{March}$*	3.90	1, 17	< 0.05	0.346 ± 0.175
		Cteno/MA	9.36	1, 17	< 0.01	-0.073 ± 0.024
Summer PGR	July-November	Intercept	21.12	1, 24	< 0.001	1.925 ± 0.419
		$MA\text{-}D_{July}$*	35.48	1, 24	< 0.001	-0.824 ± 0.138
		Fleas/MA	6.68	1, 24	< 0.01	-0.158 ± 0.061
Summer PGR [2]	July-November	Intercept	19.58	1, 24	< 0.001	2.000 ± 0.452
		$MA\text{-}D_{July}$*	34.88	1, 24	< 0.001	-0.840 ± 0.142
		Nosop/MA	6.13	1, 24	< 0.05	-0.246 ± 0.099
Winter PGR	November-March	Intercept	6.56	1, 23	< 0.05	1.170 ± 0.457
		$MA\text{-}D_{November}$*	24.69	1, 23	< 0.001	-0.750 ± 0.151
		Fleas/MA	4.86	1, 23	< 0.05	-0.214 ± 0.097
Winter PGR [3]	November-March	Intercept	8.76	1, 23	< 0.01	1.164 ± 0.393
		$MA\text{-}D_{November}$*	26.25	1, 23	< 0.001	-0.699 ± 0.137
		Cteno/MA	7.34	1, 23	< 0.05	-0.337 ± 0.124

1.- The variables *Nosop*/*MA* and *Lepto*/MA were dropped from the final model.

2.- The variables *Cteno*/MA and *Lepto*/MA were dropped from the final model.

3.- The variables *Nosop*/*MA* and *Lepto*/MA were dropped from the final model.

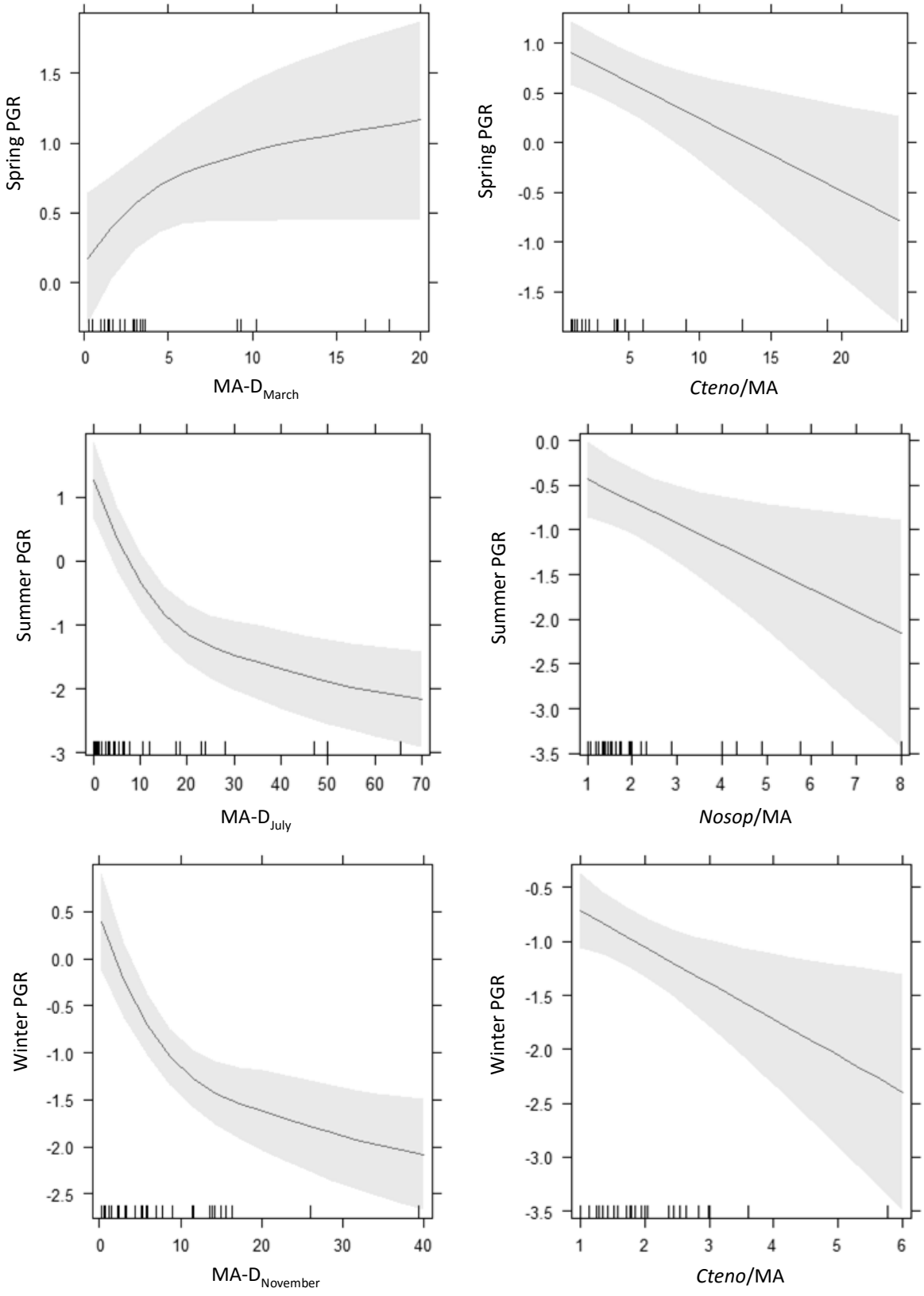

Figure 3. Associations between Population growth rates (PGR) of common vole in spring, summer and winter and contemporary density (MA-D); and flea burden (number of *Ctenophthalmus apertus gilcollidai*, of *Nososyllus fasciatus* and of *Leptopsylla taschenbergi amitina* (*Cteno*/MA, *Nosop*/MA and *Lepto*/MA, respectively). Grey areas denote 95% confidence intervals of the predicted curves.

4. Discussion

In this study, we provided new evidences about the role that fleas might play in the dynamics of their rodent hosts by studying a natural system of parasite-host populations. In particular, we found that high burdens of *Cteno* fleas may contribute to impair vole population growth. We found a delayed density-dependence response of flea burden to common vole density, consistent with the hypothesis that ectoparasites such as fleas, alone or in combination with the pathogens they vector, may contribute to maintain a low population density phase after a peak in common vole abundance. Two flea species (*Cteno* and *Nosop*) seem to have negative effects on vole body condition, which can vary between host sexes. Remarkably, the increase in the burden of one flea species (*Cteno*) was associated with a reduction in the number of embryos carried by vole females. Finally, fleas seem to limit seasonal population growth of common voles, as evidenced by the negative correlations found between flea burden and population growth rate in spring, summer and winter.

Despite the harm that arthropod ectoparasites such as fleas can usually cause to their hosts (Devevey and Christe, 2009; Devevey et al., 2008, 2010), their role in influencing host population dynamics is difficult to demonstrate empirically. Since arthropod parasites (ticks, fleas) are frequently vectors of pathogens, the effects of parasites on the host may be masked by the effects from the pathogen, or their interaction. This issue continues to be one of the major unresolved questions in ecology. Many examples have shown that parasites can affect directly host demographic rates, i.e. death and birth, and can reduce the survival and fecundity of the hosts, but, is this enough to destabilize host populations?. The association between parasite burden and population growth may be density-dependent or delayed density-dependent, but only the latter could contribute to multi-annual fluctuations in numbers (Krebs, 2013). However, few studies have evaluated the ecological role of arthropod parasites in modulating rodent host populations with *boom-bust* dynamics. Factors different from parasitism have been pointed as the cause of vole dynamics elsewhere, such as variation of predation (Hansson and Henttonen, 1985; Hanski et al., 2001; Gilg et al., 2003; Turchin, 2003; Begon et al., 2006, but see Krebs, 1996 and Ergon et al., 2011) or food resources (e.g. Krebs et al., 2010, Boonstra and Krebs, 2012). Yet, a delayed density-dependence response has been reported in the case of pathogens and decline phase of fluctuating vole populations in northern Europe (Soveri *et al.* 2000).

In our study, we show that flea burden in common voles is positively correlated with vole densities attained 8 months earlier, i.e., 8-month time lag between the increase in common vole abundance and a synchronous increase in the number of fleas per vole. In particular, the monitoring

of common vole populations during a 6-year period (July 2009 to July 2015) suggests that vole abundance peaks every 3 years (which is the common trend elsewhere in Europe; Jacob and Tkadlec, 2010), and that after vole population peaks (July 2011 and July 2014 in this study), flea burden on common voles increase (March 2012 and March 2015, respectively), and through negative effects on individual performance may contribute to maintain vole population densities low eight months later. The basic mechanistic loop boosted by our data suggests that flea population size increases during vole population increases (*boom* phase), bottom-up process, but since vole numbers rapidly collapse (*bust* phase), the enhanced flea population re-distributes among the remaining few and spatially scattered voles in the environment. These remaining voles with high loads of fleas concentrating on them result relatively impaired in terms of survival and reproductive performance, which may maintain vole numbers down during a variable period of time (low phase in-between *boom-bust* outbreaks). These intermittent episodes in which the flea population dilutes or concentrates into their also contracting-expanding main resource (*boom-bust* rodent host population) suggest a potential naturally regulating feed-back scenario based on trophic ecological interactions. Yet, whether the delayed impact of fluctuating fleas on vole PGR is quantitatively enough to avoid density re-bounds, and for how long, remains a fundamental question to be addressed.

The observed dynamic patterns of fleas in our system were detected not only at community level (overall mean number of fleas), but also at the species level (in *Cteno* and *Nosop*; Fig. 2). Thus, these results suggest that some flea species, the most abundant in the studied system, may play a role in shaping common vole dynamics. A limitation of our study, however, is that we cannot infer precisely how the total flea population size varied over time, because we could only estimate it by considering fleas that are on, carried by, their rodent hosts, but not those fleas that remain in the nests and burrows. In any case, and as we do with their rodent hosts, here we assume that the proportion of the flea community sampled in the studied system is a representation (proxy) of the total flea population in the environment. The few previous empirical studies on flea-rodent study systems yielded similar findings. In in the area between the Amur and Bureya Rivers (Russia), the increase in abundance of two flea species (*Megabothris advenarius* and *M. asio*) followed after the increase in the abundance of their small mammalian hosts, showing a one-year time-lag pattern (Krasnov, 2008). A delayed density-dependence with a lag of 1 year has been also found between field voles (*M. agrestis*) densities and the probability of flea infestation in northwest England (Telfer et al., 2007), although flea infestation was based on the presence or absence of fleas as a binary response variable. A possible explanation for such delayed response may be a dilution effect. Dilution occurs when high host numbers dilute the distribution (and thus impact) of fleas among hosts, reducing vole-flea interactions and potential vectored-disease risk. Thus, such pattern may not be a true delayed density-

dependence in common voles. It is possible that the delay in flea abundance is indirectly related to a delay in the fleas' reproductive response to changes in host density, i.e., the rates of flea reproduction and transmission can be lower than the rate of reproduction and dispersal of the hosts (Krasnov, 2008). For example, the development time of many fleas is longer than the time of pregnancy and postnatal development of many small mammals (Krasnov, 2008). The negative association between flea burden and current vole densities is consistent with a dilution effect (Krasnov et al., 2007, 2002; Telfer et al., 2007). High common vole densities imply a great number of hosts, decreasing the probability that each will be infested by fleas because the fleas can homogenize their distribution within the common vole population. Moreover, the decrease or the lack of increase in flea abundance in relation to an increase in host density could be the result of anti-parasitic behavioural activities of spatially-clumped hosts, such as repelling or killing fleas by grooming (Stanko et al., 2002).

We showed a negative correlation between body condition of common voles and *Cteno* and *Noso*p flea burdens in spring and winter. The lack of correlation between *Cteno* fleas and condition of females may be due to the inclusion of pregnant females in the data. During the reproductive season males increase their androgen levels, which consequently supress the immune function leading to a higher infestation by ectoparasites (Folstad and Karter, 1992). By now, we do not have certainty of the intensity of reproduction through the different seasons. Basing on reproductive sign in captured females, we could infer that the main breeding season occurred in spring for common voles. Similarly, a study conducted in northeast Spain found that the intensity of parasitization by fleas was higher in males of *A. sylvaticus* during spring but not in winter, while no such differences were observed for females (Cevidanes et al., 2016). We can suggest that the negative consequences of fleas on the body condition of males during spring could be the result of breeding activities (e.g. increase in the home range and movements of males looking after females and mattings). As consequence, there would be an increase in the frequency of encounters with more ectoparasites, as well as with more direct or indirect contacts with other common vole individuals or with other rodent species, for example, due to more visits to burrows. Males are also more solitary than female common voles, which typically clump together, and thus may not benefit of mutual grooming for removing of parasites.

Body condition of both vole genders was negatively correlated to the number of particular flea species during all seasons, which is a reflection in nature of what experiments have shown with *Nosop* and *M. arvalis*. Under experimental conditions, residual body condition in common vole males infested by *Nosop* tended to be lower than in non-parasitized voles (Devevey and Christe, 2009). During reproductive season, females of bank vole (*Myodes glareolus*) are characterised by higher level of residency, especially during pregnancy and lactation (Gliwicz, 1988). Thus, a higher flea burden in

females than in males may be due to the accumulation of fleas in their burrows or nests, i.e., females will be more infested because they will spend more time in the nests. Our results showed that the reproductive activity of females, i.e., number of embryos and litters per female, varied seasonally and covaried with by flea infestation, in particular by the species *Cteno*. Thus, *Cteno* seems to affect breeding performance of females and then, PGR. This specie has a key ecological role.

These negative effects of fleas on individual voles could have repercussions at population level. Indeed, population growth rates of common voles were negatively correlated to flea burden in spring, summer and winter. The positive relationship between common vole abundance and spring growth rate could points that there may be other factors, apart from parasites, which may also regulate common vole populations during the spring, such as food, habitat availability and/or winter temperature. On the contrary, summer and winter growth rates were negative related to both vole density (negative density-dependence) and flea abundance suggesting that during these seasons parasites may have a role on vole populations. *Cteno* was the only flea species that was negatively associated with common vole PGR during all seasons, which could be due to its highest abundance compared with the other flea species.

Overall, our results demonstrate that flea burden varied dynamically in fluctuating common vole populations. Fleas likely adversely affect common vole body condition, pregnancy probability and the vole population growth rates, and also exhibit an apparent delayed-density dependence that may occur through dilution effects. Therefore, it is likely that the higher flea burdens observed on voles 8 months after a population peak contribute to maintaining a low phase, preventing vole population to quickly rise again. Our observations are consistent with the hypothesis that common vole fluctuations could be caused by density-dependence overcompensation, like in western France (Barraquand et al., 2014), where fleas have a role to play. Our results also highlight a need to better understand the roles of pathogens transmitted by fleas in natural populations, and to evaluate if pathogen and flea interactions together can be the true cause of delayed density-dependence in vole populations.

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work (JJLL, FM and RRP held animal experimentation permits of level B for Spain) and euthanize was made following a protocol approved by our institution ethics committee (CEEBA, Universidad de Valladolid; authorisation code: 4801646). Capture permit was provided by the Consejería de Fomento y Medio Ambiente, Junta de Castilla y León (Expte: EP/CYL/665/2014).

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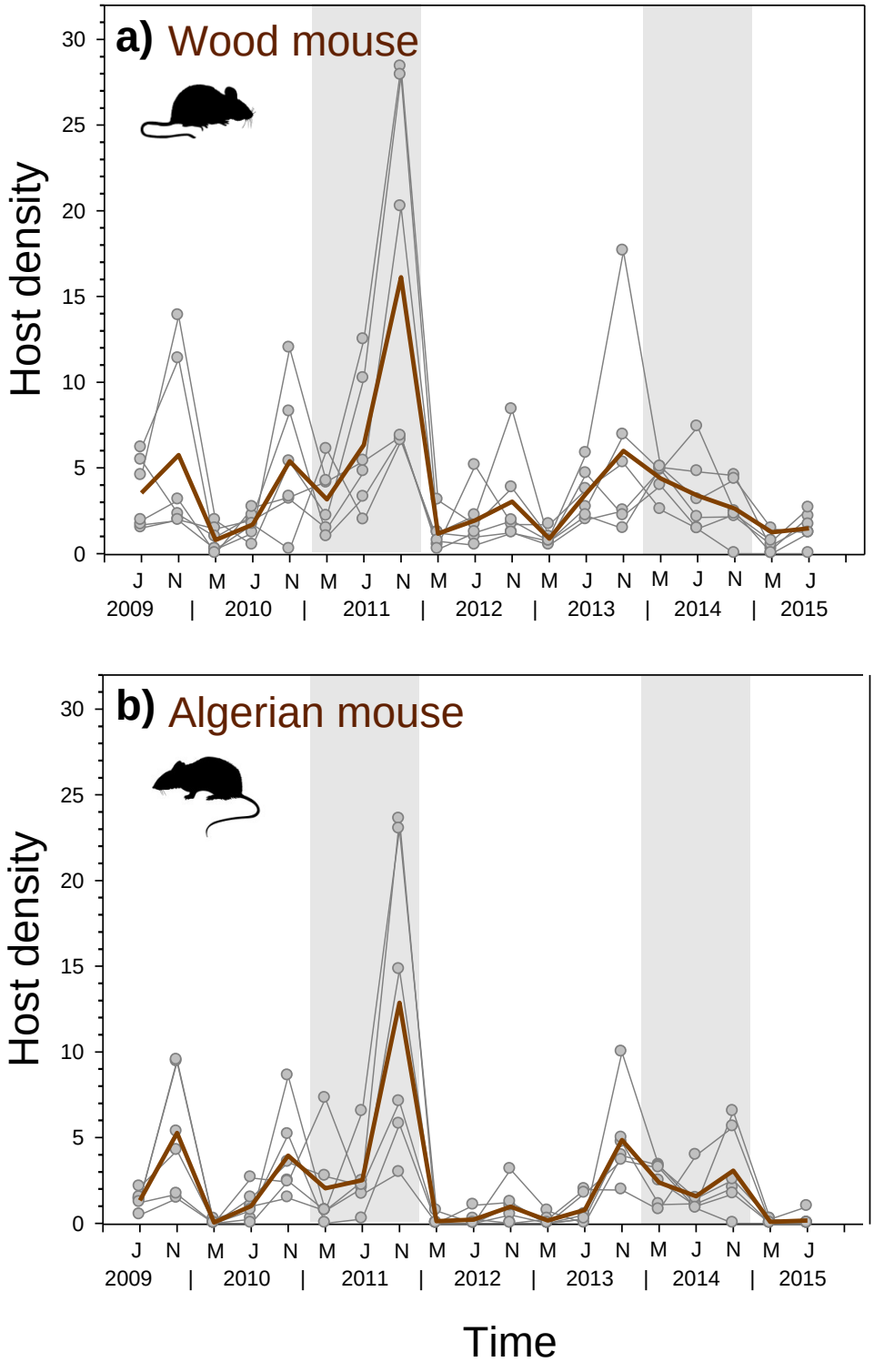
SUPPLEMENTARY MATERIAL

Table S1. Number of flea species identified per rodent host (AS: *Apodemus sylvaticus*; MA: *Microtus arvalis*; MS: *Mus spretus*) from July 2009 to July 2015 in the study area. *Cteno*: *Ctenophthalmus apertus gilcollidai*, *Lepto*: *Leptopsylla taschenbergi amitina* and *Nosop*: *Nosopsyllus fasciatus*.

Host species	Host examined	Host infested	Number of fleas collected	Flea prevalence (%)	Number of *Cteno* collected	*Cteno* prevalence (%)	*Cteno* mean intensity ± SD	Number of *Lepto* collected	*Lepto* prevalence (%)	*Lepto* mean intensity ± SD	Number of *Nosop* collected	*Nosop* prevalence (%)	*Nosop* mean intensity ± SD
AS	522	238	686	45.59	116	14.4	1.55 ± 1.17	387	26.8	2.76 ± 2.98	183	22.2	1.57 ± 1.09
MA	1381	942	3446	68.21	1731	39.1	3.21 ± 5.02	34	2.1	1.17 ± 0.60	1681	46.6	2.61 ± 2.07
MS	304	49	81	16.12	13	3.6	1.18 ± 0.60	39	5.9	2.17± 1.65	29	6.9	1.38 ± 0.92

Figure S1. Temporal variations in wood mouse (*Apodemus sylvaticus*) density **(a)** and Algerian mouse (*Mus spretus*) density **(b)**, from July 2009 to July 2015. Graphs show the population dynamic in each of the six studied populations separately (grey dots-solid lines) and in overall populations (bold solid line). Grey areas indicate the population peaks of common vole (*Microtus arvalis*).



GENERAL DISCUSSION

Humans and rodents cohabit and share infectious diseases. In agrarian ecosystems, changing landscapes can contribute to the invasion of these anthropogenically-transformed habitats by wild animals. Such invasion can in turn promote exposition to new infectious agents and increase infection risk for humans and domestic animals. These contemporary functional and numeric changes in farming ecosystems often translate into local economic and social consequences (impacts): for agriculture in the form of plant damage, and for public health through epidemic risk. Trying to avoid or reduce the impact of zoonotic diseases now and in the future requires an integrative and interdisciplinary view that includes the study, at landscape level, of the variation of distribution and abundance of animals with substantial epidemiological roles (vectors, hosts, reservoirs) and how it influences the variation of risky-pathogen populations in nature.

It is thus necessary to implement a dynamic approach to any ecological study on disease, taking into account both the spatial and temporal variations in the abundance of vectors, pathogens and hosts, which may greatly change over time, particularly so if some of the organisms involved in the epidemiology of disease display boom-bust population dynamics. Taking into account such spatial–temporal interactions can greatly contribute to mechanistically understand, and thus explain, dynamic patterns of infection risk of irruptive diseases linked to wildlife such as zoonoses. The scientific approach of adding community ecology to understand and manage zoonotic disease risk is the basic aim and strength of global initiatives pursuing a fully integrated approach of dealing with global health in the planet: the “Eco-Health” concept and scientific discipline of Conservation Medicine. Such worldwide concept assumes that human, wildlife and environmental health are linked because humans, wild animals and companion animals cohabit and interact with each other in the ecosystem. Thus, the study of the biological and demographic processes is fundamental in order to promote and improve the health and welfare of all species.

In this thesis I used a dynamic and interdisciplinary approach to the study of interactions between agrarian habitats, boom-bust populations of rodent hosts, vectors and zoonotic pathogens. For this purpose, I combined the ecological and epidemiological views of disease. Based on a well-structured seasonal monitoring scheme of vole populations, I was able to: (i) identify the reservoir habitats of common voles at low densities, as well as their spatial spreading patterns in the landscape when abundances rise (outbreaks); (ii) confirm that the studied vole populations are infected with two main bacterial zoonotic pathogens of risk to humans (*F. tularensis* and *Bartonella* spp.), and that the fluctuations in abundance of the rodent populations were associated with a co-varying prevalence of both pathogens among voles; (iii) provide new evidence that vole outbreaks contribute to the amplification and spill-over of tularemia in the environment and that voles are the dynamic driver of

tularemia epidemic outbreaks among humans in the studied system, irrespective of whether *F. tularensis* cycles in terrestrial and/or aquatic habitats; (iv) show that fleas collected on voles also carry these pathogens, suggesting a role as vectors of the zoonotic pathogens detected in voles; and finally, (viii) show that flea burden on voles varied in a delayed-density dependent manner that can be explained by a dilution effect and that can contribute to maintaining the low density phases of common vole populations (Fig. 4).

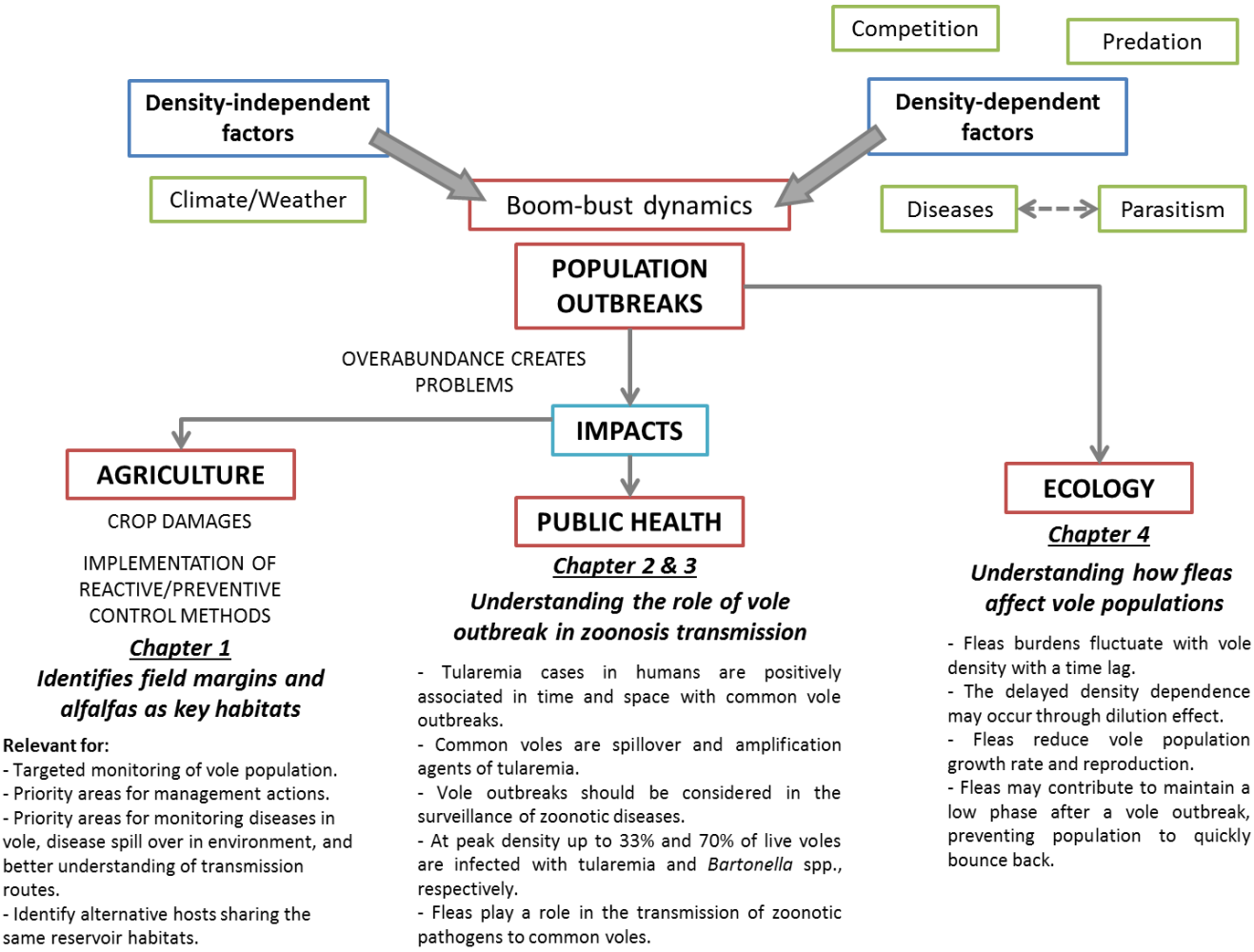


Figure 4. Graphical summary of the main findings of the different chapters of this thesis and of their implications. Grey lines represent the interactions studied.

My technical research approach (global methodology applied through the thesis) aimed to be in line with the "*ideal world of population data*" paradigm described by Krebs (1999), which is based in the quantitative monitoring of study systems at temporal, spatial, individual and community scales. This thesis sets up the observational knowledge baseline for progressing towards the next fundamental step in understanding rodent population dynamics: the experimental approach.

Dynamics of space use by common voles: implications for crop protection and public health

The seasonal monitoring of common vole populations has permitted to identify the reservoir habitats of common voles in the farmland of northwest Spain (Castilla-y-León region), in particular, field margins and alfalfa crops. Field margins harboured vole abundances more than twice higher than inside crop fields, despite being a limited habitat in occurrence (<5% of the agricultural surface) in the study area (intensified farming landscape). One of the reasons for the higher abundance of voles in margins is that vegetation characteristics of margins hardly change seasonally and the soil remains undisturbed (Briner et al., 2005; Butet et al., 2006; de Redon et al., 2010; Delattre et al., 2009). Perennial alfalfa crops are also a suitable and most preferred habitat for common vole in farmlands (Heroldová et al., 2004; Luque-Larena et al., 2018). The soil of this fodder crop typically remains undisturbed in the absence of yearly ploughing for several successive years, which allows the burrowing of stable colonies by voles. Alfalfas supply vole colonies with protective cover and important quantities of protein-rich biomass for most of the year. In fact, this leguminous crop is considered a key driver of the recent vole colonization of the study region (Jareño et al., 2015; Luque-Larena et al., 2018, 2013). Field margins can sustain high population densities from where voles can spread inside adjacent fields during population outbreaks. Hence, vole abundance typically shows a decay from the margin towards the inside of crop fields. However, the magnitude of this edge effect varied depending on crop type, season and vole abundance (density-dependence) (Fig. 4 in **Chapter 1**). The incursion of voles inside fields may be due to margins being not large enough areas to permit substantial population growth and consequently, voles spread inside fields causing damage to crops. Because voles are hosts of different pathogens, the incursion of voles inside fields also favours the spill-over of pathogens in the environment. Some of these pathogens are prejudicial for human health and other animals. Thus, finding out where the reservoir habitats are located, and how voles spread in the farmland is relevant to identify the ecological niche of pathogens and areas of elevated risk for human exposure to bacteria within endemic regions (i.e., identify the hotspots or local focus where pathogens occur). This also contributes to a better understanding of the possible routes of pathogen transmission.

The dominant crops in the study area are cereals (wheat, barley). Cereal crops represent about 48% of the whole agricultural surface in the study area, which is in turn the main land use in the region (Jareño et al., 2015). However, these habitats are the least optimal for voles, yet with an important spill-over of colonizing voles from the neighbouring field margins occurring in summer during the largest population increases (boom phase). This finding should be taken into account when surveying zoonotic pathogens in nature and planning prevention. Expectations are that, during a year with an

overabundance of voles, cereal fields will be progressively invaded by voles, as well as their pathogens (spatial displacement), potentially increasing in space the infection risk for humans and other animals, also favouring new transmission routes. During the 2011 vole peak of moderate amplitude in the study area, I recorded increasing densities of voles in field margins, alfalfas and fallows in 2011, but not a massive invasion of cereal fields (Fig. 3 in **Chapter 4**). During that year, very few cases of tularemia in humans were reported (2 official cases; Fig. 1 in **Chapter 2.a**). An explanation for the low number of human cases registered in one of the regions of Europe with lower human density (Castilla-y-León region) could be the lack of invasion of voles inside cereals, the dominant habitat type, and an overall reduced environmental contamination of farmlands by the pathogen (Fig. 3 in **Chapter 4**). By contrast, during 2013-2014, a comparatively larger vole outbreak greatly increased abundance of rodents in all four habitats (Fig. 1 **Chapter 2.a**), with a concomitant increase in *F. tularensis* prevalence in voles (Fig. in **Chapter 2.b**). During 2014 (vole peak numbers in July), a significant peak in the number of human tularemia cases was also recorded, with 96 officially declared cases, when the yearly average during years outside outbreaks is 2-3 (Fig. 1 in **Chapter 2.a**). The temporal association between vole population peaks and human outbreaks of tularemia, as well as the increased tularemia prevalence in overabundant voles suggests that their boom-bust populations have a key role in the transmission cycles of tularemia in the studied region.

Rodents have the potential to transfer zoonotic enteric foodborne pathogens to human food crops by faecal contamination (Jay-Russell, 2013). Crop contamination should be considered during certain agrarian practices, like for example during cereal harvest in summer, which may contribute to spread the bacteria in the environment through aerosols, favouring its airborne transmission. This is in agreement with the form of tularemia infection detected in humans during 2007-2008, when >65% of case-patients had typhoidal and pneumonic forms of tularemia (Allue et al., 2008). During those years, there was also a coincidence between a major regional vole outbreak and increased numbers of tularemia cases in humans (**Chapter 2.a**). The same temporal link was recorded in July 2014 at the same time as the main cereal harvesting season. During 2014, I recorded extremely high vole densities inside cereals, as well as in margins, alfalfas and fallows (see Figs. 3 in **Chapter 1**), and tularemia cases in humans also increased significantly (**Chapter 2.a**). This accumulated evidence supports the idea of an increased human infection risk occurs during outbreaks with transmission by inhalation of the bacteria in aerosols as a consequence of the harvest of cereals, when fields are infested with dead contaminated voles at the time of harvesting. My findings provide empirical clues to improve prevention and reduce infection risk for humans, like, for instance, strongly recommending the use of breathing masks during harvest when and where voles have invaded cereal fields (Fig. 1 in **Chapter 2.c**).

The role of voles in the spill over process of *F. tularensis* has been also documented in Finland, where the temporal occurrence of vole peak years clearly predicted human tularemia outbreaks (Rossow et al., 2015). In Finland, tularemia outbreaks in humans mostly occurred during the year immediately after the vole peak years, i.e., when vole population declined on year after (Rossow et al., 2015). In Spain, I found that vole peaks and human tularemia outbreaks were simultaneous in time and space, which suggests that different transmission scenarios may be involved, as well as different types of vector/s and climatic or environmental conditions. In northwest Spain, I found quick and progressive outbreaking episodes of tularemia concomitant with vole outbreaks in the region. Thus, it would be advisable to adopt preventive measures when there is objective evidence that voles are going to be overabundant in the near future. Previous research suggests that *F. tularensis* has the ability to replicate very fast within voles, but this should be confirmed under laboratory conditions, as well as the degree of its pestilence in field populations (i.e., application of SIR models with proportions of susceptible-infected-recovered individuals). Based on the results of **Chapter 1**, I suggest that targeting control actions in cereal field margins in spring or autumn, before vole density increases, may contribute to reduce the damage to cereals, and potentially decreasing the imminent infection risk for humans and other animals. Yet, field margins represent the very last patches of semi-natural habitats in the study region, and its role in preserving biodiversity and functioning services in agroecosystems should be technically seized against any rodent control measure short and long term global effects. In addition, alfalfa crops are a highly attractive reservoir habitat for rodents worldwide (Luque-Larena et al., 2018) and the evidence suggests that those landscapes planted with alfalfa and hosting boom-bust rodent species should record more recurrently tularemia cases in humans where the disease is endemic (**Chapter 2.c**). Again, this suggested causative correlation should be tested in future studies.

Bacterial zoonotic diseases in intensive farmland from Northwest Spain: dynamic epidemiological roles of boom-bust rodents and ecological interactions.

Recent research conducted in Castilla-y-León compared the genotypes of isolates of *F. tularensis* from the two outbreaks occurred in 1997-1998 and 2007-2008 in the region (Ariza-Miguel et al., 2014). The genetic study indicates that the re-emergence of tularemia in Spain ten years after the first outbreak recorded (1997) was not caused by the reintroduction of exotic strains of *F. tularensis*, but was probably due to the persistence of an endemic bacterial population, i.e., local reservoirs of infection, that has been circulating in the region (Ariza-Miguel et al., 2014). This suggests that the bacteria could

have been persisting in local environmental reservoirs such as water, sediments or other animal hosts and vectors between the two epidemic periods. In **Chapter 2.c**, I suggested that previously-proposed independent aquatic and terrestrial cycles of tularemia may in fact be connected by expanding-contracting (boom-bust) vole populations. In the study area, the presence of an extensive irrigation network of canals and diches along the field margins provides suitable conditions for the occurrence of *F. tularensis* in the water and potentially increases the contact between water and voles. The continental Mediterranean climate of the region is characterised by humid and cold winters, and dry and hot summers with a variable drought period, which creates a hostile environment for the maintenance of *F. tularensis* across farmland during summer periods. Thus, the watering of naturally-dry landscapes has provided suitable environmental conditions for the bacterium, and the vole. In addition, during outbreaks, dead voles frequently end up in the irrigation canals, where their carcasses accumulate and can contribute to contaminate water, sediments as well as animals living in water, such as amebae or crayfish. Overall, the evidence from Spain points to an aquatic cycle with a wide range of vectors involved and different transmission routes. According to this, another interesting perspective would be to use geographic information systems and statistical models in order to identify environmental risk factors for tularemia by using landscape, vegetation, and meteorological variables. The resulting models could provide disease risk maps allowing for the visualization of disease risk areas where surveillance should be prioritized.

Besides voles, other infectious and dead animals coexisting with voles likely contaminate the environment (water and soils) resulting in local hotspots of *F. tularensis*. In particular, *F. tularensis* biovar *palaearctica* was detected as the agent of a waterborne outbreak of tularemia in Spain, as well as crayfish as reservoirs of the bacteria due to they acquired the bacteria from contaminated water and maintained it in their internal organs (Anda et al., 2001). Later, one human clinical case was linked to the contact with crayfish in the study area during an inter-epizootic period, supporting the idea that crayfish can be reservoirs of *F. tularensis* in Castilla-y-León (Ordax, 2003). Thus, it will be interesting to detect and map spatially the local hotspots of the bacteria in the environment in order to understand possible transmission routes, and thereby apply preventive measures. In northern Europe (Sweden) tularemia incidence has been shown to be greater near lakes and rivers (Desvars et al., 2015). A recent study has also linked tularemia cases and water samples that tested positive, urging the need to strategically monitor the spatial and temporal distribution of the causative agent of tularemia (Janse et al., 2018).

Considering wild animal vectors of tularemia, mosquitoes are putatively responsible of the main transmission route of the pathogen to humans in Sweden and Finland, as evidenced by clinical

experience and epidemiological data (Christenson, 1984; Eliasson et al., 2002). Indeed, a novel and recent experiment demonstrated that adult mosquitoes having acquired the bacteria from their aquatic larval habitats can transmit the bacteria, and that the virulence of the bacteria is retained during the development of the mosquito (Bäckman et al., 2015). This confirms that *F. tularensis* has a great adaptability to different environments and vectors, which is not surprising considering this is the most infectious zoonotic pathogen described to date, and that the bacteria persists in the larva and in the adult mosquito that could infect susceptible hosts during blood-feeding. The massive irrigation canal network in my study area in Spain provides suitable conditions for mosquitoes too, which are abundant in summer. However, a role for mosquitos as vectors of tularemia has not been yet empirically proved here. Other arthropods such as ticks and deerflies are also known reservoirs of tularemia in other (more humid) regions in the Northern Hemisphere. However, I found that common voles are mainly infested by fleas in the study area.

Seasonally arid climatic conditions, reduced mammal communities and lack of vegetation other than crops may favour the occurrence of fleas rather than ticks in voles. Thus, fleas could potentially have an important role to play in the transmission and circulation of tularemia in nature. I found fleas infected with *F. tularensis* (**Chapter 3**), and they all came from voles also infected by the pathogen. This suggest that fleas could indeed act as vectors of tularemia, but, given the low proportion of fleas found infected, the existence of an effective pathogen transmission process between fleas and voles should be confirmed. The low *F. tularensis* prevalence detected in fleas should be taken with caution because the detection of a pathogen in an arthropod does not necessary imply a functional role as a vector of the pathogen. Thus, further investigations should fill this knowledge gap, aiming at demonstrating that a positive flea can transmit the bacteria to a negative vole or alternative host. It would also be useful to quantify the bacterial concentration that the flea should acquire through a blood meal to become infected with *F. tularensis,* and to determine where do bacteria multiply inside the fleas. For example, *Yersinia pestis* colonize and multiply within the midgut and proventriculus of fleas. The multiplication of *Y. pestis* within the proventriculus can cause an occlusion or blockage that prevents newly ingested blood from reaching the midgut. The blockage is likely to increase the probability of transmission, but such blockage is not possible in all flea species, and this capacity determines their role as vector (Eisen and Gage, 2012). What is happening in fleas that parasitize common voles in Spain should be studied to clarify the possible pathogen transmission mechanisms between parasite and host.

With the available data to date, fleas seem to play little role in transmitting tularemia under natural conditions. However, fleas have been reported to be capable of transmitting *F. tularensis*

infrequently under some circumstances (Hopla, 1977, 1974), although they are known to be more efficient transmitting other pathogens (e.g., *Bartonella* spp.).

I investigated the occurrence of other zoonotic pathogens of bacterial nature and of risk to humans in common voles and their fleas. I specifically screened voles and fleas for *Anaplasma phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii,* and *Rikettsia* spp., but I only detected *Bartonella* spp. in flea or vole DNA (in addition to *F. tularensis*). *A. phagocytophilum* is a tick-borne bacterium that has been described as the agent of the human granulocytic ehrlichiosis (Dumler et al., 2001). Lyme disease (borreliosis) is a multisystemic zoonotic disorder caused by *Borrelia* spp. and transmitted by ticks (Escudero et al., 2000; Oteo et al., 1998). *C. burnetii* may cause severe infections such as Q fever, and is thought to be transmitted by ticks to small rodents and other mammals. Humans mainly become infected by inhalation of aerosols or dust containing spore-like forms of *C. burnetii*. *Rickettsia* spp. are obligate intracellular bacteria transmitted by lice, ticks, fleas, mites and are pathogenic for humans. The distinct symptoms of human rickettsioses are different for every *Rickettsia* species and the severity of infection ranges from mild to life-threatening disease (Parola et al., 2005). *Bartonella* spp. are emerging zoonotic bacteria which are transmitted by hematophagous arthropod vectors and maintained in nature by different reservoir hosts (Harms and Dehio, 2012). Currently more than 30 different *Bartonella* species have been described and over half of them have been associated with human diseases (Chomel et al., 2009). The lack of detection of *Rikettsia* spp., *A. phagocytophilum*, *Borrelia* spp., and *C. burnetii* in the studied voles could be due to the climatic conditions (seasonally semi-arid Mediterranean climate with variable summer drought periods) and the habitat type (intensive agricultural landscape with scarce grassy vegetation) of the study area, influencing the absence of other more suitable vectors, such as ticks, which in my studies were found to infect less than 5% of the sampled voles (**Chapter 2.d**). For example, *B. burgdorferi* has been previously detected in *A. sylvaticus* and *C. russula* in Spain, although these rodent hosts were infested by ticks (Gil et al., 2005). To have a wider and more complete picture, it would be useful to test the occurrence of these zoonotic bacteria in other small mammal coexisting with common voles and which are potential host species, such as *A. sylvaticus*, *Mus spretus* and *C. russula*, as well as in their fleas.

Experimental studies have demonstrated that fleas can transmit different species of *Bartonella* between rodents (Bown et al., 2004) and field studies have shown that fleas are important vectors for the maintenance and transmission of many *Bartonella* species among populations of small mammals (Chomel et al., 2009; Gutiérrez et al., 2015; Lipatova et al., 2015). The close contact between humans and rodent populations can influence the transmission of *Bartonella* spp. from animals to

humans (Morick et al., 2010). Some species of *Bartonella* are causative agent of infectious diseases in human, so there is a necessity to study the relationship between vole population dynamics and these different species of *Bartonella* that occur in voles. For example, from the five *Bartonella* species detected in the voles I studied (*B. taylorii*, *B. grahamii*, *B. rochalimae*, *B. doshiae*, and *B. clarridgeiae*), only two species can infect humans: *B. grahamii* causes neuroretinitis, and *B. rochalimae* causes bacteraemia and fever (Angelakis and Raoult, 2014). Importantly, the dynamic prevalence of each species of *Bartonella* was different, which affects host-pathogen interaction and disease transmission (**Chapter 2.d**).

Most theoretical models in epidemiology have focused on the dynamics of pathogens in relatively stable host populations (populations that do not experience boom-bust dynamics), but this approach is not reasonable when natural host population abundances widely change over time (but see Telfer et al., 2007). As zoonotic disease risk to humans is linked to rodent density, to understand what leads to irruptive spill-over and involved transmission routes, it is necessary to analyse rodent population dynamics within a framework of ecological interactions, and to determine the relative contributions of intrinsic and extrinsic factors influencing the observed demographic dynamics. Overall, it is expected that an increase in vole density could contribute to an increase in the risk of human exposure to pathogens. Risk expectations also vary, because susceptibility to pathogens in the human population also vary in time and place (Meerburg et al., 2009). Yet, epidemiological relationships and pathogenicity of microorganisms in nature are often difficult to prove (Glass et al. 2000; Ostfeld and Holt 2004), due to the high complexity of host-pathogen and host-vector interactions. Here, I provided clear evidences that the abundance of organisms interacting with voles at two different ecological niches in a distinct trophic level (parasites), i.e., pathogen prevalence (*F. tularensis* and *Bartonella* spp.) and flea burden, vary seasonally and inter-annually together with the multi-annual fluctuations in abundance of common vole populations (**Chapters 2.b, 2.d and 4**). Prevalence of the pathogen *Bartonella* spp. varied according to rodent host (vole and mouse) density, seasonality, and vector prevalence, specifically flea prevalence in this system (**Chapter 2.d**). Differences in the density-dependence relationship with the host were found among species of *Bartonella*. *Bartonella* spp. may not be virulent enough to kill voles, as reflected by the high infection prevalence found in the voles (a mean overall prevalence of 47%, which reached 70% during the vole abundance peak in July 2014). However, this bacterium causes chronic systemic infections in field voles (Telfer et al., 2007), so this may also happen in common voles. *Bartonella* spp. may persist in voles and voles may become infected several times or become resistant to the pathogen, but by now, we do not know for how long this group of bacteria persist in voles.

Integral epidemiological studies considering SIR (susceptible-infected-recovered) models as well as pathogenicity among voles are needed to better understand the dynamics and mechanisms operating in this multi pathogen-host-vector system. A pivotal question is how vole health and condition, and thus breeding performance and survival, may be affected by the infection. Another interesting aspect is what happens with the bacteria when an infected vole dies. For example, in the case of *Y. pestis*, it has been suggested that the apparent cost for the bacteria to kill the host is balanced by the benefit of increased probabilities that at least some fleas complete feeding prior to the host's death and, thus, acquire sufficient bacterial concentration to become infectious to other animals during subsequent blood meals (Gage and Kosoy, 2005). In addition, plague-induced host mortality increases the likelihood of transmission to another host of the same or different species either through transmission by direct contact with the infectious carcass or by forcing newly infected fleas to seek alternative hosts (Eisen and Gage, 2009; Hinnebusch, 2005). Yet, most of the voles that tested positive to infection by *Francisella* and *Bartonella* in my study area were apparently healthy (based on observations of their general body aspect both externally during field trapping and internally during post-mortem necropsies). This suggests that the voles may not develop the disease or suffer immediately after infection. So, how infective and lethal are these pathogens for voles in wild populations is a next logical question to address, in order to improve knowledge on this multiple pathogen-host dynamic system. In the case of *Bartonella*, I tentatively suggest that fleas may transmit the bacteria to voles when they abandon dead *Bartonella*-infected voles, and then transmit the bacteria to other susceptible hosts they parasitize next, which mostly are other rodents but could potentially be humans. As occurs with *F. tularensis*, it will be interesting to quantify the bacterial concentration in fleas to know the vector efficiency and how the bacteria are maintained in the flea before being transmitted to another host.

Pathogen (*Bartonella* or *F. tularensis*) and flea dynamics have not been studied yet together in fluctuating vole populations. The same occur with the prevalence of *Bartonella* spp. and *F. tularensis* in other rodent species, as is the case of *A. sylvaticus* and *M. spretus*. Both rodent species cohabit with common voles and share burrows and fleas. Thus, it is likely that mice will be infected by pathogen-positive voles either by direct contact or vectored by fleas. Most importantly, the three flea species that infect studied common voles (i.e., *Ctenophthalmus apertus*, *Nosopsyllus fasciatus* and *Leptopsylla taschenbergi*) are shared by voles and mice in nature (**Chapter 4**), but I do not know whether these three flea species have the same potential to transmit pathogens, and thus act as functional vectors of the disease. This should be tested in future studies. Factors other than fleas need also to be considered in the infection of voles by *Bartonella* spp. For example, the bacteria may be vertically transmitted from the vole mother to the embryos. A recent study detected high rates (66%) of vertical

transmission in wood mouse (Cevidanes et al., 2017). This result is consistent with a study conducted under laboratory conditions in which *Bartonella* was cultured from foetuses and new-borns (Kosoy et al., 1998). Thus, the vertical transmission detected in wild populations encourages examining this possibility in vole populations and whether infected females may transfer maternal antibodies to the progeny to be protected against the pathogen.

The two bacterial zoonotic pathogens of risk to humans found circulating in my study system, *F. tularensis* and *Bartonella* spp., both coinfect voles, and this is of significance from the epidemiological perspective. Both bacteria simultaneously occurred in voles, although the occurrence of one did not appear to depend on the occurrence of the other. However, potential bacterial interactions in voles could be tested with more detail in future studies, i.e. whether the interactions are synergistic or antagonistic: facilitation vs. competition (Cox, 2001). More studies of interactions among parasites in natural populations (i.e., focus at community level) would also be required to fully understand the mechanisms underlying their coupled delayed-dynamics with rodent hosts (Pedersen and Fenton, 2007). As interactions between parasites are largely dependent on the resources provided by the host individual, as well as by its age, sex, body condition, and immune system, it is necessary to understand whether bacterial interaction is dependent or not on host features and to identify whether interactions that have potential fitness implications for the host (morbidity and/or mortality). However, interactions between parasites and the processes that shape within-host parasite communities remain unclear in natural populations (but see Telfer et al., 2008). The direction and magnitude of effects of parasite interactions within hosts can vary considerably among systems, but, until now, there has been no general framework to explain this variation and the consequences of these interactions for host health.

The variation of extrinsic factors such as parasitism has been suggested to cause or influence host population dynamics. The quantitative understanding of how ectoparasites affect vole dynamics thus helps to predict zoonotic outbreaks and shape prevention strategies. I also studied the potential effects that fleas have on common voles and suggest that fleas play a role in maintaining the low density phases of voles (**Chapter 4**). At the same time as vole populations increase, so should do flea-infestation rates. However, I found that this relationship occurred but with a marked delay in time (i.e., flea burden increased with an 8-month time lag). This delayed-density dependence pattern between flea burden and common vole abundance may be the result of a “dilution effect”, that is, reduced flea burdens are expected when host density is high, but an increased individual burden when vole numbers decrease and fleas concentrate on fewer available vole host, or switch to alternative hosts, or remain in rodent nests (the fleas detected in voles spend most of their life cycle inside a

nest). Noteworthy, while the overall abundance of voles strongly changes throughout time (boom-bust dynamics), flea numbers seem to fluctuate through that time; yet, flea aggregation patterns (burden size on individuals) strongly respond (diluting or concentrating) to host abundance and distribution (expanding-contracting resource availability). I suggest the hypothesis that the direct density-dependence between vole density and pathogen prevalence could potentially bring down the vole populations when they are overabundant (i.e., tularemia spreads through vole populations rapidly increasing mortality rates). Then, after the number of voles has decreased, the remaining few and aggregated voles in field margins and alfalfas will be parasitized by proportionally more fleas (larger flea burdens), which in turn, may affect their condition, reproduction and survival (as it has been already shown experimentally), thus maintaining low densities in vole population and thereby preventing a rapid vole population "rebound" (increase). If true, this process mediated by dynamic ecological interactions in the assemblage host-vector-pathogen may contribute to explain why we observe boom-bust outbreak dynamics interspaced by non-outbreak years of low density (Fig. 1 in **General introduction**; Fig. 3 in **Chapter 1**). In **Chapter 4**, I provide empirical evidence that supports this mechanistic hypothesis, and show that a greater flea burden in wild common voles is associated with a poorer body condition, a reduced reproduction rate (fewer embryos) and a reduced population growth rate. These effects on reproduction and growth rates are potential causes of population declines, but in the studied system, vole density appeared to decline before flea burdens increased. Hence, fleas might be tracking ("passengers") rather than causing ("drivers") changes in vole numbers, but what they likely do is prevent vole population from quickly bouncing back after a population peak (i.e., fleas contribute to keep a low population growth rate when concentrated on few and scattered vole hosts).

Throughout the monitoring of wild common vole populations, I have highlighted that changes in host abundance influence both parasite and pathogen prevalence. I also suggest that such changes may in turn feed-back and affect, directly (pathogens) or with a time-delay (fleas), the abundance of the host. Many epidemiological and ecological studies that focus on host-pathogen systems have been traditionally developed at the level of a single species of host infected with a single species of pathogen. Yet, a dynamic multi-species approach is always needed when addressing the dynamics of zoonotic diseases in ecosystems at landscape level, since ecological interactions between organisms and environment are by nature complex and dynamic.

Conclusions

1. The recent recording of common vole outbreaks in extensive farming landscapes in northwest Spain provides an unprecedented geographical scenario to study unstable population dynamics of a boom-bust herbivore rodent and, more specifically, its role in shaping the transmission of zoonotic diseases in human-modified environments.

2. I found that common vole habitat use in intensive Mediterranean farmlands is dynamic, characterized by multiannual expansion-contraction processes at local and landscape levels. I report an overall greater abundance of voles in field margins and alfalfas, and an invasion of neighbouring predominant cereal crops initiated from field margins during population increases. Any vole control method could thus consider the cereal field margins in spring or autumn as source-patches of voles during population increasing periods.

3. Tularemia is a zoonotic infectious disease caused by the highly infectious pathogen *Francisella tularensis,* a gram-negative bacterium of wide distribution in temperate regions across the northern hemisphere. Human cases of tularemia (or tularaemia) registered in Spain, since their first epidemic appearance in 1997, are concentrated in farming regions of the northwest where common vole thrive. I show that large human tularemia outbreaks (>500 cases/outbreak) in Spain coincide in space and time with large common vole outbreaks at regional scale. This is consistent with the hypothesis that voles are involved with the spread of the disease through the farming landscapes.

4. I show how *F. tularensis* infect common voles and how its prevalence directly increases with vole abundance during their boom-bust density outbreaks. As the population of this key rodent host rapidly increases in size (boom phase), so does the population of the bacteria, therefore amplifying and contaminating the whole environment on irruptive pulses. Common voles are thus true amplifiers and spill-over agents of tularemia in Spain during epizootic periods.

5. Small mammalian herbivores such as lagomorphs and rodents are preferred hosts for *F. tularensis* to multiply and grow. I propose that the dynamic nature of key host mammalian populations determines the type of epidemiological pattern displayed by this disease across Europe. I specifically underlie that the dynamics of strongly fluctuating rodent host

populations shape the epidemiology of tularemia in Spain, where *F. tularensis* simultaneously cycles in terrestrial and aquatic environments that characterize intensive irrigated farmlands. In practical terms, my findings underlie that: (i) tularemia surveillance and prevention efforts should include the monitoring of unstable rodent populations, and that (ii) effective prevention measures should be promoted during vole outbreaks.

6. As the range of rodent-borne pathogens is diverse, I explored the occurrence of other zoonotic bacteria of risk to humans in the studied fluctuating vole populations. I found that several species of *Bartonella* occurred at high prevalence in common voles. Four *Bartonella* species infected voles: *B. taylorii*, *B. grahamii, B. doshiae* and *B. rochalimae*, whose prevalence were dynamic, and varied seasonally and with the density of rodent hosts (voles and mice). *Bartonella* spp. and *F. tularensis* prevalence in common voles average 47% and 20.4%, respectively, with coinfection rates as expected from the prevalence of each pathogen.

7. The transmission of the two zoonotic diseases of risk to humans found in common voles (tularemia and bartonellosis) can be vectored through parasitic blood-sucking arthropods. I found that the main ectoparasites of studied voles are fleas (Siphonaptera). I detected *F. tularensis* and *Bartonella* spp. in fleas parasitizing common voles, suggesting a potential vectoring role in nature. No coinfection with the two zoonotic bacteria was detected in fleas. Future molecular research should examine how the two bacteria interact within fleas and between fleas and vole hosts.

8. I found that flea burden on voles varied with vole density in a delayed density-dependent manner, registering higher parasitic burdens 8 months after a vole population peak (maximum density). Such pattern could be explained by a dilution effect, as fleas may concentrate on fewer hosts during declines. I also found that greater flea burdens were associated with reduced reproduction outputs and vole population growth rate, suggesting that fleas could contribute to maintain low density phases of common voles. The direct (blood-sucking action) versus indirect (pathogen transmission) effects of fleas on vole fitness and dynamics should be clarified in future experimental studies.

9. The results of this thesis highlight the relevance of considering the ecological context to better understand the consequences of wild rodent boom-bust dynamics. Ecological studies are basic essential tools to prevent and/or minimize the food security (e.g., crop damage)

and public health (e.g., disease spill-over) impacts associated to the biological phenomenon of strongly fluctuating rodent numbers. This thesis shows that considering the dynamic interactions between host, vectors and pathogens provides rigorous and realistic information to improve predictions of zoonotic disease emergence, disease control programs and bio-control initiatives.

Conclusiones

1. Los brotes demográficos del topillo campesino, recientemente registrados en los ecosistemas agrarios extensivos del noroeste de España, proporcionan un escenario geográfico sin precedentes para estudiar las dinámicas poblacionales inestables de un roedor herbívoro, el topillo campesino, y, específicamente, su papel en modelar la transmisión de enfermedades zoonóticas en medios agrarios modificados por los seres humanos.

2. El uso del hábitat por parte del topillo campesino en el ecosistema agario Mediterráneo es dinámico, y está caracterizado por procesos multianuales de expansión-contracción a nivel local y de paisaje. Además, encontré que hay una gran abundancia global de topillos en las linderas de los cultivos y en las alfalfas y que se produce una invasión de los cultivos de cereales desde las lindes contiguas durante la fase de crecimiento de la población de topillo campesino. Por lo tanto, cualquier método para controlar a esta especie debería considerar que las lindes de los cultivos de cereales actúan como parches fuente de topillos durante periodos de crecimiento poblacional, en particular, en primavera o en otoño.

3. La tularemia es una enfermedad infecciosa zoonótica causada por un patógeno altamente contagioso *Francisella tularensis*, una bacteria gram-negativa ampliamente distribuida en regiones templadas del hemisferio norte. En España, los casos de tularemia en humanos, registrados desde su primera aparición epidémica en 1997, se han concentrado en regiones agrícolas del noroeste, donde el topillo campesino prospera. He demostrado que en España las grandes epidemias de tularemia en humanos (>500 casos/brote epidémico) coinciden en el espacio y en el tiempo con grandes explosiones demográficas de topillo campesino a escala regional. Este resultado es consistente con la hipótesis de que los topillos están implicados en la propagación de la enfermedad en el paisaje agrícola.

4. Por un lado, he demostrado cómo *F. tularensis* infecta al topillo campesino y, por otro lado, cómo la prevalencia de dicha bacteria aumenta directamente con la abundancia

de este roedor durante sus explosiones demográficas de "auge y caída" (*boom and bust*). Cuando la población de este roedor hospedador aumenta en tamaño (fase de auge o de incremento), aumenta la población bacteriana. Este hecho contribuye a la amplificación y a la contaminación del medio ambiente durante las explosiones demográficas del roedor. Por lo tanto, el topillo campesino es un verdadero agente amplificador y de propagación de tularemia en España durante los periodos epizoóticos.

5. Los micromamíferos herbívoros como los lagomorfos y roedores son los hospedadores preferidos de *F. tularensis* para multiplicarse y crecer. En esta tesis propongo que la dinámica natural de las poblaciones de mamíferos hospedadores determina el tipo de patrón epidemiológico que exhibe esta enfermedad a lo largo de Europa. Específicamente, sostengo que las dinámicas de aquellos roedores que fuertemente fluctúan van a modelar la epidemiología de la tularemia en España, donde simultáneamente ocurren ciclos de *F. tularensis*, tanto en medios acuáticos como terrestres, caracterizados por el sistema de regadío en el medio agrícola. En términos prácticos, mis resultados sostienen que: (i) los esfuerzos de vigilancia y de prevención de la tularemia deberían incluir la monitorización de poblaciones inestables de roedores, y que, (ii) deberían promoverse medidas preventivas más efectivas durante las explosiones demográficas del topillo campesino.

6. Debido a que el rango de patógenos que pueden ser trasmitidos por los roedores es muy amplio, he explorado la presencia de otras bacterias zoonóticas de riesgo para los humanos en las poblaciones fluctuantes de topillo campesino. En concreto, he encontrado que varias especies de *Bartonella* spp. pueden ocurrir con alta prevalencia en el topillo campesino. De esta forma, existen 4 especies capaces de infectar a los topillos: *B. taylorii*, *B. grahamii, B. doshiae* and *B. rochalimae*, cuyas prevalencias eran dinámicas y variaban estacionalmente con la densidad de diferentes especies de roedores hospedadores (topillos y ratones). La prevalencia media de *Bartonella* spp. y *F. tularensis* en los topillos era del 47% y 20.4%, respectivamente, con tasas de coinfección esperadas a partir de la prevalencia de cada uno de los patógenos.

7. La transmisión de las dos enfermedades zoonóticas de riesgo para los humanos encontradas en los topillos, tularemia y bartonelosis, pueden ser vectorizadas a través de artrópodos parásitos chupadores de sangre. El principal ectoparásito encontrado en las poblaciones de los topillos estudiados eran las pulgas (Siphonaptera). Detecté que *F. tularensis* y *Bartonella* spp. estaban en las pulgas que a su vez parasitan a los topillos. Esto sugiere que las pulgas tienen un papel potencial como vectores en la naturaleza. Por el contario, no encontré coinfección de las dos bacterias en las pulgas. Por lo tanto, los trabajos a nivel molecular que se desarrollen en el futuro deberán examinar cómo estas dos bacterias interactúan entre sí dentro de las pulgas y entre las pulgas y los topillos hospedadores.

8. Encontré que la carga parasitaria de pulgas variaba con la densidad de los topillos de una forma denso-dependiente retrasada, registrando altas cargas parasitarias 8 meses después de un pico poblacional de topillos (densidad máxima). Este patrón podría explicarse por un "efecto dilución", ya que las pulgas pueden concentrarse en menos hospedadores durante los descensos poblacionales. También encontré que una mayor carga parasitaria de pulgas estaba asociada con una menor producción reproductiva y una disminución de la tasa de crecimiento poblacional del topillo campesino, sugiriendo que las pulgas pueden contribuir a mantener las fases de baja densidad del topillo. Tanto los efectos directos (la acción de chupar sangre) como los indirectos (transmisión de patógenos) de las pulgas sobre el *fitness* y las dinámicas del topillo deberían ser clarificados en futuros estudios experimentales.

9. Los resultados de esta tesis destacan la relevancia de considerar el contexto ecológico para conseguir un mejor conocimiento de las consecuencias que conllevan las dinámicas de "auge y caída" de roedores salvajes. Los estudios ecológicos son una herramienta básica y esencial para prevenir y/o minimizar los impactos a la seguridad alimentaria, por ejemplo, el daño a los cultivos, y a la salud pública, por ejemplo, la propagación de enfermedades, asociados al fenómeno biológico de abundancias de roedores que fluctúan ampliamente. Esta tesis muestra que considerar las interacciones dinámicas entre hospedador, vectores y patógenos proporciona una información rigurosa y realista que permite mejorar las predicciones de enfermedades zoonóticas emergentes, los programas de control y las iniciativas de control biológico.

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