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

Authorship

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SUMMARY

Diseases and host dynamics are linked, but their associations may vary in strength, be time-lagged, and 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 varies according to host density, seasonality, and vector prevalence. We detected *Francisella tularensis* and four species of *Bartonella*, but not *Anaplasma*, *Borrelia*, *Coxiella* or *Rickettsia*. *B. taylorii* and *B. grahamii* prevalence increased and decreased with current host (vole and mice) density, respectively, and increased with flea prevalence. *B. doshiae* prevalence decreased with mice 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* spp. occurred as expected from respective prevalence.

INTRODUCTION

The reservoir of an infectious agent is the natural habitat in which the agent normally lives and multiplies. Reservoirs may include humans, animals, and environmental sources, and they may or may not be the source from which an agent is transferred to a host (CDC, 2012; Bonita *et al*., 2006). Rodents are important reservoirs of diseases of relevance to livestock and human health (Han *et al*. 2015), so there is considerable interest in understanding endemic infections in natural rodent host populations. 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). Understanding natural infections requires knowledge on how host density, which may be very variable in space or time, and seasonality influence pathogen prevalence, as well as knowledge on the role that vectors play in pathogen transmission (e.g. Telfer *et al.* 2007*a*). 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. Rodents are frequently exposed to ectoparasites that transmit pathogens (Gratz, 1994). These 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. 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 occur when a host is infected by different parasites, at the same time or sequentially. Parasite interactions can result in co-occurrence or in competition between parasites for a shared resource, such as food or habitat, thus affecting host population and

74 resulting in direct interactions. The immune response of the host to one parasite may affect the
75 host's ability to control a second parasite species, and coinfection may favour the transmission
76 and progression of other diseases (Jolles *et al.* 2008; Telfer *et al*. 2010). In this case, the presence
77 of a parasite can increase the host susceptibility to be infected with a second parasite or, on the
78 contrary, decrease the probability of infection by another parasite due to an immune response
79 (Cox, 2001). Coinfections not only result from the interactions among parasites, but also from
80 shared risk factors such as environmental and climatic conditions, vectors or groups of vectors,
81 host density or host physiological conditions. Many studies have shown that rodents can be
82 simultaneously infected by more than one pathogen (Meerburg *et al.* 2009; Buffet *et al.* 2012;
83 Kallio *et al.* 2014; Razzauti *et al.* 2015; Koskela *et al.* 2017). However, the existence and types of
84 interactions between parasites in natural systems, which may be essential to predict disease
85 dynamics and control parasites, remains poorly known (but see Telfer *et al.* 2010).

86 The common vole (*M. arvalis*) is one of the most abundant and widespread mammals in
87 continental Europe (Jacob and Tkadlec, 2010). Throughout its range, common vole populations
88 typically exhibit regular fluctuations in abundance or irruptive outbreaks (Tkadlec and Stenseth,
89 2001; Lambin *et al.* 2006). The species recently colonized ca. 5 million ha of farmland in
90 northwest Spain during a rapid range expansion (<20 years), coinciding with an increase in the
91 surface area of irrigated herbaceous crops, in particular alfalfa (Luque-Larena *et al.* 2013; Jareño
92 *et al.* 2015). Large-scale regional vole outbreaks followed this colonisation and occurred every
93 ca. 5 years since early 1980s (Luque-Larena *et al.* 2013), with very high vole abundances (>1,000
94 individuals/ha) during peak phases. These outbreaks have caused unprecedented public health
95 risks because voles carry and amplify the bacterium *Francisella tularensis*, a highly infectious
96 agent causing tularemia (Rossow *et al.* 2015; Luque-Larena *et al.* 2017). *F. tularensis* prevalence
97 in voles was found to increase with vole abundance (direct-density dependence; Rodríguez-
98 Pastor *et al.* 2017) and human cases of tularemia were found to greatly increase during vole
99 outbreak years (Luque-Larena *et al.* 2015). As 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, and both vectors can potentially transmit *F. tularensis* (Hopla, 1974; Bibikova, 1977) as well as other pathogens. Therefore, to obtain a complete understanding of the dynamics of pathogens, it is necessary to take into account not only the dynamics of the hosts, but also the dynamics of vectors, pathogen interactions (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 across a sectional study of two years. Specifically, we screened every four months 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 voles species (including the common vole) across Europe (Barandinka *et al*. 2007; Telfer *et al*. 2010; Buffet *et al*. 2012; Silaghi *et al*. 2012; Kallio *et al*. 2014; Rossow *et al*. 2014; Rodríguez-Pastor *et al*. 2017; Fischer *et al*. 2018). We also investigated whether the prevalence of these pathogens in common 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 than 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 vector (flea) and pathogen prevalence to assess whether vectors participated in pathogen transmission. Finally, we investigated coinfection patterns and tested whether the infection probability by a given pathogen varied depending on the presence of a second pathogen.

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-C for Spain, and capture permission (permit number 4801646) was provided by the Dirección General del Medio Natural, Junta de Castilla-y-León, Spain.

Study area

The study was conducted in an 80-km^2 area of farmland located in Palencia province, Castilla-y-León autonomous region, north-western 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 (Rodríguez-Pastor *et al.* 2016), allowing us to investigate delayed-density dependent patterns.

Bacterial zoonoses and small mammals in Spain: background

Six vector-borne pathogens (*A. phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *C. burnetii*, *F. tularensis* and *Rickettsia* spp.) were studied in common voles. Empirical data about the role of the common vole as reservoir of zoonotic bacteria are very scarce in Spain, although information is available from other sympatric small mammals (Oporto *et al*. 2003; Gil *et al*. 2005; Barandika *et al*. 2007). In northern Spain, tick-borne zoonotic bacteria, such as *Borrelia* spp., *A. phagocytophilum* and *C. burnetii* have been detected in small mammals, but not the spotted fever group rickettsiae (Barandika *et al*. 2007). All these pathogens are considered as agents of emerging human diseases (Table S1).

Common vole sampling

Common vole abundance, as well as pathogen and vector prevalence were monitored every 4 months during 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). Since our trapping method was extractive, we avoided sampling the same fields during consecutive trapping events in order to minimize any potential impact on host populations. Common voles live in sympatry with other rodent species in the area, but the majority of captures were voles (76%; 929/1221), followed by *A. sylvaticus* (18.5%; 226/1221) and *M. spretus* (5%; 66/1221). From a total of 929 voles captured between March 2013 and March 2015, a subset of 240 voles (105 males and 135 females) was used for pathogen and vector screening. The selection was based on a representative sample of captured voles that arrived alive at the laboratory and was stratified by seasonal sampling event and vole gender.

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 were subsequently identified to species level using a binocular microscope (x10 and x40 magnification; Nikon Optiphot-2) based on morphological

traits following Gómez *et al*. (2004). Three flea species were identified (*Ctenophthalmus apertus*, *Nosopsyllus fasciatus* and *Leptopsylla taschenbergi*). Ticks were rarely found and collected on voles, so we did not identified ticks to genus and/or species levels, or record the developmental stage (i.e., larva, nymph, or adult). 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 until used for molecular detection of pathogens.

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). Sensitivity of the multiplex PCR was between 10 and 100 GE (Genome Equivalents) and specificity with unrelated bacteria, mammals and arthropods was 100% (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.

***Detection of* F. tularensis**

We used a phylogenetically informative region of gene *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.*

197 *tularensis*); here, we screened 240 (99%) of these voles for 6 pathogens (including *F. tularensis*)
198 using the multiplex PCR (Escudero *et al.* 2008).

199 ***Identification of* Bartonella *species infecting voles***

200 *Bartonella* positive samples were further analysed using a multiplex PCR targeting the 16S rRNA
201 and the intergenic transcribed spacer (ITS) 16S-23S rRNA. Subsequently, amplicons were
202 analysed with a RLB that included 36 probes for the identification of the different genotypes and
203 species of *Bartonella* (Garcia-Esteban *et al.* 2008; Gil *et al.* 2010).

204 ***Statistical analyses***

205 We focused on *Bartonella* and *F. tularensis* because the other pathogens screened were not
206 detected in voles. We used Generalized Linear Models (GLM) with a binomial error structure
207 and logit link for all the analyses of prevalence, which were done with R v3.4.1 (R Development
208 Core Team, 2017). Model selection was performed using the Aikake Information Criterion for
209 small sample size (Δ-AICc) with the "AICcmodavg" package in R and compared. In order to
210 evaluate hypotheses on pathogen prevalence, we calculated time-varying host population-level
211 covariates and individual-level vole host covariates. The former included mean vole abundance,
212 mean mouse abundance (wood mouse and Algerian mouse pooled) per seasonal sampling
213 (mean rodent abundances were estimated as the average number of captures per 100 traps per
214 24h for a given seasonal sampling period), and mean prevalence of *F. tularensis* and *Bartonella*
215 spp. for each seasonal sampling (hereafter, *Bartonella* spp. refers to all species of *Bartonella*).
216 Seasonal sampling-specific pathogen prevalence was calculated as the number of voles positive
217 for a particular pathogen, over the total number of voles analysed. Individual level covariates
218 included vole sex; *F. tularensis* PCR result (0/1); *Bartonella* spp. PCR result (0/1); overall flea
219 prevalence (0/1) and flea burden (number per host); species-specific flea prevalence and flea
220 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.

Density-dependence: host-pathogen interactions

The probability of a vole being infected (categorical variable: "0" vs. "1", as dependent variable) at time $_t$ was tested according to vole abundance (at time $_t$), previous vole abundance (4 months before, times $_{t-4}$) and mouse abundance (wood mouse 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 initial models. Correlation between vole and mouse abundances at times $_t$ and $_{t-4}$ was tested. In order to address collinearity issues and improve model fitting to the data, vole abundances were log-transformed when included as explanatory variables (*rho* between Log (vole abundance $_t$) and Log (vole abundance $_{t-4}$) = 0.28; *rho* between Log (vole abundance $_t$) and mouse abundance $_t$ = 0.46; *rho* between Log (vole abundance $_{t-4}$) and mouse abundance $_t$ = -0.35). We built a series of GLMs with a binomial error including these different explanatory variables.

Flea-pathogen interactions

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 that of each *Bartonella* species separately.

Pathogen-pathogen interactions

We used *Bartonella* spp. prevalence as dependent variable and *F. tularensis* prevalence, vole abundance at time $_t$ 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.

RESULTS

Pathogens prevalence in common vole

Among the six pathogens screened, only *F. tularensis* and *Bartonella* spp. were detected using PCRs. *Bartonella* spp. prevalence averaged 47% (112/240), with marked differences between seasonal samplings: prevalence was maximum during the summer peak in vole density (July 2014), when 69% (70/101) of voles were infected (Fig. 1). For *F. tularensis*, we also confirmed that 20% (49/240) of voles were infected on average, and that in July 2014, prevalence peaked at 34% (34/101; Fig. 1).

Bartonella *species 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 65% (72/111) of the *Bartonella*-positive voles. Mixed infections with different *Bartonella* species were detected in 59% (65/111) of the positive voles (Table 1). Moreover, a mix of three different *Bartonella* species was found in 8% (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.

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 abundance and vole abundance were statistically significant: vole abundance influenced prevalence positively

(slope ± standard error (S.E.): 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).

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 ± S.E.: -0.19 ± 0.09), was higher in winter (estimate ± S.E.: 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 ± S.E. = 0.88 ± 0.48) and with vole density 4 months before (slope ± S.E. = 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 ± S.E.: 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 ± S.E.: 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 ± S.E. = -2.27 ± 0.79) than in winter (1.54 ± 0.69) and there was a null effect in summer (coefficient not significant).

Flea-pathogen interaction

Almost all (94%; 225/240) the voles that were screened during this study arrived alive to the laboratory. Among them, 56% (125/225) were females and 44% (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%) 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%), followed by *N. fasciatus* (37%), and with *L. taschenbergi* (1%) occurring in a minor proportion. Details about flea prevalence and tick prevalence on voles at each sampling period are shown in Table 3.

Bartonella spp. prevalence was positively correlated with flea prevalence (estimate ± S.E. = 0.60 ± 0.29). *Bartonella* spp. prevalence was 1.4 fold higher in voles carrying fleas than in voles without fleas. Considering species-specific prevalence, *B. doshiae* and *B. rochalimae* prevalence were not related to flea prevalence, while *B. grahamii* and with *B. taylorii* prevalences were 3.5 and 1.8 fold higher when voles had fleas as compared with voles without fleas, respectively (*B. grahamii*: estimate ± S.E. = 1.49 ± 0.46; *B. taylorii*: 0.79 ± 0.34).

At flea species level, *Bartonella* spp. prevalence was 1.4 fold higher when voles had *N. fasciatus*, but did not differ according to the prevalence of other flea species. This positive association between *Bartonella* prevalence and *N. fasciatus* was found in *B. grahamii* (estimate ± S.E. = 0.75 ± 0.33) and in *B. taylorii*, but marginally significant, (estimate ± S.E. = 0.51 ± 0.29; p = 0.07). There was a positive association between *B. doshiae* prevalence and *C. apertus*, but marginally significant (estimate ± S.E. = 1.14 ± 0.64, p = 0.07).

Pathogen-pathogen interaction

The presence of both *F. tularensis* and *Bartonella* spp. was detected in 13% (31/240) of the screened voles (Table 3). Coinfection rate (*F. tularensis* and *Bartonella* spp) reached a maximum of 24% (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

317 from the prevalence of each pathogen at a given sampling time (Table 3). Coinfection rate was
318 15% (20/135) in female voles and 10% (11/105) in male voles (Table 4). We observed that the
319 probability of being infected with both pathogens was not different from the predicted
320 prevalence of each pathogen in voles (χ^2_1= 6.81, p < 0.05; Table 3). Evidences for association
321 were found for *B. grahamii* with *F. tularensis* (χ^2_1= 8.24, p < 0.05), *B. taylorii* with *F. tularensis*
322 (χ^2_1= 7.94, p < 0.05), *B. grahamii* with *B. taylorii* (χ^2_1= 34.24, p < 0.05), *B. doshiae* with *B.*
323 *rochalimae* (χ^2_1= 9.12, p < 0.05) and *B. rochalimae* with *B. taylorii* (χ^2_1= 15.40, p < 0.05). When
324 vole abundance and sex were considered in the model, the probability of infection with
325 *Bartonella* spp. did not depend on *F. tularensis* prevalence, but only depended on vole density
326 (slope ± S.E. = 0.03 ± 0.01). This positive association with vole density was found for *B. grahamii*
327 (slope ± S.E. = 0.05 ± 0.01) and *B. taylorii* (0.03 ± 0.01).

328 **DISCUSSION**

329 Prevalence of *F. tularensis* and *Bartonella* spp. has been studied in small mammals other than
330 common voles from Mediterranean areas (Márquez *et al.* 2008; Gil *et al.* 2010; Cevidanes *et al.*
331 2017; Rodríguez-Pastor *et al.* 2017), although the relationship between the dynamics of hosts,
332 pathogens and vectors, as well as the interactions between pathogens, have not been studied
333 previously. Our study shown a significant association between host and pathogen dynamics, and
334 that the probability of infection with *Bartonella* spp. increased with flea prevalence, which is
335 consistent with *Bartonella* spp. being a flea-borne pathogen. We also provided evidence that
336 the occurrence of one zoonotic pathogen (*Bartonella* spp.) was not dependent on the
337 occurrence of the other (*F. tularensis*) in vole populations. Our study was cross-sectional and
338 relatively limited in terms of duration (2 years), so, in order to better tease apart the relative
339 importance of density-dependence and seasonality, a longer-term investigation of host-
340 pathogen dynamics should follow up, complemented by longitudinal studies that follow

infection dynamics at individual level over time. Despite these limitations, we were able to provide novel insights that we discuss below.

Bartonella ***infection in voles***

Bartonella spp. was the most prevalent bacteria in voles, infecting almost half (47%) of all the voles analysed, while just a fifth (20%) of all the voles were infected with *F. tularensis*. This *Bartonella* spp. prevalence falls within the range (between 11 and 72 %) of those 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 (59%), with dual infections among *B. taylorii* and *B. grahamii* being most frequent. 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.

Density-dependence of **Bartonella** ***prevalence in 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 without voles, *Bartonella* spp. occurrence was positively correlated with wood mouse abundance, the most abundant small mammal of the 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 may have been masked by the most prevalent species of *Bartonella*. In another study in a moist Atlantic climate using long-term data from field voles, which 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* increased with wood mouse density. In

366 another study with different rodent hosts (bank voles, *Myodes glareolus*, and wood mice), *B.*
367 *taylorii* and *B. doshiae* were more prevalent in wood mouse, while *B. birtlesii* was more prevalent
368 in bank vole (Telfer *et al.* 2007*b*). This suggests that the distribution and abundance of each
369 *Bartonella* species do not follow common patterns and that their response to host density
370 depends on the most abundant, preferred host. These findings highlight that each species of
371 *Bartonella* has its distribution pattern and abundance, host specificity, seasonality and response
372 to host density. Therefore, studying the relationship between pathogen and host dynamics
373 requires considering each species of *Bartonella* separately (Telfer *et al.* 2007*b*). In agreement
374 with previous findings by Telfer *et al*. (2007*a*, *b*), we provided evidence for a density-dependence
375 response that differed among *Bartonella* species and rodent hosts: i.e., *B*. *taylorii* and *B.*
376 *grahamii* responded to both vole and mouse densities, while *B. doshiae* responded to mouse
377 density (direct response), and *B*. *rochalimae* to vole density (direct and delayed responses). The
378 positive direct density-dependence to vole density suggests that the pathogen spreads quickly
379 between individuals, and that voles may have low resistance to pathogen infection. Moreover,
380 the negative relationship with mouse density suggests that voles may influence infection
381 prevalence in other coexisting rodent species.

382 ***Seasonal variations of* Bartonella *prevalence in voles***

383 Factors such as seasonality can also determine variation of pathogen prevalence in reservoir
384 hosts. *Bartonella* spp. prevalence in small mammals follows a seasonal pattern, although results
385 differ among studies: *Bartonella* spp. prevalence can peak in summer (Paziewska *et al.* 2012) or
386 in autumn (Cevidanes *et al.* 2017). However, these seasonal patterns are based on a pool of
387 *Bartonella* spp., not on the prevalence at species level (but see Telfer *et al.* 2007*b*). Overall, we
388 found that *Bartonella* spp. prevalence in voles was highest during winter (Fig. 2) when taking
389 into account host densities. Altogether, more fleas were collected in spring and summer than
390 during winter. An increase in the infection probability with *Bartonella* spp. in winter could be

391 the result of an increase in the occurrence of infected alternative hosts, increasing the infection
392 probability in voles. However, we need to know the *Bartonella* spp. prevalence of the alternative
393 rodent hosts (mice) as well as prevalence in the main vector (fleas) in order to better understand
394 these interactions. At the species level, the infection probability with *B*. *grahamii*, *B*. *taylorii* and
395 *B*. *doshiae* in voles followed a marked seasonal variation, i.e., increased in winter and lowest in
396 spring. *B*. *rochalimae* was the only species whose prevalence did not vary seasonally, but was
397 also the one with the lowest prevalence in voles. A seasonal pattern for *B. grahamii* has been
398 also found in other vole species, but not for *B*. *taylorii* and *B*. *doshiae* (Telfer *et al.* 2007*a*). Such
399 seasonal differences may be due to the dynamics and phenology of the fleas that transmit
400 *Bartonella* spp.

401 ***Ectoparasite vectors and* Bartonella *prevalence in voles***

402 Pathogen prevalence also varies with vector dynamics. *Bartonella* spp. prevalence has been
403 previously shown to be higher in mice carrying greater flea burdens (Cevidanes *et al.* 2017). In
404 our studied common vole population, *B. taylorii* and *B. grahamii* were the most prevalent
405 species and the infection probability increased when voles were infested by fleas, independently
406 of the flea burden. This positive relationship between flea and pathogen was found between *N.*
407 *fasciatus* and both species of *Bartonella*, providing evidence for vector specificity: these bacteria
408 were likely transmitted by *N. fasci*atus. Indeed, both *B. taylorii* and *B. grahamii* have been
409 previously detected in *N. fasciatus* collected from rodents (Silaghi *et al.* 2016). However, we
410 need to confirm the role of fleas in the transmission process, because when host density and
411 flea prevalence were simultaneously considered, variation in pathogen infection was explained
412 by host dynamics rather than flea prevalence. A lack of effect of flea prevalence on *Bartonella*
413 dynamics has been previously shown in voles (Telfer *et al.* 2007*a*). Therefore, our findings should
414 be considered with caution because we do not know which proportion of fleas becomes
415 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 modulating *Bartonella* prevalence over time could also be explained by a delayed-density dependence response of flea burden to common vole density as we observed in our study system (a lag of 8 months; unpublished data), but more work is needed to test this hypothesis.

***Coinfections with* Bartonella *and* F. tularensis**

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 24% during the population peak in July 2014 (see Table 3). The high percentage of individuals infected 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. According to Rossow *et al*. (2014), field voles and bank voles experimentally and naturally infected with *F. tularensis* ssp. *holarctica* readily developed lethal tularemia with similar severity and lesions, which suggests that there is not a chronic or latent infection in voles. On the other hand, experimentation has showed that common voles are less susceptible to be infected with a wild strain of *F. tularensis* ssp. *holarctica* than either BALB/c mice (*M. domesticus*) or yellow-necked mouse (*A. flavicollis*) (Bandouchova *et al*. 2009). Thus, these experimental studies suggest that *F. tularensis* can be potentially fatal to common voles. *Bartonella* spp. provokes lasting chronic infection in woodland rodents and can be detected in

rodent's blood for several weeks (Birtles *et al*. 2001). Thus, *F. tularensis* is expected to cause an acute and lethal infection in common voles, and *Bartonella* spp. a more chronic but non-lethal infection (Harms and Dehio, 2012). However, we do not know the average duration of infection by both bacteria in common voles. 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 multiplying the percentage of infected individuals by each pathogen independently (see Table 3), so we have no clear evidence of pathogen interaction. This preliminary result about coinfection should be confirmed by experimental studies focusing on interactions between *Francisella* and *Bartonella,* and some measures of infection duration in common voles.

Other pathogens

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, which may be related to the absence of other more suitable vectors, such as ticks (that infested around 2% 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 (*Crocidura russula*), 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,

465 although like in our study, *Ricketssia* spp. was not detected. In this other study, however, all the
466 small mammals were heavily infested by ticks.

467 All the results shown in our study came from one 80-km^2 area, so caution should be
468 exercised before generalizing to other common vole populations. Notwithstanding, we found
469 that voles were infected with four species of *Bartonella*, which had different dynamics according
470 to host density (vole and mice), season and flea infestation. Moreover, voles were infected with
471 *Bartonella* spp. and *F. tularensis*, but we did not find a clear pattern of association among
472 pathogens. Future studies could focus on identifying other suitable reservoirs as well as the
473 effect that these pathogens may have on individual voles and how the infective process
474 happens.

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484 **COMPETING INTERESTS**

485 The authors declare that they have no competing interests.

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671 **TABLES**

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

Bartonella species	N (%)
B. taylorii	19 (17)
with *B. grahamii*	27 (24)
with *B. rochalimae*	17 (15)
with *B. rochalimae* and *B. grahamii*	4 (4)
with *B. rochalimae* and *B. doshiae*	3 (3)
with *B. doshiae* and *B. grahamii*	2 (2)
B. rochalimae	14 (13)
with *B. doshiae*	4 (4)
with *B. grahamii*	3 (3)
with *B. clarridgeae*	1 (1)
B. grahamii	11 (10)
with *B. doshiae*	4 (4)
B. doshiae	1 (1)
Bartonella spp.	1 (1)
Total	111 (100)

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

682 **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
683 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
684 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	0	4	0	0	0	0	0	0	0	0
July 2013	14	71	7	15	0	0	0	0	0	0	0	0
November 2013	31	35	0	31	16	16	6	3	3	3	13	3
March 2014	58	43	0	63	14	40	6	6	0	8	24	25
July 2014	101	87	4	101	34	69	24	23	8	42	23	48
November 2014	12	92	0	18	6	50	6	3	22	14	22	33
March 2015	7	100	0	8	0	38	0	0	13	13	0	25
Total	225	68	2	240	20	47	13	95	6	22	19	30

685

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

			F. tularensis		Total
			Negative	Positive	
Bartonella spp.	Female	Negative	59 (25)	14 (6)	73
		Positive	42 (18)	20 (8)	62
	Male	Negative	51 (21)	4 (2)	55
		Positive	39 (16)	11 (5)	50
	Total		191	49	240

689

690 **FIGURE CAPTION**

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

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

701 **SUPPLEMENTARY MATERIAL**

702 **Table S1.** Background information on the pathogens screened in the studied common voles,
703 based on the best available knowledge for infections in rodents.

Pathogen	Mode of transmission	Infection length	References
A. phagocytophilum	Ticks	Self-limiting (4 to 8 weeks)	Jonhs *et al*. 2009
Bartonella spp.	Hematophagous arthropods (fleas)	Self-limiting (4 to 8 weeks)	Birtles R.J. 2005; Telfer *et al*. 2007*a*
B. burgdorferi	Ticks	Prolonged persistence	Gern *et al*. 1994
C. burnetii	Ticks	Murine rodents, used as animal models, are poorly susceptible to *C. burnetii* infection, and consequently a high dose of this bacterium is necessary to induce organ lesions.	Barandika *et al*. 2007
F. tularensis	Arthropods, water and air	Rapid infection (5-10 days)	Rossow *et al*. 2014 ; Bandouchova *et al*. 2009
Rickettsia spp.	Ticks, fleas, lice, mites	The epidemiology of *Rickettsia* species has not been investigated in detail, but small mammals are considered to play a role as reservoirs of the rickettsioses	Fischer *et al*. 2018

Figure 1.

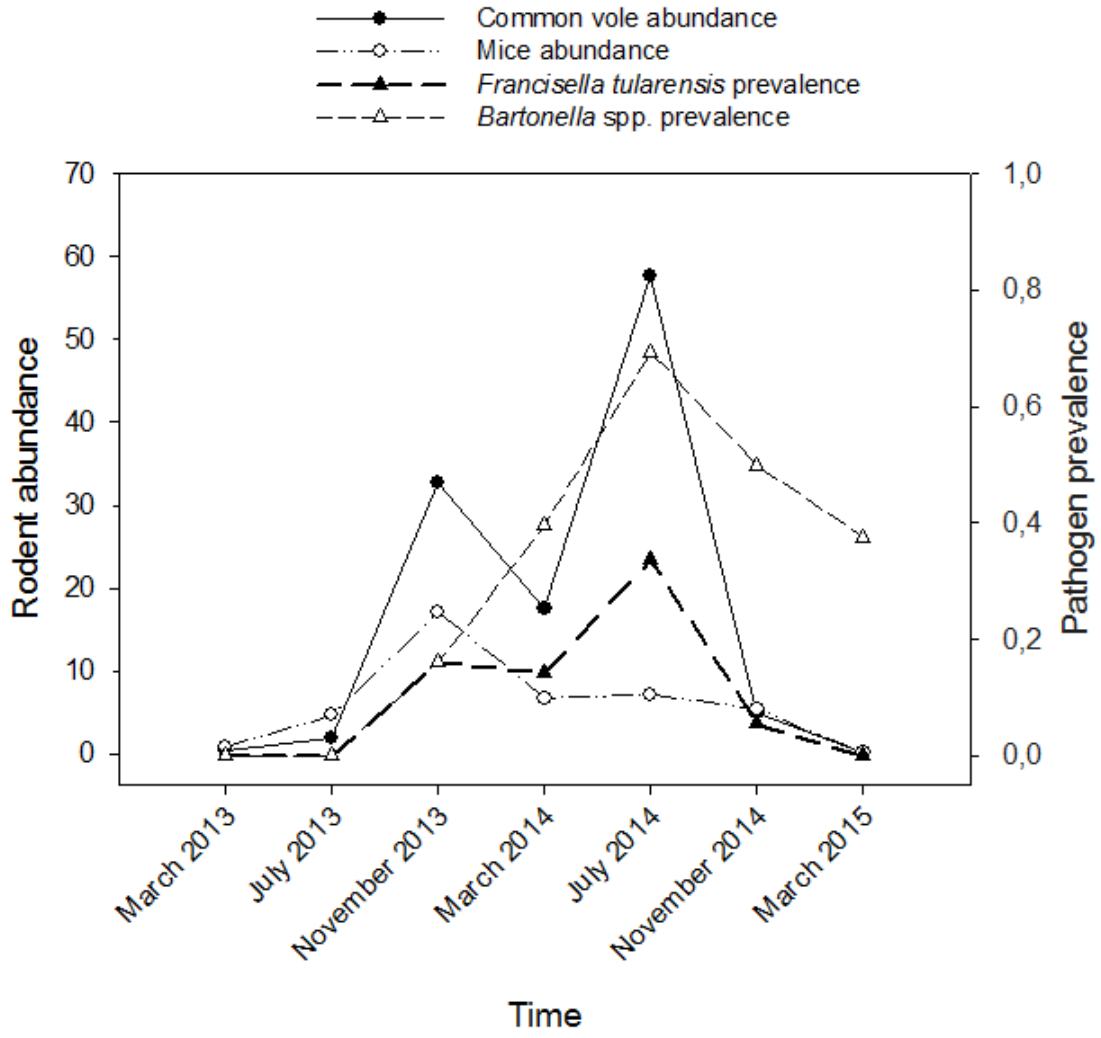
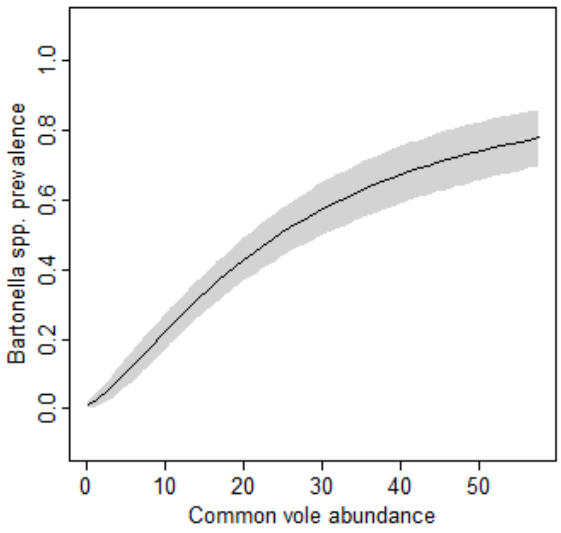
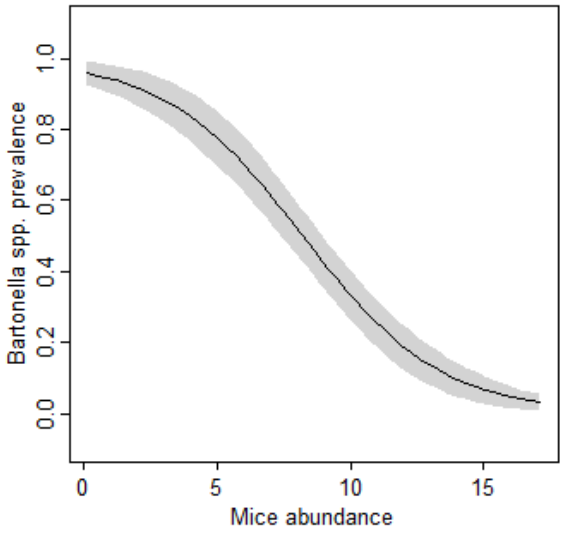
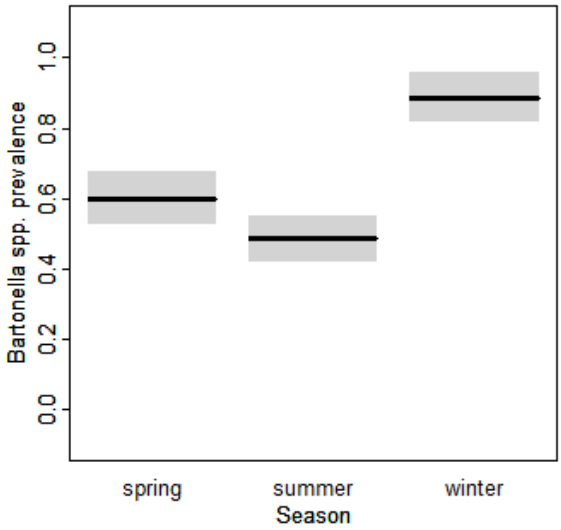

Common vole abundance
Mice abundance
Francisella tularensis prevalence
Bartonella spp. prevalence
Rodent abundance
Pathogen prevalence
70
60
50
40
30
20
10
0
1,0
0,8
0,6
0,4
0,2
0,0
March 2013
July 2013
November 2013
March 2014
July 2014
November 2014
March 2015
Time

Figure 2.


Bartonella spp. prevalence
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
Common vole abundance


Bartonella spp. prevalence
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
Mice abundance


Bartonella spp. prevalence
0.0
0.2
0.4
0.6
0.8
1.0
spring
summer
winter
Season