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# FREQUENT *LEPTOSPIRA* SPP. DETECTION BUT ABSENCE OF TULA ORTHOHANTAVIRUS IN *MICROTUS* SPP. VOLES, NORTHWESTERN SPAIN

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ABSTRACT: The common vole (*Microtus arvalis*) is a major agricultural pest in Europe and is a reservoir for several zoonotic agents, such as *Leptospira* spp. and Tula orthohantavirus (TULV). However, little is known about the occurrence of those pathogens in voles from Spain, where the species has largely expanded its distribution range in the past decades, causing agricultural pests and zoonotic diseases. For a molecular survey, 580 common voles and six Lusitanian pine voles (*Microtus lusitanicus*) were collected in 26 localities from four provinces of northwestern Spain. We assessed the presence of *Leptospira* spp. DNA in kidney tissue by PCR targeting the *lipL32* gene, detecting a prevalence of 7.9% (95% confidence interval, 5.9–10.4) for common voles and of 33.3% (95% confidence interval, 4.3–77.7) for Lusitanian pine voles. We identified *Leptospira kirschneri* in 24 animals and *Leptospira borgpetersenii* in two animals, using *secY* gene–specific PCR. We analyzed environmental and demographic factors (such as age class, weight, and sex) and population dynamics data for their potential effect on the *Leptospira* spp. prevalence in those voles. The *Leptospira* spp. DNA detection rate in common voles increased significantly with maximum air temperature, vole weight, and amount of accumulated rainfall during the 90 d before capture and within the peak phase of the population cycle. We assessed the presence of TULV in lung tissue of 389 voles by reverse-transcription PCR, with no positive results. The absence of TULV might be explained by the evolutionary isolation of the common vole in Spain. The detection of two *Leptospira* genomospecies underlines the necessity for further typing efforts to understand the epidemiology of leptospiral infection in the common vole and the potential risk for human health in Spain.

*Key words:* Age, common vole, hantavirus, *Leptospira* spp., Spain, weight, zoonoses.

## INTRODUCTION

The common vole (*Microtus arvalis*) is broadly distributed in major parts of Europe and features massive population explosions in certain regions of central and western Europe (Jacob and Tkadlec 2010). These population booms can be associated with human leptospirosis and tularemia disease clusters (Desai et al. 2009; Luque-Larena et al. 2015). Leptospirosis, considered a (re)-emerging zoonotic disease in humans, is caused by pathogenic species of gram-negative bacteria of the genus *Leptospira*, such as *Leptospira kirschneri*, *Leptospira borgpetersenii*, and *Leptospira interrogans*. The main means of spread within one host species, be it rodent or livestock, is by direct transmission within their nests or territories (Faine et al. 1999). Zoonotic infection with these pathogenic *Leptospira* spp. results from direct or indirect exposure to carrier animals that shed the bacteria in their urine. The spectrum of

human disease is variable and can range from subclinical infections to fatal multiorgan dysfunction (Karpagam and Ganesh 2020). Rodents have recently been linked, directly or through epidemiological studies, to leptospirosis outbreaks in livestock (Fávero et al. 2017; Marquez et al. 2019). The transmission depends on several factors, including climatic conditions, agricultural and livestock system factors, and the natural range of movement of the animals (Mwachui et al. 2015).

Additionally, the common vole is the main reservoir of Tula orthohantavirus (TULV), which is broadly distributed in various parts of Europe, with low to moderate prevalence (Schmidt et al. 2016; Maas et al. 2017). Only a few cases of human disease have been reported so far, with high fever, diffuse pain, headache (Reynes et al. 2015), renal syndrome, and pneumonia (Klempa et al. 2003), as well as dyspnea in an immunocompromised patient (Zelená et al. 2013). Hantaviruses, including TULV, are enveloped, with a segmented, negative-stranded RNA genome (Elliott 1990; Kukkonen et al. 1998). The small (S) segment and the large (L) segment encode the nucleocapsid protein and the RNA-dependent RNA polymerase, respectively, and are frequently used for molecular diagnostics and phylogenetic investigations (Sibold et al. 1999; Nikolic et al. 2014). Phylogenetic analysis of TULV sequences indicated a strong genetic structuring that is partially explained by their association to different evolutionary lineages of the common vole (Saxenhofer et al. 2017, 2019; Hiltbrunner and Heckel 2020).

There is no previous information about the infection with *Leptospira* and TULV in voles from Spain. However, pathogenic leptospires have been detected in other rodents in Spain (Arent et al. 2017; Millan et al. 2018), and hantavirus-reactive antibodies have been detected in humans and red foxes (*Vulpes vulpes*; Sanfeliu et al. 2011; Lledó et al. 2020). Therefore, our molecular survey aimed to evaluate the presence of *Leptospira* spp. and hantaviruses in voles from northwestern Spain, where the common vole has enlarged its distribution range in the past decades (García et al. 2020), and to identify potential factors related to the presence of these pathogens.

## MATERIALS AND METHODS

Between 2011 and 2014, voles were trapped at 26 sampling sites in four provinces of northwestern Spain within the western part of the Duero River basin (Fig. 1 and Supplementary Material Table S1). For each animal, trapping data and site, including phase of the population cycle and distance to the nearest water point (in meters), species, weight, age class (determined by the classification scheme of Morris [1972]), sex, and various biometric measurements were recorded; further details of trapping, phases of the population cycle, necropsy and sample collection, and storage and shipment are provided in the Supplementary Material. All handling procedures were approved by the University of Castilla-La Mancha Ethics Committee (reference no. CEEA: PR20170201) and are in accordance with the Spanish and European policy for animal protection and experimentation.

For detection of pathogenic *Leptospira* spp., DNA was extracted from kidney samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Samples of DNA were initially analyzed by conventional *lipL32* PCR (Mayer-Scholl et al. 2011). All PCR-positive samples were then subjected to *sec*Y sequence typing (Victoria et al. 2008) followed by multilocus sequence typing (MLST) of selected *secY* PCR-positive samples (Boonsilp et al. 2013). We used a mitochondrial cytochrome *b* gene-specific PCR (Schlegel et al. 2012a) to identify or confirm vole species and as a control for the quality of the nucleic acid preparation.

For hantavirus detection, RNA was extracted from lung samples with QIAzol reagent (Schmidt et al. 2016) and validated by β-actin reverse transcription (RT)-PCR (Wakeley et al. 2005). The RNA samples were screened by conventional S segment–specific RT-PCR (Schmidt et al. 2016) and SYBR green–based L segment RT real-time PCR (RT-rtPCR; Schlegel et al. 2012b) using the QuantiTect SYBR Green RT-PCR Kit (QIAGEN).

We used univariate and multivariable generalized linear mixed-effects models (GLMMs) to investigate the influence of several factors, including climatic, common vole individual factors, and population cycle-phase information (see Supplementary Material Table S2) on individual test status for *lipL32* PCR-positive or -negative common voles (binomial response variable).

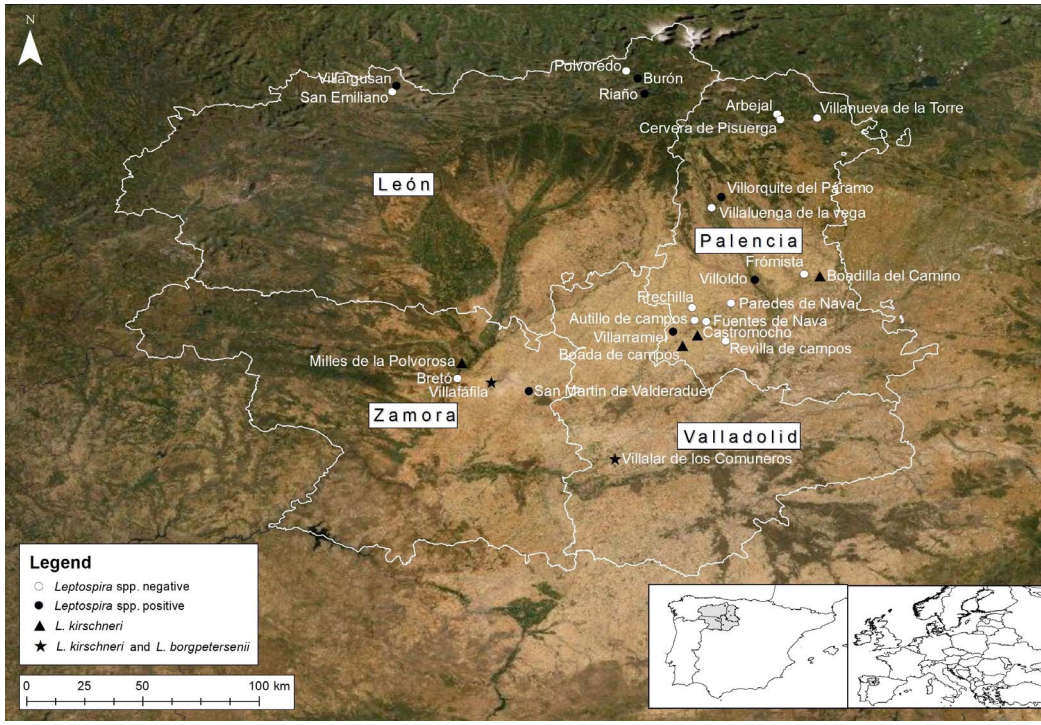


FIGURE 1. Map of northwestern Spain with the trapping sites for common voles (*Microtus arvalis*) and Lusitanian pine voles (*Microtus lusitanicus*) in provinces León, Palencia, Zamora, and Valladolid, with inserts of the localization of the trapping region in Spain and Europe. Black dots mark locations where voles tested positive for *Leptospira* spp. DNA; white dots indicate locations without *Leptospira* DNA detection in voles. Detection of *L. kirschneri* and of both *L. kirschneri* and *L. borgpetersenii* is indicated at the vole trapping sites by triangles and stars, respectively. *Leptospira* spp. DNA was detected by PCR targeting the *lipL32* gene; genomospecies identification used *secY* PCR and sequence determination. The map was generated using ArcMap 10.5.1 (ESRI 2011).

The trapping location (site) was included as a random factor, accounting for the spatial design of the study. To account for missing values in the explanatory variables, we removed all rows with at least one missing value from the dataset (pruning). The loss of information was high (33%, 195 of 599 rows); thus, models were fitted also with datasets in which only the respective rows with missing values in the explanatory variable considered were excluded (not pruning). The results of both approaches were qualitatively similar (data not shown). Here, we only present the results of the analyses performed with the dataset without pruning. Because some explanatory variable values were not available, we did not perform Akaike information criterion-based model selection because comparison across different models is only meaningful when fitted with the same dataset. Therefore, stepwise-backward and -forward model selection was used to determine the final regression models and to obtain a single minimum adequate model in which all variables were significant ($P<0.10$; Crawley 2007). All analyses were performed in R software (version 3.6.1; R Development Core Team 2019) using the package lme4 (Bates et al. 2014).

## RESULTS

Screening of 580 common voles and six Lusitanian pine voles (*Microtus lusitanicus*) with *lipL32* PCR allowed us to detect pathogenic *Leptospira* spp. in 46 common voles (7.9%; 95% confidence interval [CI], 5.9–10.4) and two Lusitanian pine voles (33.3%; 95% CI, 4.3–77.7; Supplementary Material Table S1). Infected voles were detected at 13 of 26 sites, in all four provinces (Fig. 1). In general, the DNA detection rate varied between the years (lowest in 2012 with 4.2%; 95% CI, 2.3–7.0; highest in 2014 with

TABLE 1. Results of univariate binomial generalized linear mixed-effects models for the individual infection of common voles (*Microtus arvalis*) with *Leptospira* spp. collected in the provinces of León, Palencia, Zamora, and Valladolid, northwestern Spain, 2011 to 2014.

| Variables tested in univariable models | Explanatory variables | | | |
|---|---|---|---|---|
| | Estimate | SE | Z value | *P* value[a] |
| Rainfall | 0.014026 | 0.005855 | 2.396 | **0.017** |
| Weight | 0.05496 | 0.01365 | 4.026 | **<0.001** |
| Maximum temperature | 0.06247 | 0.03762 | 1.660 | 0.097 |
| Relative air humidity | −0.02311 | 0.01775 | −1.302 | 0.193 |
| Phase population cycle (reference level: peak phase) | −0.9742 | 0.4766 | −2.044 | **0.041** |
| Sex, male (reference level: female) | 0.5347 | 0.3145 | 1.700 | 0.089 |
| Distance to water point log (water point meter) | 0.0807 | 0.1220 | 0.662 | 0.508 |
| Age class (reference level: adult) | | | | |
| Juvenile | −1.5978 | 0.7571 | −2.110 | **0.035** |
| Subadult | −1.1088 | 0.6275 | −1.767 | 0.077 |
| Year (reference level: 2011) | | | | |
| 2012 | −1.0183 | 1.3423 | −0.759 | 0.448 |
| 2013 | 0.2543 | 1.7659 | 0.144 | 0.886 |
| 2014 | 0.2267 | 1.4111 | 0.161 | 0.872 |

[a] Significant values are highlighted in bold ($P<0.05$).

13.1%; 95% CI, 9.2–18.3) and province (highest in León: 23.8%; 95% CI, 8.2–47.2; lowest in Valladolid: 5.1%; 95% CI, 0.6–17.3; Supplementary Material Table S1). The highest DNA detection rate at sites with at least 10 voles sampled was found in Palencia, Boada de Campos, and the average for the entire observation period was 16.4% (95% CI, 10.5–24.0) and, in 2014, was 20% (95% CI, 12.7–29.2). Typing by *secY* PCR revealed two *Leptospira* species in the common vole: *Leptospira kirschneri* ($n=24$) and *L. borgpetersenii* ($n=2$). All *L. kirschneri* samples selected were identified as ST 110 strains by MLST (Supplementary Material Table S1); MLST for *L. borgpetersenii* failed.

Results of the univariate analyses showed that the prevalence of *Leptospira* spp. was significantly associated with common vole weight, age class, rainfall, and phase of the population cycle (Table 1). Weight and age class had significant effects on *Leptospira* spp. prevalence when they were included separately in univariate GLMMs: adults had significantly higher prevalence than juveniles had ($P=0.035$) but not significantly higher prevalence than subadults had ($P=0.078$); weight effect was also significant ($P=0.000057$; see Table 1). However, when both variables were included together into models, only weight was significant ($P=0.000981$; other $P>0.05$). The variable "phase of the population cycle" was significant in the model when included alone ($P=0.041$), but when it was included with other variables, the model did not converge. The best minimum adequate model showed positive effects of vole weight and rainfall ($P<0.05$; Table 2). The *P* value of maximum temperature ($P=0.097$) was slightly above the chosen significance (Table 2). Results of this model showed that, for each one-unit increase in weight, we expect about 6% increase in the probability of the common vole being infected (i.e., *P=lipL32*–PCR positive; Fig. 2). The model predicts that, maintaining a constant maximum air temperature at its mean value (27 C), the probability of infection of common voles with *Leptospira* spp. was very high (0.70–0.90) for weights >40 g and high amount of accumulated rainfall (250 mm) during the 90 d before capture (Fig. 2). We tested two- and three-way interactions of all the variables that were statistically significant in univariate GLMMs (see Table 1). In addition, interactions between each of these significant variables with the rest of the nonsignificant

TABLE 2. The minimum adequate generalized linear mixed-effects model for the probability of individual infection of common voles (*Microtus arvalis*) with *Leptospira* spp. collected in northwestern Spain during 2011 to 2014. The vole trapping site was used as a random factor.

| Variables tested in multivariable model | Explanatory variables | | | |
|---|---|---|---|---|
| | Estimate | SE | Z value | *P* value[a] |
| Intercept | 7.250217 | 1.395408 | 5.196 | **<0.001** |
| Weight | 0.059013 | 0.013359 | 4.417 | **<0.001** |
| Maximum temperature | 0.069124 | 0.039647 | 1.744 | 0.081 |
| Rainfall | 0.015472 | 0.006141 | 2.520 | **0.012** |

[a] Significant values are highlighted in bold ($P<0.05$).

variables were tested and were found to be nonsignificant (data not shown).

For 384 common vole and four Lusitanian pine vole samples, the β-actin RT-PCR (internal control) was positive. However, the parallel hantavirus S- and L-segment RT-PCR assays had negative results for all samples (Supplementary Material Table S1).

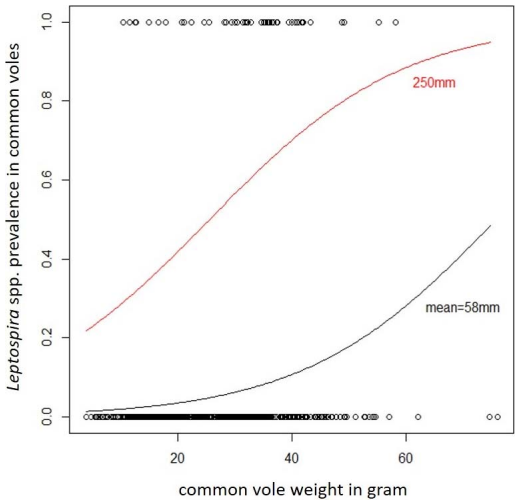


FIGURE 2. Probability of common vole (*Microtus arvalis*) being *lipL32*-PCR–positive (i.e., probability of vole being infected) as a function of vole weight and different amounts of accumulated rainfall during the 90 d before capture, for voles captured 2011–14 in provinces León, Palencia, Zamora, and Valladolid, northwestern Spain. Points are observed values. Lines are predictions of the best multivariable generalized linear mixed-effects model for the probability of common vole being *lipL32*-PCR–positive at specific amounts of rainfall (mean=58 mm; maximum=250 mm), which was calculated as follows:

$$p = \frac{\exp(\beta_0 + \beta_1 x_1 + \cdots + \beta_k x_k)}{1 + \exp(\beta_0 + \beta_1 x_1 + \cdots + \beta_k x_k)}.$$

For the predictions, $T_{max}$ was held constant at its mean value (27 C).

## DISCUSSION

Our molecular survey of voles from Spain for *Leptospira* spp. and TULV detected pathogenic *Leptospira* DNA in 46 of 580 (7.9%) common voles and in two of six (33%) Lusitanian pine voles. In previous studies in Spain, *Leptospira* DNA has been detected in various murine species, such as the wood mouse (*Apodemus sylvaticus*), the black rat (*Rattus rattus*), the house mouse (*Mus musculus*), and shrew species with a rate of 8% to 13% (Arent et al. 2017; Millan et al. 2018). Investigations in other European countries indicated DNA detection rates of 6.6% to 30% in common voles (Mayer-Scholl et al. 2014; Schmidt et al. 2014; Fischer et al. 2018; Kurucz et al. 2018; Blagojevic et al. 2019). Differences in the DNA detection rates might be due to different environmental conditions in Spain compared with those in central and southeastern European countries, which may influence survival of *Leptospira* spp. in the environment, transmission within the reservoir vole populations, and dynamics of vole populations and, thereby, the probability of pathogen transmission.

Our almost-exclusive detection of *L. kirschneri* and ST110 in common voles in Spain is in line with surveys in Germany (Mayer-Scholl et al. 2014; Fischer et al. 2018) and Austria (Jeske et al. in press). The additional detection of *L. borgpetersenii* in our study may imply a

spillover from another rodent or insectivore species, as reported previously (Mayer-Scholl et al. 2014). Wood mice were reported as a carrier of *L. borgpetersenii* in Spain (Millan et al. 2018) and were also trapped in the same areas in which the *L. borgpetersenii*–positive common voles were detected in our study (data not shown). Field voles (*Microtus agrestis*) were found in Germany to be exclusively infected by *L. kirschneri* ST110, whereas bank voles (*Myodes glareolus*) and yellow-necked mice (*Apodemus flavicollis*) were found to be infected not only by *L. kirschneri* ST110 but also by *L. borgpetersenii* and *L. interrogans*, suggesting potential spillover infections among rodent species in grassland, agricultural, and forest habitats (Fischer et al. 2018).

The discrepancies between the number of *lipL*32-positive and *secY*/MLST-positive animals are due to the lower sensitivity of the *secY*-PCR in comparison to the *lipL*32 screening PCR and the lower sensitivity of the MLST (based on seven individual PCRs). Because both the *secY*-PCR–based genomospecies identification and MLST analysis rely on sequencing, which needs larger amounts of amplified DNA, the sensitivity of the methods is lower than that of the *lipL*32 PCR. This has been observed previously (Obiegala et al. 2016; Fischer et al. 2018).

Weight was strongly and positively correlated to *Leptospira* DNA detection rate in common voles, in line with previous studies (Cortez et al. 2018; Fischer et al. 2018). This is also seen in our study in the significantly lower infection rates in juvenile voles compared with adult voles. This relationship has also been observed in other rodent species, such as rats (Krøjgaard et al. 2009; Costa et al. 2014; Heuser et al. 2017), and explained as a consequence of a persistent infection (Benacer et al. 2016; Heuser et al. 2017; Minter et al. 2017).

Sex has been controversially discussed as a further demographic factor that influences the *Leptospira* prevalence (Benacer et al. 2016; Cortez et al. 2018). This potential influence in males might be explained by the immunosuppressive effect of androgens or by behaviors (aggressiveness, dispersal, foraging) that increase exposure to pathogens. However, neither our study nor previous studies found a significant sex effect on *Leptospira* prevalence (Benacer et al. 2016; Cortez et al. 2018).

We found a significant, positive correlation for abundance or phase of population cycle of common voles and *Leptospira* prevalence in the univariate GLMM ($P$=0.049; Table 1). During the peak phase of the population cycle with high population density of voles, spread of *Leptospira* by direct transmission among reservoir hosts (Faine et al. 1999) is enhanced. Indirect transmission may also have an important role in vole reservoirs because climatic factors, such as rainfall and temperature, were significant in the GLMM. Once the bacteria are excreted into the environment, factors such as temperature, pH-value, ultraviolet light, and moisture affect survival of the organism and, thus, transmission. The highest *Leptospira* incidences are reported in regions with a mean annual temperature of 20 C (Jensen and Magnussen 2016) because these bacteria require warm conditions for survival outside the host (Thibeaux et al. 2018). The mean annual temperature in the investigated region in Spain is lower (Climate-Data.org 2021), suggesting low *Leptospira* survival, except for the summer, in which the maximum temperature observed in our study area in Spain is close to the temperature of 30 C, which is reported to be optimal for *Leptospira* survival outside the host (Andre-Fontaine et al. 2015).

Soil humidity is also an important factor for the survival of *Leptospira* spp. outside the host (Schneider et al. 2018; Thibeaux et al. 2018) and, therefore, for its prevalence in rodents (Morand et al. 2019). This can be mediated either by rainfall, which was positively correlated to *Leptospira* prevalence in our study, or by close water bodies, such as rivers and irrigation ditches (Ganoza et al. 2006; Morand et al. 2019), for which, we found no indication. In addition, we measured relative air humidity as a proxy, which was, however, not a significant factor in this study (Table 1; $P$=0.193). Heavy rainfall and flooding have been reported in several studies to

have a positive effect on *Leptospira* spp. prevalence in rodents (Perez et al. 2011; Ivanova et al. 2012; Mason et al. 2016; Cortez et al. 2018).

In our study, no TULV or related hantavirus RNA was detected in any of the voles investigated, although both a highly sensitive conventional and a broad-spectrum RT-rtPCR were used. This is in strong contrast to the TULV RNA detection rates of 11.8% to 40.8% reported for different regions in Europe (Scharninghausen et al. 2002; Schmidt et al. 2014, 2016; Maas et al. 2017; Kurucz et al. 2018; Saxenhofer et al. 2019). A possible reason for these negative results might be the long-term isolation of common voles on the Iberian Peninsula (Heckel et al. 2005; Fischer et al. 2014; Saxenhofer et al. 2017; García et al. 2020).

In conclusion, *Leptospira* spp. are important common vole–associated zoonotic pathogens in Spain. The role of Lusitanian pine voles as a potential reservoir of leptospires needs further attention because the number of such voles we sampled was very low. Additionally, the role of interspecies transmission of *Leptospira* spp. from the main rodent reservoir to other rodents needs further evaluation. The molecular typing approach requires further improvements because the sensitivity of the *secY* PCR and MLST seems to be only moderate. The risk of *Leptospira* infection for humans may increase after rainfall during population explosions of common voles. For evaluation of human infection risk, harmonized approaches are needed in a pan-European rodent-monitoring approach (Sonnenburg et al. 2017). These investigations should incorporate analysis of environmental conditions, such as rainfall and temperature, and soil features, including moisture. Finally, additional efforts are needed to clarify whether leptospirosis is an underreported disease. The lack of TULV in voles from Spain might be explained by the evolutionary history of the isolated common vole population. Future investigations should also investigate whether the common vole in Spain acts as reservoir of additional (potential) zoonotic pathogens, such as cowpox virus (Prkno et al. 2017), hepevirus (Ryll et al. 2019), tick-borne encephalitis virus (Achazi et al. 2011), *Coxiella burnetii* (Literak 1995), *Bartonella* spp. (Rodriguez-Pastor et al. 2018), and *Francisella tularensis* (Rodriguez-Pastor et al. 2017).

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## SUPPLEMENTARY MATERIAL

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