

1 **Pathogen community composition and co-infection patterns in a wild**
2 **community of rodents**

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31 **Abstract**

32 Rodents are major reservoirs of pathogens that can cause disease in humans and livestock. It is
33 therefore important to know what pathogens naturally circulate in rodent populations, and to
34 understand the factors that may influence their distribution in the wild. Here, we describe the
35 occurrence and distribution patterns of a range of endemic and zoonotic pathogens circulating
36 among rodent communities in northern France. The community sample consisted of 713 rodents,
37 including 11 host species from diverse habitats. Rodents were screened for virus exposure
38 (hantaviruses, cowpox virus, Lymphocytic choriomeningitis virus, Tick-borne encephalitis virus)
39 using antibody assays. Bacterial communities were characterized using 16S rRNA amplicon
40 sequencing of splenic samples. Multiple correspondence (MCA), multiple regression and
41 association screening (SCN) analyses were used to determine the degree to which extrinsic factors
42 (study year and site; host habitat, species, sex and age class), contributed to pathogen community
43 structure, and to identify patterns of associations between pathogens within hosts. We found a rich
44 diversity of bacterial genera, with 36 known or suspected to be pathogenic. We revealed that host
45 species is the most important determinant of pathogen community composition, and that hosts that
46 share habitats can have very different pathogen communities. Pathogen diversity and co-infection
47 rates also vary among host species. Aggregation of pathogens responsible for zoonotic diseases
48 suggests that some rodent species may be more important for transmission risk than others.
49 Moreover, we detected positive associations between several pathogens, including *Bartonella*,
50 *Mycoplasma* species, Cowpox virus (CPXV) and hantaviruses, and these patterns were generally
51 specific to particular host species. Altogether, our results suggest that host and pathogen specificity
52 is the most important driver of pathogen community structure, and that interspecific pathogen-
53 pathogen associations also depend on host species.

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59 **Keywords (6max)**

60 16S rRNA amplicon high throughput sequencing; Disease Ecology; Microbial Interactions;

61 Pathobiome; Rodent reservoirs; Zoonoses

62 1. Introduction

63 Infectious diseases are among the most important global threats to biodiversity, wildlife and
64 human health, and are associated with potential severe socioeconomic consequences (Daszak et al.,
65 2000; Jones et al., 2008; K. F. Smith et al., 2006). Although combatting these risks is a main
66 worldwide priority, our understanding of the processes underlying disease emergence still remains
67 too limited for developing efficient prediction, prevention and management strategies. In humans,
68 the majority of emerging pathogens originate as zoonoses from animal host populations in which
69 they naturally circulate (Jones et al., 2008; Taylor et al., 2001). Thus, identifying the
70 epidemiological features (e.g., prevalence, diversity, host specificity, geographic distribution) of
71 zoonotic pathogen communities in their wild hosts, and the factors that influence pathogen
72 occurrence in those communities, is as important to human health as it is to understanding the
73 fundamentals of disease ecology (Garchitorena et al., 2017).

74 Both extrinsic and intrinsic factors can contribute to the composition of natural pathogen
75 communities within and between wild animal species, populations and individuals. Factors extrinsic
76 to the hosts include geographic location, climate, periodicity of epidemic cycles and abiotic features
77 influencing inter-specific transmission opportunities (e.g., (Burthe et al., 2006; Harvell et al., 2002;
78 Poulin et al., 2012). Factors extrinsic to the pathogens such as host species identity, sex, age, and
79 body condition as well as genetic and immunogenetic features have also been intensively studied
80 (e.g., (Beldomenico et al., 2008; Bordes et al., 2017; Charbonnel et al., 2014; Salvador et al., 2011;
81 Streicker et al., 2010, 2013)). Although less investigated, inter-specific ecological interactions (e.g.,
82 competition, facilitation) among pathogens within animal hosts are also likely to be an important
83 intrinsic force in determining the composition of pathogen communities. Ecological interactions
84 between free-living species are well-known to play a part in the distribution, abundance, and many
85 other qualitative and quantitative features of populations; the application of this basic tenant of
86 community ecology to pathogen incidence and expression of disease has become recognized as
87 imperative for assessing both risks and potential benefits posed to human health, agriculture,

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97 wildlife, and conservation (Pedersen & Fenton, 2007). Simultaneous infection by multiple parasite
98 species is ubiquitous in nature (Cox, 2001; Moutailler et al., 2016; Petney & Andrews, 1998), and
99 prior infections can have lasting effects on future susceptibility via e.g., changes to host condition
100 and behavior or through immune-mediated processes (Karvonen et al., 2019; Kumar et al., 2018;
101 Quiñones-Parra et al., 2016; Singer, 2010). Interactions among co-circulating parasites may have
102 important consequences for disease severity, pathogen transmission, host and pathogen evolution or
103 co-evolution, and community-level responses to perturbations (Abbate et al., 2018; Alizon et al.,
104 2013; Jolles et al., 2008; Seppälä & Jokela, 2016; Telfer et al., 2010). Consequences of interaction
105 may be life-long, as exposure to pathogens circulating among juveniles have been found to be
106 strongly associated with those experienced by adults (Fountain-Jones et al., 2019). Such
107 interactions can also play a role in the consequences of pathogen emergence (e.g., emerging
108 bacterial infection increasing susceptibility to an endemic virus (Beechler et al., 2015)).
109 Henceforth, and through the advent of sequencing technologies in particular, it is now possible and
110 essential to investigate disease emergence from a multi-host / multi-pathogen perspective (Galan et
111 al., 2016), considering the potential influence of pathogen interactions on current and future disease
112 distributions (Abbate et al., 2018; Budischak et al., 2015; Cattadori et al., 2008; Jolles et al., 2008).
113 Rodent communities are relevant models for developing this community ecology approach to
114 disease distribution and emergence. They harbor a wide variety of pathogenic taxa (Bordes et al.,
115 2013; Diagne et al., 2017; Koskela et al., 2016; Pilosof, Morand, Krasnov, & Nunn, 2015), and are
116 important reservoir hosts of agents of zoonoses that have severe implications for human health. Han
117 et al. (2015) have revealed that about 10% of the 2277 extant rodent species are reservoirs of 66
118 agents of zoonoses, including viruses, bacteria, fungi, helminths, and protozoa. They also described
119 79 hyper-reservoir rodent species that could be infected by multiple zoonotic agents. Strong
120 ecological interactions, such as facilitation and competition, have been shown in wild rodent
121 populations among some of these zoonotic agents (Telfer et al., 2010), as well as between non-
122 zoonotic agents and zoonotic agents (e.g., helminthes and bacteria (Carvalho-Pereira et al., 2019);

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140 helminthes and viruses (Guivier et al., 2014; Sweeny et al., 2020); helminthes and protozoa
141 (Knowles et al., 2013)). In addition, rodents share a number of habitats with humans, including
142 urban settings, agricultural lands, and forests, providing opportunities for human-rodent contact and
143 pathogen transmission (Davis et al., 2005). Describing the distribution and composition of natural
144 pathogen communities in rodent populations, and determining the drivers behind pathogen
145 associations, is imperative for understanding the risks they may pose for public health.

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Deleted: (Knowles et al., 2013)

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146 In this study, we analyzed the pathogen communities carried by rodent communities in a rural
147 area of northern France, a region known to be endemic for several rodent-borne diseases including

148 nephropathia epidemica (Puumala orthohantavirus (Sauvage et al., 2002)) and borreliosis (Razzauti
149 et al., 2015). We investigated exposure histories (via the presence of antiviral antibodies) for several

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150 viruses (hantaviruses, cowpox virus, lymphocytic choriomeningitis virus, Tick-borne encephalitis
151 virus) and current or recent exposure to bacterial pathogens (using high-throughput 16S
152 metabarcoding of host splenic tissue). We described the pathogens detected, their prevalence in the
153 community and their individual distributions among host populations. We then tested the role of
154 extrinsic factors (e.g., habitat, host species, host age) in explaining variation in pathogen
155 distributions, and for associations (non-random co-infection frequencies) between pathogens that
156 might indicate intrinsic drivers (e.g., competition, facilitation) of pathogen community composition.

157 We expected that host species and habitat would be the most important factors structuring pathogen
158 community composition because most pathogens are largely host-specific, but those sharing

159 habitats should also share opportunities for transmission (Davies & Pedersen, 2008). After
160 accounting for extrinsic factors, we expected to retrieve several pathogen-pathogen associations

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161 previously identified in the literature. This included i) positive associations between cowpox virus
162 and *Bartonella* infections (*Microtus agrestis*, (Telfer et al., 2010)); ii) positive associations between

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163 distinct *Mycoplasma* species in mammalian hosts (Fettweis et al., 2014; Sykes et al., 2008; Tagawa
164 et al., 2012; Volokhov et al., 2017); iii) associations between *Bartonella* and hemotropic

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165 *Mycoplasma* species (both positive and negative associations, as well as experimental

175 demonstration of dynamic interactions, have been described in *Gerbillus andersonii* (Eidelman et
176 al., 2019). Lastly, we also expected to find previously-undescribed associations due to the large
177 bacteria and rodent dataset included in our study. All these associations were likely to differ
178 between host species, as differences in host specificity are also likely to be accompanied by
179 differences in transmission dynamics and host responses to infection (Dallas et al., 2019; Davies &
180 Pedersen, 2008; Singer, 2010).

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182 2. Materials and methods

183 2.1. Study area and host sampling

184 Rodent sampling was conducted over two years (Autumn 2010 & 2011) in rural habitats
185 surrounding two villages (Boult-aux-Bois and Briquenay) in the Ardennes region of northern
186 France (previously described in (Gotteland et al., 2014)). Sex and age class (based on specific body
187 measurements and classed as ‘adult’ for sexually mature animals and ‘juvenile’ for both juveniles
188 and sexually immature sub-adults) were recorded for each animal, a blood sample was taken for
189 serological analyses, and animals were then euthanized using isoflurane inhalation. Spleens were
190 taken and stored in RNAlater Stabilizing Solution (Invitrogen) at -20°C. Species captured from the
191 two sites included (family: Cricetidae) 195 *Arvicola scherman* (montane water vole), 10 *Microtus*
192 *agrestis* (field vole), 66 *Microtus arvalis* (common vole), 203 *Myodes glareolus* (bank vole); and
193 (family: Muridae) 43 *Apodemus flavicollis* (yellow-necked mouse), 156 *Apodemus sylvaticus* (wood
194 mouse), 32 *Rattus norvegicus* (brown rat). These seven focal host species were collected from traps
195 placed in distinct landscapes (henceforth referred to as host ‘habitats’) (Supplemental Materials
196 Figure S1): *R. norvegicus* were found uniquely on farms, *Ar. scherman* and *Mi. arvalis* were found
197 almost entirely in meadows, and the five remaining species occupied both forests and hedgerows.
198 Demographic differences between host species were observed for sex (e.g., male bias in *Ap.*
199 *sylvaticus*; Figure S2A) and age classes (e.g., relative abundance of juveniles in *Mi. arvalis* and *Ap.*
200 *sylvaticus* hosts; Figure S2B). Five *Microtus subterraneus* (European pine vole) and one each of

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206 three additional host species (one cricetid one echimyid and one murid) were also found in these
207 communities, but excluded from analyses due to their rarity; these rare (non-focal) hosts and their
208 pathogens are described in Supplemental Materials Appendix 1.

209

210 2.2. Detecting virus exposure and bacterial infection

211 Among the 713 rodents sampled for this study, indirect fluorescent antibody tests (IFATs; see for
212 details (Kallio-Kokko et al., 2006)) were successfully performed on 677 animals to detect
213 immunoglobulin G (IgG) specific to or cross-reacting with cowpox virus (CPXV, *Orthopoxvirus*),
214 Puumala or Dobrava-Belgrade virus (respectively PUUV and DOBV, *Orthohantavirus*, collectively
215 referred to henceforth as “hantavirus”), lymphocytic choriomeningitis virus (LCMV,
216 *Mammarenavirus*), and Tick-borne encephalitis virus (TBEV, *Flavivirus*). We refer to these
217 antiviral antibody tests as indicating a history of past exposure, but antibodies against hantavirus
218 and LCMV also likely indicate continued chronic infection. In contrast, current or very recent
219 exposure to bacterial infection was tested via 16S rRNA gene amplicon sequencing of splenic
220 tissue, giving no indication of past exposure history. The spleen was chosen because this organ is
221 known to filter microbial cells in mammals, allowing the detection of a wide array of pathogenic
222 and zoonotic agents. Funding was available to test for bacteria in just half of the animals, chosen
223 haphazardly to equally represent all host species, study sites and years, resulting in successful
224 analysis for 332 rodents (see Figure 1 for a breakdown of number of individuals sampled per focal
225 host species). For each individual animal, the DNA from splenic tissue was extracted using the
226 DNeasy Blood & Tissue kit (Qiagen) following the manufacturer recommendations. Each DNA
227 extraction was analyzed twice independently. We followed the method described in Galan *et al.*
228 (2016) to perform PCR amplification, indexing, pooling, multiplexing, de-multiplexing, taxonomic
229 identification using the SILVA SSU Ref NR 119 database as a reference ([http://www.arb-](http://www.arb-silva.de/projects/ssu-ref-nr/)
230 [silva.de/projects/ssu-ref-nr/](http://www.arb-silva.de/projects/ssu-ref-nr/)). Briefly, DNA samples were amplified by PCR using universal primers
231 targeting the hyper variable region V4 of the 16S rRNA gene (251 bp) and sequencing via Illumina

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233 MiSeq. The V4 region has been proven to have reasonable taxonomic resolution at the genus level

234 ~~(Claesson et al., 2010)~~. A multiplexing strategy enabled the identification of bacterial genera in

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235 each individual sample ~~(Kozich et al., 2013)~~. Data filtering was performed as described in Galan *et*

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236 *al.* (2016) to determine presence/absence of bacterial infections (summarized in Figure S3). Briefly,

237 we discarded all bacterial OTUs containing fewer than 50 reads in the entire dataset and animals for

238 which one or both individual PCR samples produced fewer than 500 reads. A bacterial OTU was

239 considered present in an animal if the two independent PCR samples were both above a threshold

240 number of reads, defined as the greater of either 0.012% of the total number of reads in the run for

241 that OTU (i.e., filtering using the rate of indexing leak) or the maximum number of reads for that

242 OTU in any negative control sample (i.e., filtering using the presence of reads in the negative

243 controls due to contaminations) ~~(Galan et al., 2016)~~. We removed chimera ~~(Ashelford et al., 2006)~~,

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244 using the *Uchime* program implemented in *mothur*, and manually checked OTUs representing

245 suspected chimera not identified by the program. For each OTU suspected as pathogenic, Basic

246 Local Alignment Search Tool (BLAST) searches of the most common sequences were conducted to

247 infer species identity where possible. ~~Given that rare, low abundance taxa tend to show high~~

248 ~~dissimilarity between sample replicates (D. P. Smith & Peay, 2014)~~, we assumed that only OTUs

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249 with at least 500 reads across all animals in the dataset were considered reliably detectable,

250 allowing us to assign absent status to these OTUs in animals failing to meet the criteria for OTU

251 presence. ~~Thus, only~~ OTUs for which there were at least 500 reads across all animals in the dataset

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252 (for which present and absent statuses could be assigned), and where reasonable certainty of

253 pathogenicity could be established from the literature ~~(see Table 1)~~, were considered in analyses of

254 the pathogen community.

255

256 2.3. Statistical Methods

257 All statistical analyses were implemented in R version 3.2.2 ~~(R Core Team, 2015)~~. Throughout

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258 our analyses, we refer to simultaneous bacterial infections as “co-infection”, while analyses

266 involving simultaneous presence of antiviral antibodies and bacterial infection are referred to as
267 evaluating “co-exposure”. While we can only be sure that antiviral antibodies represent past
268 exposure, we cannot rule out simultaneous viral and bacterial “co-infection”, particularly for viruses
269 known to cause chronic infections. Likewise, detection of current (or very recent) bacterial
270 infection, particularly for taxa known to cause chronic infections, cannot tell us how long the
271 animal has carried the infection. Thus, we refrain from assuming sequence of infection for statistical
272 tests unless specified by an *a priori* hypothesis from the literature.

273
274 *2.3.1. Testing for extrinsic drivers of pathogen community composition across the rodent*
275 *community*

276 We analyzed pathogen community composition across the whole rodent community. We use the
277 term “pathogen community” to refer to the group of viruses and pathogenic bacteria for which we
278 had the means to include, which was not exhaustive; thus measures of diversity are to be considered
279 relative and not absolute. We first estimated pathogen community richness using the Shannon
280 diversity index (alpha diversity) considering pathogenic bacterial OTUs and antiviral antibodies
281 found in each study year, study site, habitat, host species, host sex and host age group (default
282 options (natural logarithm Shannon index) in ‘diversity’ function from the *vegan* package). We
283 evaluated a linear regression model using analysis of deviance (‘lm’ and ‘drop1’ functions from the
284 basic *stats* package) to test for significance of differences in pathogen diversity due to the fixed
285 factors listed above after first correcting for all other factors in the model (marginal error tested
286 against the *F*-distribution). Post-hoc comparisons and correction for multiple tests were performed
287 using function ‘TukeyHSD’ from package *stats* and ‘HSD.test’ from package *agricolae* to group
288 factor levels that were not significantly different. An additional Akaike Information Criterion
289 (AIC)-based model selection analysis was performed to assess any qualitative influence of spurious
290 predictors on these comparisons using the ‘glmulti’ and ‘weightable’ function from package
291 glmulti. The impact of host species diversity (Shannon diversity with Chao’s estimator correction

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294 using 'Shannon' in package *entropart*) on pathogen species diversity was tested for by correlation
295 ('cor.test', package *stats*) across each year x site x habitat community.

296 We next tested for differences in pathogen community composition (beta diversity) between host
297 species, habitats, study sites, years, age ~~classes~~, and sexes by applying a permutational multivariate
298 analysis of variance (PERMANOVA) on a Bray-Curtis dissimilarity matrix ('adonis2' function in
299 the *vegan* package). To explore how intrinsic factors (pathogen-pathogen associations) contributed
300 to the structure of the pathogen community, we used multiple correspondence analysis (MCA) to
301 reduce variance in presence/absence of each bacterial pathogen species and antiviral antibody,
302 implemented with the function 'MCA' in the *FactoMinR* package and visualization tools found in
303 the *factoextra* package. This produces a set of quantitative and orthogonal descriptors (dimensions)
304 describing the pathogen community composition, revealing correlated variables. With each MCA
305 dimension as a continuous dependent response variable, we then evaluated linear regression models
306 using analysis of deviance with post-hoc comparisons (as detailed above) to understand how the
307 variation described by each MCA dimension was influenced by the extrinsic factors.

308

309 2.3.2. Testing for associations between co-circulating pathogens

310 Because we identified a large number of pathogens, the number of potential association
311 combinations to consider was excessively high, especially with regard to the relatively small
312 number of rodents sampled. We therefore decided to test the significance only of those
313 associations (i) clearly suggested by the community-wide MCA or (ii) previously described in
314 the literature: positive association between *Bartonella* spp. and CPXV (Telfer *et al.* 2010), positive
315 associations between *Mycoplasma* species (Fettweis *et al.*, 2014; Sykes *et al.*, 2008; Tagawa *et al.*,
316 2012; Volokhov *et al.*, 2017), and both positive (Eidelman *et al.*, 2019; Kedem *et al.*, 2014), and
317 negative (Cohen *et al.*, 2015), associations between *Bartonella* spp. and hemotropic *Mycoplasma*
318 species. Given the *a priori* assumption that associations would differ between host species, we
319 analyzed each host species separately; where evidence suggested no significant differences between

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Deleted: (Eidelman *et al.*, 2019; Kedem *et al.*, 2014)

Deleted: (Cohen *et al.*, 2015)

325 host species (non-significant variation in the MCA dimension among host species or non-significant
326 host species identity x explanatory pathogen term in logistic regressions), we pooled individuals
327 into a single analysis to gain statistical power.

328 We tested the significance of each association using both association screening (SCN) analysis

329 ~~(Vaumourin et al., 2014), and multiple logistic regression analysis (GLMs, modeling the binomial~~

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330 ‘presence/absence’ status of each pathogen as a function of the occurrence of other pathogens) on

331 the subset of host species in which the pathogens were found to circulate. We first performed SCN

332 analysis, as this approach is among the most suitable for detecting pathogen associations in cross-

333 sectional studies (Vaumourin et al. 2014). Briefly, given the prevalence of each pathogen species in

334 the study population, SCN analysis generates a simulation-based 95% confidence envelope around

335 the expected frequency of each possible combination of concurrent infection status (a total of 2^{NP}

336 combinations, where NP = the number of pathogen species) under the null hypothesis of random

337 pathogen associations. Observed frequencies of co-infection combinations falling above or below

338 this envelope are considered to occur more or less frequently, respectively, than in 95% of the

339 random simulations. Significance of the association is given as a *p*-value, calculated as the number

340 of instances in which the simulated co-infection frequency differed (above or below the upper or

341 lower threshold, respectively) from the observed frequency divided by the total number of

342 simulations ~~(Vaumourin et al., 2014).~~

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343 The benefit of the SCN approach is a relatively high level of statistical power and the ability to

344 identify precisely which combinations of pathogens occur outside the random expectations

345 (Vaumourin et al. (2014)). However, the SCN is sensitive to heterogeneity in the data due to

346 extrinsic factors (e.g., host specificity, or structuring in space, time, age or sex), which can both

347 create and mask true associations. A multiple logistic regression (GLM) approach was thus also

348 systematically employed, as it has the benefit of explicitly taking into account potentially

349 confounding extrinsic factors. Binomial exposure (presence/absence of either bacterial infection or

350 antiviral antibodies) to a single pathogen was set as the dependent variable with exposure to the

353 hypothetically associated pathogen(s) treated as independent explanatory variable(s) and extrinsic
354 factors (host sex, host age, study year, study site, and where appropriate, habitat) were specified as
355 covariates using function ‘glm’ in the *stats* package with a binomial logit link. When the multiple
356 host species were involved, we tested an interaction term (host species identity x explanatory
357 pathogen), and either (if $p < 0.05$) performed separate analyses for each host species or (if $p \geq 0.05$)
358 simply added host species identity as another covariate in the model. When there was no *a priori*
359 assumption concerning timing of exposure (e.g., antiviral antibody presence is more likely to affect
360 current acute bacterial infection than the reverse), the occurrence of each pathogen involved in a
361 given association was set as the dependent variable in reciprocal GLMs. ~~In contrast to the MCA~~
362 ~~dimensions above, a model selection step was first performed using the ‘glmulti’ and ‘weightable’~~
363 ~~function from package *glmulti* to find the best model among top-ranking models (small-sample size~~
364 ~~corrected Akaike Information Criterion (AICc) score less than 2 + lowest AICc) that retained all~~
365 ~~explanatory pathogens of interest, in an effort to limit the appearance of associations due to the~~
366 ~~inclusion of spurious predictors. Statistical~~ significance of the association was then assessed after
367 first correcting for all remaining covariates in the best model using the ‘drop1’ function (-2 log
368 likelihood ratio tests via single-term deletions compared to the full model). Despite a large number
369 of *a priori* hypotheses, we regarded a p-value of < 0.05 as significant due to the very low number of
370 individuals of each host species sampled. Though conceivably important, we also did not have
371 sufficient power to test for additional interaction terms.

372

373 2.3.3. Evaluating false discovery

374 Given the large number of significance tests performed on this single dataset, we compiled all
375 relevant p-values (N=77) and applied a Benjamini-Hochberg correction procedure to estimate how
376 many of the significant results may fall within the false discovery zone (using function ‘p.adjust’ in
377 the *stats* package). ~~Where model selection was performed, p-values were taken from full models~~
378 ~~prior to model selection.~~ Among tests of positively correlated hypotheses (e.g., pairwise tests of

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382 intrinsic pathogen-pathogen associations N=20), only one of the p-values from the pair was
383 included in calculating the false discovery rate (selected randomly). SCN analysis results were not
384 included, because they were also expected to be positively correlated with the logistic regressions,
385 and because the method inherently performs correction for multiple tests. Between hypotheses, the
386 data were often composed of different non-overlapping subsets of varying sizes, and sample sizes
387 varied widely with some being very small. Thus, application of this procedure likely indicates a
388 conservative (low) estimate for how many null hypotheses should truly be rejected.

389

390 **3. Results**

391 *3.1. Taxonomic identification and prevalence of pathogens*

392 *3.1.1. Viral exposure*

393 The most abundant virus detected was CPXV, with 222 (32.8%) positive sera of the 677 animals
394 tested for anti-CPXV antibodies. It was detected in all focal host species. However, significant
395 variation in prevalence was observed between focal host species (highly prevalent (43-70%) in *Ar.*
396 *scherman*, *Mi. agrestis*, and *My. glareolus*; Figure 1; $\chi^2 = 119.5$, $df = 6$, $p < 10^{-15}$). Anti-hantavirus
397 antibodies were detected in 16 animals (2.4%), and were significantly structured among host
398 species (with exposure highest in *Mi. arvalis* (9.7%), *R. norvegicus* (3.3%) and *My. glareolus*
399 (3.1%); Figure 1; $\chi^2 = 19.4$, $df = 6$, $p = 0.0036$). Anti-LCMV antibodies were detected in two *Mi.*
400 *arvalis* individuals (Figure 1). No animals were positive for anti-TBE antibodies.

401

402 *3.1.2. Bacterial pathogens*

403 Out of 952 bacterial OTUs represented by at least 50 reads in the dataset, 498 were considered
404 positive in at least one animal after data filtering (presented in Supplemental Materials Table S1).
405 Two OTUs (00024 & 00037) identified as *Bartonella* with low bootstrap values (74 and 92
406 respectively) appeared to represent chimeric sequences between the two highly amplified genera
407 (*Bartonella* and *Mycoplasma*) in co-infected samples. Two OTUs (00009 & 00117) which were

408 unclassified but which had a large number of reads in positive animals were also found to represent
409 chimeric sequences between the two genera, despite high bootstrap values (100). Three additional
410 chimeric *Mycoplasma* OTUs with under 500 reads were also excluded (OTUs 00076, 00159, and
411 00316). Two OTUs (00002 & 00059) were found to be redundant with OTUs Myco1 and Myco3,
412 respectively, and two more (00134 & 00220) were chimera between Myco OTUs. These 11 OTUs
413 were manually removed from the database, and are not included in Table S1.

414 We identified 43 OTUs belonging to bacterial genera with members known or thought to be
415 pathogenic in mammals (Table 1). After BLAST queries, we found 16 of these OTUs (representing
416 7 distinct genera) which could be considered as reliably detectable pathogens in the focal host
417 species (Figure 1). An additional 24 OTUs were considered potentially pathogenic but excluded
418 from analyses because they were only observed in rare host species, because presence-absence
419 could not be reliably established due to a low total number of reads (<500 in the dataset, e.g.,
420 *Borrelia* spp. and *Leptospira* spp.), because we could not rule out contamination by natural sources
421 of non-pathogenic flora during dissection (e.g., *Helicobacter* spp., *Streptococcus* spp.) or by known
422 contaminants of sequencing reagents (e.g., *Williamsia* spp.; (Salter et al., 2014)), or because their
423 identity to a pathogenic species was uncertain due to insufficient genetic variation at the 16S rRNA
424 locus (e.g., *Yersinia* spp.) (Table 1). We also identified three OTUs belonging to the eukaryotic
425 family Sarcocystidae (98% sequence similarity to the coccidian parasite *Sarcocystis muris*); though
426 each OTU was represented by >500 reads, there are currently no data on the reliability of this
427 method for detection (Table 1). Individual infection status for each of these OTUs is given in Table
428 S2.

429 The 16 reliably detectable pathogenic OTUs included *Bartonella* spp., 10 *Mycoplasma* spp.
430 OTUs, *Rickettsia canadensis*, “*Candidatus* Neoehrlichia mikurensis”, *Orientia* spp., *Brevinema*
431 *andersonii*, and *Spiroplasma* spp. Phylogenetic analysis including published sequences from
432 BLAST queries revealed that the 10 *Mycoplasma* spp. OTUs belonged to three distinct species:
433 *Myco. haemomuris* (Myco1-3,5,7-9), *Myco. coccoides* (Myco4 and Mco6), and “*Candidatus* Myco.

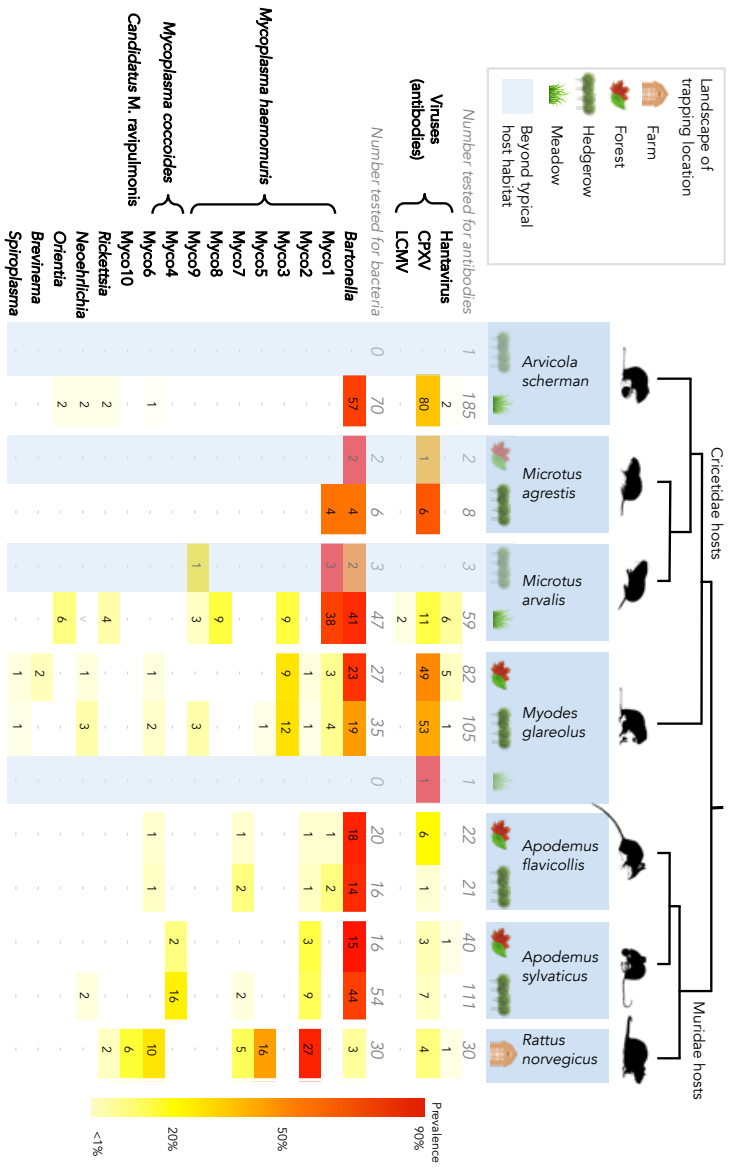
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435 ravipulmonis” (Myc10) (Figure S4). In general, these bacterial infections were present in all but 30
436 of the 332 animals tested (91.0% prevalent), and were not concentrated in any particular focal host
437 species ($\chi^2 = 9.7$, $df = 6$, $p = 0.139$). Prevalence of each pathogen in each focal host species is
438 presented in Figure 1.

439
440 3.2. Extrinsic drivers of pathogen community diversity and composition within rodent community

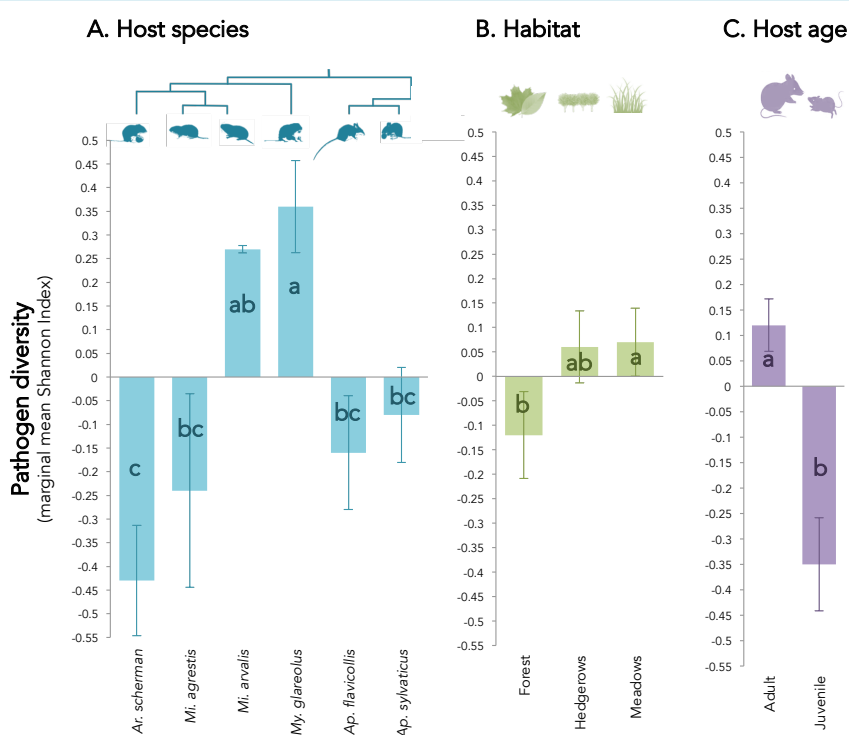
441 3.2.1. Analyses of pathogen diversity

442 We found evidence for the co-circulation of between 3 (in *Mi. agrestis*) and 12 (in *My.*
443 *glareolus*) pathogen taxa per host species across the rodent community (Figure 1). Using multiple
444 regression analysis on the Shannon diversity index, we found that marginal mean pathogen diversity
445 differed significantly between host species ($F_{5,69} = 6.86$, $p < 10^{-4}$) and habitats ($F_{2,69} = 4.97$, $p =$
446 0.0096), and it was significantly higher in adults than in juveniles ($F_{1,69} = 21.43$, $p < 10^{-4}$). Pathogen
447 diversity did not, however, differ between study sites, years, or host sexes (Figure 2, Table S3).
448 Post-hoc Tukey tests showed that after correcting for all other factors in the model, meadow
449 habitats had higher diversity of pathogen exposure than forest habitats, and the diversity of



451 pathogen communities in host species fell along a continuum between *My. glareolus* (high) and *Ar.*
 452 *scherman* (low) extremes (Figure 2, Table S3). The results were qualitatively identical when non-

Figure 2: Extrinsic drivers of pathogen diversity in a rodent species community. Differences in Shannon diversity index was tested on marginal means for each factor in the multiple regression model. Different letters signify statistically significant differences at $p < 0.05$, with post-hoc Tukey adjustments for multi-level factors.



453 significant predictors were removed from the model following model selection (Figure S5). Host
 454 species diversity in each community (year x site x habitat) was positively correlated with pathogen
 455 diversity ($r = 0.62$, $t = 2.5$, $df = 10$, $p = 0.032$).

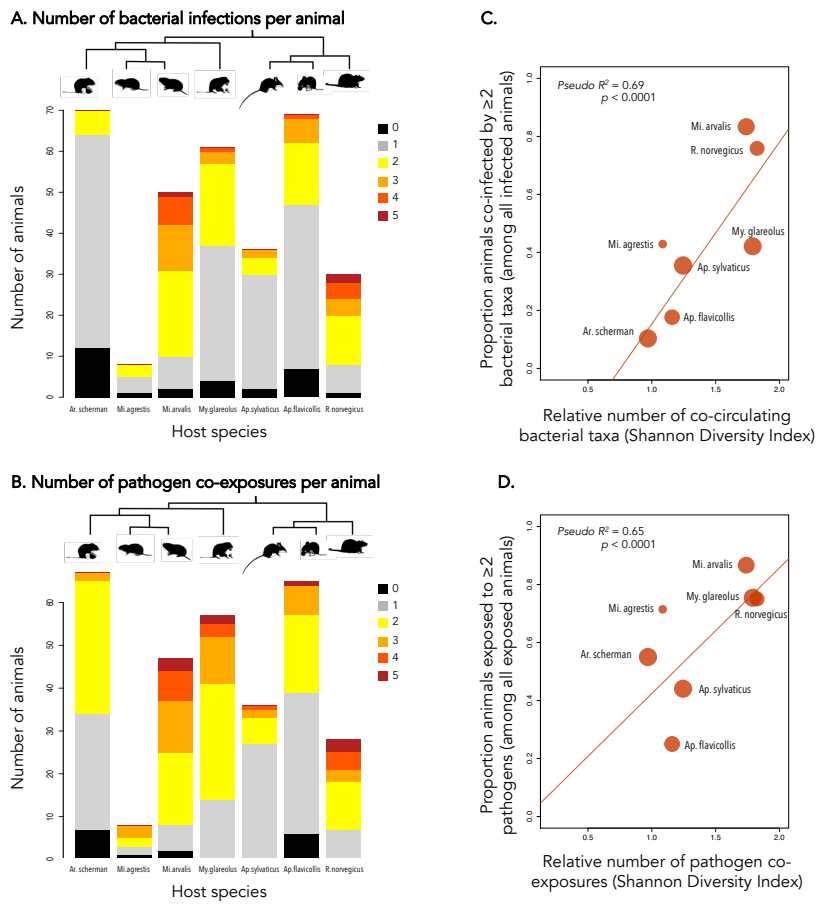
456 To understand the relative pathogen diversity of *R. norvegicus* hosts, not included in the above
 457 analysis because they were entirely confounded with farm habitats, we analyzed a modified model,
 458 excluding host habitat and including all seven focal host species. Post-hoc Tukey tests from this

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467 model showed that *R. norvegicus* hosts had the second most diverse pathogen community (Table
 468 S4).

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Figure 3: Bacterial co-infection and co-exposure patterns across host species.



469
 470 We also found an enormous amount of both bacterial co-infections and concurrent history of
 471 viral exposures (Figures 3A, 3B). The percentage of animals co-infected with two or more reliably
 472 detectable pathogenic bacterial OTUs among all those infected in each host species ranged between
 473 84.4% (in *Mi. arvalis*) and 10.5% (in *Ar. scherman*). This co-infection frequency was significantly
 474 correlated with the diversity (Shannon Index) of bacteria circulating in each rodent species (Figure

479 3C; analysis of deviance $Pseudo-R^2 = 0.69$, $p < 10^{-11}$, calculated using logistic regression weighted
480 by the number of infected animals per species). Bacterial co-infections were more frequent than
481 expected in *Mi. arvalis*, and less frequent than expected in *My. glareolus* (according to Cook's
482 Distance, Figure S6A). Results were similar when co-occurrence of antiviral antibodies was
483 considered along with bacterial OTU exposure (Figure 3D; $Pseudo-R^2 = 0.65$, $p < 10^{-7}$). While *Mi.*
484 *arvalis* had both more bacterial co-infections and slightly more pathogen co-exposures than
485 expected based on pathogen diversity, other outliers differed between the two measures (Figure
486 S6B): *My. glareolus* co-exposure frequencies were not lower than expected, and both *Apodemus*
487 species had lower than expected co-exposures. Host species diversity in each community did not
488 correlate with bacterial co-infection ($r = 0.33$, $t = 1.11$, $df = 10$, $p = 0.29$) or pathogen co-exposure
489 ($r = 0.022$, $t = 0.071$, $df = 10$, $p = 0.95$) frequencies.

490

491 3.2.2. Analyses of pathogen community composition

492 Many pathogen taxa were found only in a single host species (*Mycoplasma haemomuris* OTU
493 Myco8, “*Candidatus Mycoplasma ravigulmonis*” (Myco10), *Brevinema* spp., *Spiroplasma* spp.,
494 LCMV), and each host species had a unique combination of co-circulating pathogens (Figure 1). In
495 order to best identify extrinsic and intrinsic factors potentially driving the composition of pathogen
496 communities within the rodent community, we reduced the dataset to limit biases. We excluded *R.*
497 *norvegicus* individuals due to competing *a priori* hypotheses that host species and habitat would be
498 important factors (as this host species was the only one found in farm habitats, confounding these
499 two variables; but see MCA results when *R. norvegicus* was included in Figures S7, S8). Likewise,
500 we excluded pathogens that occurred only in one habitat type of one host species (not including *R.*
501 *norvegicus*). Two additional individuals were excluded due to missing sex and age information.
502 Analyses were performed on the remaining 280 individuals from six host species and their 14
503 pathogens (*Bartonella*, Myco1, Myco2, Myco3, Myco4, Myco6, Myco7, Myco9, *Rickettsia*,
504 *Neoehrlichia*, *Orientia*, *Spiroplasma*, and antibodies against CPXV and hantaviruses) (Figure 4).

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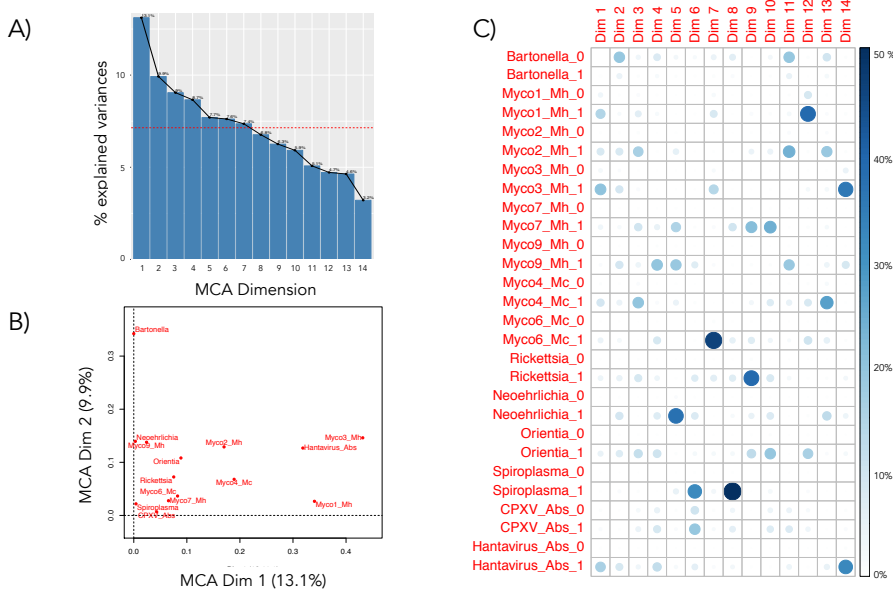
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Figure 4: Results of multiple correspondence analysis (MCA) for pathogen community composition in rodents (excluding *R. norvegicus*) are described by (A) the contribution of each dimension to the overall variance in the data, (B) variable correlations with the first two dimensions of the MCA, and (C) variable contributions to each orthogonal MCA dimension. Horizontal line in (A) represents the percent variance expected due to chance $(100/14) = 7.14\%$.



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513

514 Overall, pathogen species composition was significantly structured by host species identity
 515 ($F_{5,252} = 16.23$, $p = 0.001$) and habitat type ($F_{2,252} = 2.51$, $p = 0.024$; Table S5). Out of 14
 516 orthogonal dimensions returned by the MCA, the first two captured 23.0% of the variation in
 517 pathogen and antibody occurrence, and the first seven explained a cumulative 63.4% of the total
 518 variance (Figure 4A). Further dimensions captured less variance than would be expected if all
 519 dimensions contributed equally to overall inertia in the data. Dimension 1 (MCA Dim1; explaining
 520 13.1% of the variation in pathogen community and loading heavily with the presence of Myco1,
 521 Myco3 and anti-hantavirus antibodies) differed significantly between host species ($F_{5,268} = 23.83$, p
 522 < 0.0001) and age classes ($F_{1,268} = 6.27$, $p = 0.013$; Table S6). Dimension 2 (MCA Dim2;
 523 explaining 9.9% of the variation in pathogen community and primarily describing the occurrence of
 524 *Bartonella*) was also structured significantly by host species ($F_{5,268} = 3.89$, $p = 0.002$; Table S6).

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527 While these first two dimensions varied by host species (Figure S10A) and host age class (Figure
528 S10B), variance in host habitats (Figure S10C) was not significant after accounting for the other
529 factors. Host species was the most consistently important extrinsic driver of pathogen community
530 composition, significantly explaining variation captured in six of the first seven dimensions, MCA
531 Dim1 – MCA Dim7 (except for MCA Dim 5; Table S6).

532

533 3.3. Associations between pathogens

534 3.3.1. Validation of the associations detected by MCA

535 We applied SCN and GLM analyses to further characterize patterns detected using MCA. Strong
536 and relatively equal loading of MCA Dim1 with Myco1, Myco3, and anti-hantavirus antibody
537 presence indicated that these three pathogens were positively associated with one-another. Indeed,
538 the six animals with anti-hantavirus antibodies were found exclusively in animals infected with
539 Myco3, and Myco1 was found in 2/3 of hantavirus-exposed animals but in just 1/3 of those without
540 hantavirus exposure. The MCA also revealed significant differences among host species and host
541 age classes for Dim 1; hantavirus and Myco3 only circulated in two host species (*Mi. arvalis* and
542 *My. glareolus*) and 34 of those 35 occurrences were in adults. To exclude positive associations
543 arising from mutual host specificity and age-related accumulation of exposure probability, we
544 focused our analyses on the dataset restricted to adults of the two host species in which all three
545 pathogens co-circulated (*Mi. arvalis* and *My. glareolus*). Individual SCN analyses performed on
546 adults of each host species revealed no associations (Table S7A). However, since values of MCA
547 Dim 1 did not differ between the two host species (according to post-hoc tests given in Table S6),
548 we also ran a single SCN analysis on the pooled data from adults of both species to improve
549 statistical power (Table S7A). According to this pooled SCN analysis, the three-way co-occurrence
550 of Myco1, Myco3 and anti-hantavirus antibodies was significantly more frequent than would be
551 expected by random chance ($p = 0.008$), with a trend for anti-hantavirus antibodies occurring by
552 themselves more rarely than expected (sitting on the lower bound at zero; $p = 0.13$; Table S7A,

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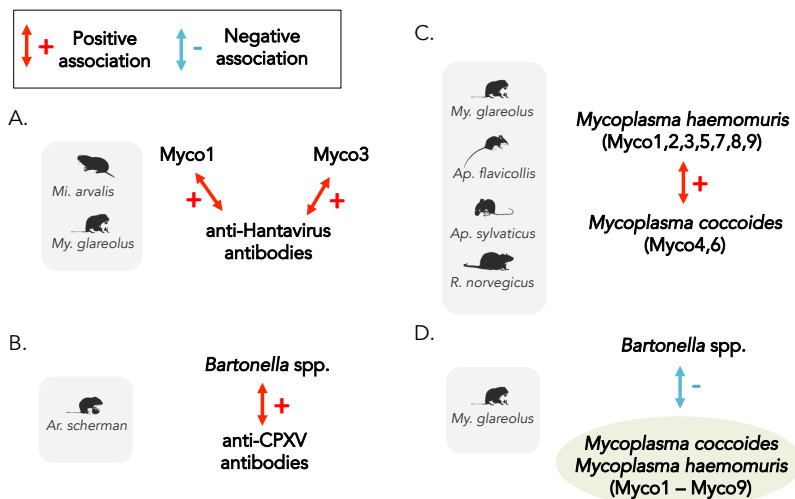
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560 Figure S11). We also investigated this association using GLM. However, given the small number of
 561 hantavirus exposures and perfect association with Myco3 infection, there was insufficient statistical
 562 power to explicitly test for an association between all three pathogens and extrinsic factors. We
 563 therefore ran three reciprocal GLM models on the restricted dataset, one for each pathogen as a
 564 function of extrinsic factors to control for heterogeneous host groups (host species, host sex, study
 565 site, habitat, and year sampled) and exposure to the two other pathogens (Table S7B). [The number](#)
 566 [of extrinsic variables in each model was reduced using model selection \(results in Figure S12\).](#)
 567 These models showed that there remained significant unexplained positive associations between
 568 hantavirus exposure and Myco1 infection (anti-hantavirus antibodies ~ Myco1: $\chi^2 = 5.80$, $p =$
 569 0.016) and between hantavirus exposure and Myco3 infection (Myco3 ~ anti-hantavirus antibodies:
 570 $\chi^2 = 13.66$, $p < 0.001$), but that there was no evidence of direct association between Myco1 and
 571 Myco3 infections (Myco1 ~ Myco3: $\chi^2 = 0.01$, $p = 0.94$; Myco3 ~ Myco1: $\chi^2 < 0.37$, $p = 0.54$;
 572 Figure 5A).

Figure 5: Associations between pathogens in a community of rodents. Association hypotheses were generated by multiple correspondence analysis (A) or previously noted in the literature (B, C, D). Only associations supported by significant statistical tests ($p < 0.05$) are illustrated. Red arrows represent positive associations, blue arrows represent negative associations.



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586 The third MCA dimension also presented a clear hypothesis with sufficient statistical power to
587 be tested. MCA Dim3 was characterized by co-variation in Myco2 (*Myco. haemomuris*) and Myco4
588 (*Myco. coccoides*) infections suggesting a positive association between members of these two
589 *Mycoplasma* species. Myco2 and Myco4 OTUs co-circulated only in *Ap. sylvaticus* hosts, thus we
590 limited our analysis to this host species. There was no significant association between the two
591 OTUs detected by SCN analysis (Table S8A), and after correcting for extrinsic factors remaining in
592 the models after model selection (Figure S13), there remained only a non-significant trend (Myco2
593 ~ Myco4: $\chi^2 = 2.79$, $p = 0.12$; Myco4 ~ Myco2: $\chi^2 = 2.34$, $p = 0.13$; Table S8B) for a positive
594 association between the two OTUs. No additional associations with sufficient variance for statistical
595 tests were clearly suggested by the MCA analysis.

596

597 3.3.2. Validation of associations described in the literature

598 We tested the *a priori* hypothesis that seropositivity to CPXV would be positively associated
599 with *Bartonella* infection, previously detected in *Mi. agrestis* (Telfer et al., 2010). The whole
600 dataset was considered as these two pathogens co-circulated in all host species (Figure 1). SCN
601 analyses performed independently for each host species revealed no associations (Table S9A), and
602 the MCA results suggested that pooling data across host species would be inappropriate. After
603 correcting for extrinsic factors using GLM, we found reciprocal evidence for a positive association
604 in *Ar. scherman* hosts (*Bartonella* ~ anti-CPXV antibodies: $\chi^2 = 5.07$, $p = 0.024$; anti-CPXV
605 antibodies ~ *Bartonella*: $\chi^2 = 5.07$, $p = 0.024$; Figure 5B), but not in any other host species (Figure
606 S14; Table S9B). It is of note that there were only eight *Mi. agrestis* individuals, rendering
607 statistical power to test for the association while controlling for extrinsic factors insufficient in this
608 host species where the association was previously described. While prevalence of both pathogens in
609 *Mi. agrestis* was relatively high compared to other host species, one of the two animals without

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620 *Bartonella* infection was positive for anti-CPXV antibodies, also precluding evidence for a within-
621 species trend.

622 We next focused on the potential associations between OTUs identified as belonging to two
623 different species of hemotropic *Mycoplasma*, *Myco. haemomuris* (HM) and *Myco. coccoides* (HC),
624 within the four host species in which they both circulated (*My. glareolus*, *Ap. flavicollis*, *Ap.*
625 *sylvaticus*, *R. norvegicus*; Figure 1; Figure S4). We found no significant associations using
626 independent SCN analyses for each host species (Table S10A). However, after controlling for
627 extrinsic factors using GLM, a significant positive association was detected (HM ~ HC: $\chi^2 = 9.5$, p
628 = 0.0021; HC ~ HM: $\chi^2 = 9.59$, $p = 0.002$), and did not differ between host species (non-significant
629 interaction term between host species by explanatory pathogen occurrence in each reciprocal model,
630 [Figure S15](#); Table S10B; Figure 5C). We note that only one *R. norvegicus* animal was uninfected
631 with *Myco. haemomuris*, and that animal also had no *Myco. coccoides* infection; thus the trend for
632 the association in this host species was also positive but lacked sufficient variance for independent
633 statistical analysis.

634 Finally, we tested for associations between *Bartonella* spp. and hemotropic *Mycoplasma* species,
635 grouping the occurrence of different OTUs of the latter (Myco1 – Myco9) into a single presence-
636 absence variable. There was no association detected by SCN analyses (Table S11A), and marginal
637 evidence that any association may differ by host species after correcting for extrinsic factors using
638 GLM with model selection ([Figure S16](#); Table S11B). After controlling for extrinsic factors using
639 independent GLMs for each host species (where possible), we found a negative association between
640 the two pathogen groups only in *My. glareolus* hosts (*Bartonella* ~ *Mycoplasma*: $\chi^2 = 4.14$, $p =$
641 [0.042](#); *Mycoplasma* ~ *Bartonella*: $\chi^2 = 6.59$, $p = 0.010$; Table S11B, Figure 5D).

642
643 3.4. Evaluating false discovery

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651 Benjamini-Hochberg correction of p-values from hypothesis tests throughout the study suggested
652 that those above ~0.01 may lie above the false discovery cutoff for statistical significance (Figure
653 S17), and that null hypotheses rejected with smaller p-values have been rejected with confidence.

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655 4. Discussion

656 Rodents have long been recognized as important reservoirs of infectious agents, with a high
657 transmission potential to humans and domestic animals (Kruse et al., 2004), Europe is identified as
658 a hotspot of rodent reservoir diversity and one third of rodent species are considered hyper-
659 reservoirs, carrying up to 11 zoonotic agents (Han et al., 2015). Nevertheless, associations between
660 these pathogens have still only rarely been investigated (but see, for example, studies from field
661 voles in the UK (Telfer et al., 2010), and in Poland (Pawelczyk et al., 2004), gerbils in Israel (Cohen
662 et al., 2015), across a rodent community in North America (Dallas et al., 2019), and co-infection
663 frequencies of zoonotic pathogens from rodents in Croatia (Tadin et al., 2012)).

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664 In this study, we confirmed that rodent communities in northern France may harbor a large
665 diversity of potential zoonotic pathogens, with at least 10 bacterial genera and antibodies against at
666 least four genera of viruses. Some of these pathogens have already been reported in the study region
667 or in geographic proximity, including viruses (*Orthohantavirus*, *Orthopoxvirus*, *Mammarenavirus*
668 (*Charbonnel et al., 2008; Salvador et al., 2011*)) and bacteria (e.g., *Bartonella*, *Mycoplasma*,
669 *Rickettsia*, "*Candidatus Neohrlichia*", *Orientia*, *Spiroplasma*, *Treponema*, *Leptospira*, *Borrelia*,
670 *Neisseria*, *Pasteurella*; see (*Razzauti et al., 2015; Vayssier-Taussat et al., 2012*)). A previously
671 undetected relative of the putatively pathogenic spirochaete *Brevinema andersonii* that infects
672 short-tailed shrews and white-footed mice in North America (*Defosse et al., 1995*), was among our
673 findings, and TBEV is not known to circulate this far east (*Lindquist & Vapalahti, 2008*). The high
674 prevalence of anti-hantavirus antibodies in *Mi. arvalis* is likely explained by cross-reactivity
675 between the anti-PUUV antibodies used in our assay and those elicited against the related *Tula*

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690 *orthohantavirus* (TULA) virus common to European voles (Deter et al., 2007; Tegshduuren et al.,
691 2010).

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692 Three zoonotic pathogens were particularly prevalent: *Orthopoxvirus*, *Bartonella* spp., and
693 *Mycoplasma* spp. The wide range of hosts with anti-*Orthopoxvirus* antibodies corroborates prior
694 evidence that cowpox virus could be widespread in European rodents, particularly voles (Bennett et
695 al., 1997; Essbauer et al., 2010; Forbes et al., 2014). An astounding 77% of all individuals in the
696 study were infected by *Bartonella* spp., a diverse group of hemotrophs known to commonly infect
697 rodents and other mammals (Bai et al., 2009; Breitschwerdt & Kordick, 2000), and which have also
698 been implicated in both zoonotic and human-specific disease (Breitschwerdt, 2014; Iralu et al.,
699 2006; Vayssier-Taussat et al., 2016). We could not assess the specific diversity of *Bartonella* spp.

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700 circulating in these rodent communities because accurate resolution in this genus requires additional
701 genetic markers (L. Guy et al., 2013; Matar et al., 1999). Hemotropic and pneumotropic

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702 *Mycoplasma* spp. were also highly prevalent across all host species, though surprisingly lower than
703 expected in *Ar. scherman* (Villette et al., 2017). These *Mycoplasma* species are also known
704 pathogens of humans and rodents (Baker, 1998; Harwick et al., 1972). Here, we found two distinct

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705 hemotropic *Mycoplasma* species (*Myco. haemomuris* and *Myco. coccoides*) and the pneumotropic
706 *Mycoplasma* species *Myco. pulmonis* and “*Candidatus Myco. ravidulmonis*”. The former two are
707 both hemotropic mycoplasmas responsible for vector-transmitted infectious anaemia of wild mice,
708 rats, and other rodent species (Messick, 2004; Neimark et al., 2001, 2005). In contrast, *Myco.*
709 *pulmonis* and “*Candidatus Myco. ravidulmonis*” cause respiratory infections, are more closely
710 related to other pneumotropic mycoplasmas, and “*Candidatus Myco. ravidulmonis*” has only ever
711 before been described in laboratory mice (formerly termed Grey Lung virus (Andrews & Glover,
712 1945; Graham & Schoeb, 2011; Neimark et al., 1998; Piasecki et al., 2017)).

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713 Our results also corroborated the status of hyper-reservoir (more than two zoonotic pathogens
714 carried by a reservoir species) for all seven of the focal rodent species studied here (Han et al.,
715 2015). Even the rare host species *Mi. subterraneus* also carried two potentially zoonotic pathogens

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730 (*Bartonella* spp. and *Brevinema* spp.; Appendix 1). Overall, we found a high variability in the
731 number of pathogens circulating in each species despite correction for sampling effort, with low
732 levels observed in *Apodemus* species and *Arvicola scherman*, and high levels detected in *Mi.*
733 *arvalis*, *My. glareolus*, and *R. norvegicus*. While physiology, genetics, and behavior can contribute
734 to the number of pathogen species able to infect a given host species, larger geographic range size is

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735 highly correlated with higher pathogen species diversity (Morand, 2015); this explanation matches
736 the pattern among hosts in the communities sampled here (i.e., *Ar. scherman* and *Apodemus* spp.
737 have small geographic ranges compared to those of *Mi. arvalis*, *My. glareolus*, and *R. norvegicus*).
738 Several studies have emphasized the influence of host habitat specialization on parasite species
739 richness, low habitat specialization being associated with both high species richness of macro- and
740 micro-parasites (e.g., (Morand & Bordes, 2015)). Our results did not fully corroborate this
741 association; while the grassland-specific *Ar. scherman* had the lowest pathogen diversity and the
742 multi-habitat spanning *My. glareolus* had the highest pathogen diversity, entirely farm-dwelling *R.*
743 *norvegicus* had high pathogen diversity nearly equal to that of *My. glareolus*, and the two
744 *Apodemus* hosts (neither with significantly higher pathogen diversity than *Ar. scherman*) were
745 found across both meadows and hedgerows. Instead, we found that more diverse host species
746 communities hosted more diverse pathogen communities. However, the implications of that result
747 are complex because while exposure to diverse (i.e., potentially novel) pathogens is a risk for
748 disease emergence, diverse host species communities are thought to keep individual pathogen
749 prevalence low due to the dilution effect – which should limit risk of zoonoses (Keesing et al.,
750 2010).

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751 The search for factors that drive parasite species richness, diversity and community composition
752 has been at the core of numerous studies (Krasnov et al., 2010; Mouillot et al., 2005; Nunn et al.,
753 2003; Poulin, 1995; Poulin & Morand, 2000; Sallinen et al., 2020). Here, we emphasized that both
754 pathogen diversity and community composition was mainly structured by host species identity,
755 despite both shared habitats and shared pathogen taxa. Pathogen beta diversity was also structured

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762 by habitat, which could result from particular environmental suitability (e.g., for vectors) or
763 opportunities for cross-species transmission. We found no evidence that any specific pathogen-
764 pathogen associations were likely to be as important as host species identity in determining
765 pathogen distributions across the community of rodents. The strong influence of host characteristics
766 (Cohen et al., 2015), and host species identity (Dallas et al., 2019), on pathogen community
767 composition has recently been described in comparison to intrinsic pathogen-pathogen associations
768 in other rodents. Moreover, the pathogen community composition provided a unique signature for
769 each rodent species, even among those most closely related (e.g., *Ap. flavicolis* and *Ap. sylvaticus*).
770 This result is in line with the conclusions of meta-analyses showing that phylogeny, over other host
771 traits, has a minimal impact on pathogen diversity in rodent species (C. Guy et al., 2019; Luis et al.,
772 2013).

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773 The importance of host species identity in shaping pathogen community composition may not
774 stem from strict host-pathogen specificity, as most pathogens were found to infect multiple host
775 species – a broad result echoed across animal communities (Cleaveland et al., 2001; Pedersen et al.,
776 2005; Streicker et al., 2013; Taylor et al., 2001; Woolhouse et al., 2001). However, we might be
777 cautious as more precise molecular analyses are necessary to test whether different species of a
778 bacteria genus or divergent populations of the same bacteria species may circulate independently in
779 different rodent host species, with little or no transmission. For example, two genera seemed to be
780 largely shared among the rodent species studied here, *Bartonella* and *Mycoplasma*. But previous
781 studies have shown strong host-specificity when considering the genetic variants of *Bartonella*
782 (Brook et al., 2017; Buffet et al., 2013; Withenshaw et al., 2016). Evidence in the literature for host
783 specificity of *Mycoplasma* species has led to a mix of conclusions (Pitcher & Nicholas, 2005), as
784 cases of cross-species transmission are commonly reported – particularly in humans – despite a
785 general consensus that most species are highly host-specific. We found that some *Mycoplasma* taxa
786 were dominant contributors to prevalence in a single host species, and that when shared, they were
787 shared with just a few other specific host species. Rare infections in unexpected host species (e.g.,

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797 Myco6 in *Ar. scherman* and Myco1 in *Ap. flavicollis*) were represented by fewer sequence reads
798 compared to positive samples in host species where they were more prevalent, suggesting a low
799 potential for amplification and sustained transmission from these occasional hosts (Figure S4). On
800 the other hand, while the Cricetidae appeared to be susceptible only – with rare exception – to taxa
801 within the *Myco. haemomuris* group, host species in the Muridae family were susceptible to all
802 three distinct *Mycoplasma* species detected. The biggest exception to this pattern was that three of
803 62 *Myodes glareolus* (sister to all other sampled Cricetidae in the study) animals were found to be
804 infected by both hemotropic *Mycoplasma* species. These results both support the observation that
805 cross-species transmission naturally occurs among wild rodents and suggest that the degree of host
806 specificity may be driven by both host and pathogen factors.

807 Concurrent exposure to multiple pathogens within individuals was also frequent, as high as 89 %
808 (in *Mi. arvalis* hosts), in line with recent studies that have shown that co-infections by multiple
809 pathogens are common in natural populations (e.g., in mammals, birds, amphibians, ticks, humans
810 [\(Clark et al., 2016; Griffiths et al., 2011; Moutailler et al., 2016; Stutz et al., 2018; Telfer et al.,](#)
811 [2010\)](#)). Variation in the frequency of pathogen co-exposure was highly correlated to the diversity of
812 pathogens circulating in each host species, suggesting the dominance of a random process of
813 pathogen exposure for each individual. However, there were a few intriguing outliers: *My. glareolus*
814 hosts were less co-infected than expected based on diversity of bacterial taxa, but not when viral
815 antibodies were included; conversely, *Ar. scherman* hosts were more co-exposed when viruses were
816 considered, but not when only bacteria were considered; and *Mi. arvalis* hosts had consistently
817 higher proportions of co-exposures whether viruses were or were not considered along with
818 bacteria. The non-random grouping of pathogen exposures within individuals (as in *Mi. arvalis*)
819 may result from heterogeneity in extrinsic transmission, environmental, or susceptibility factors
820 [\(Beldomenico et al., 2008; Beldomenico & Begon, 2010; Cattadori et al., 2006; Fenton et al., 2010;](#)
821 [Swanson et al., 2006\)](#), or from intrinsic interactions between pathogens (e.g., facilitation mediated
822 by hosts immune response). Differences in the pattern of co-exposure frequencies when including

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828 or excluding antiviral antibodies (as with *My. glareolus* and *Ar. scherman*) could result from
829 different mechanisms (e.g., bacterial manipulation of innate immunity (Diacovich & Gorvel, 2010))
830 affecting pathogen community assemblage. However, a lack of deviance from the expected co-
831 exposure frequency does not exclude the possibility that both extrinsic and intrinsic processes may
832 be occurring.

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833 We found evidence in support of three previously identified pathogen-pathogen associations
834 (positive association between *Myco. haemomuris* and *Myco. coccoides* infections; positive
835 association between *Bartonella* spp. infection and the presence of anti-CPXV antibodies; negative
836 association between *Bartonella* spp. and hemotropic *Mycoplasma* spp. infections) and characterized
837 one set of associations not previously described (positive associations between the presence of anti-

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838 hantavirus antibodies and infections by two specific *Myco. haemomuris* OTUs) – each in a unique
839 subset of host species. *Mycoplasma* spp. blood infections are likely transmitted through bites of
840 blood-sucking arthropod vectors (Volokhov et al., 2017), meaning vectors could prefer some
841 individuals over others (Malmqvist et al., 2004). Positive associations detected between *Myco.*
842 *haemomuris* and *Myco. coccoides* could also result from similarities in rodent susceptibility. Indeed

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843 *Mycoplasma* spp. infection can lead to acute or chronic infection, and the establishment of chronic
844 bacteremia seems to occur in immunosuppressed or immunocompromised individuals (Cohen et al.,
845 2018). Co-infections with multiple *Mycoplasma* spp. might therefore be more likely to be detected
846 in these immunocompromised rodents with chronic infections. The existence of chronic infections
847 might also lead to additional co-infections and positive associations as a result of disease-induced
848 changes in population dynamics, immune system function, or through direct pathogen-pathogen
849 interactions (Aivelo & Norberg, 2018; Fenton, 2008; Fountain-Jones et al., 2019).

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850 Whether through the accumulation of exposure probabilities or increased susceptibility, the
851 previously-undocumented positive association we found here between *Myco. haemomuris* OTUs
852 (*Myco1* and *Myco3*) and anti-hantavirus antibodies may similarly be explained by the chronic
853 nature of both *Mycoplasma* spp. and hantavirus infections in rodents (e.g., for Puumala hantavirus

860 in bank voles ([Meyer & Schmaljohn, 2000](#); [Vaehri et al., 2013](#); [Yanagihara et al., 1985](#)). This
861 positive association was found in both host species where the majority of hantavirus exposures
862 occurred (*Microtus arvalis* and *Myodes glareolus*), consistent with the generality of association
863 between *Mycoplasma* species across host taxa detailed above, suggesting the intrinsic ecology of
864 these pathogens contributes to shaping variation in the pathogen community. Curiously, we found
865 no evidence for direct associations between OTUs of the same *Mycoplasma* species, thus
866 facilitation interactions are unlikely to explain the high diversity of *Mycoplasma* taxa both within
867 and between host species.

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868 Infections by *Bartonella* species are also known to often result in subclinical and persistent
869 bacteremia in mammals, including rodents ([Birtles et al., 2001](#); [Kosoy et al., 2004](#)). The positive
870 association detected in *Ar. scherman* between *Bartonella* spp. and anti-CPXV antibodies might
871 therefore be explained by, for example, joint accumulation of both chronic bacterial infections and
872 long-lived antiviral antibodies used to test for prior exposure to relatively short-lived CPXV
873 infections. However, if the same processes governing association of the chronic infections described
874 above were at play here, we would have expected to find both pathogens implicated in positive
875 associations (i) with other chronic infections, and (ii) across host species given their ubiquitous
876 prevalence. While the failure to recover the association in *Mi. agrestis* (previously described ([Telfer](#)
877 [et al., 2010](#))) was likely due to low statistical power, the lack of a general pattern across other host
878 species despite adequate sampling suggests a more specific, and potentially immune-mediated,
879 ecological process between these two pathogens. Indeed, pox virus infections, including CPXV,
880 have been shown to induce immunomodulation that increases host susceptibility to other parasites

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881 ([Johnston & McFadden, 2003](#)). These interactions could be of variable intensities according to the
882 rodent species considered, due to potential differences in impacts of CPXV infection on immunity
883 across host species, or to the influence of other infections not examined here on host immune
884 responses during pox infections (e.g., helminths ([Cattadori et al., 2007](#)), protozoa ([Telfer et al.,](#)
885 [2010](#))). Furthermore, *Bartonella* spp. infection was negatively associated with *Mycoplasma* spp.

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893 infections in *My. glareolus*, corroborating negative interactions reported in co-infection experiments
894 in gerbils (Eidelman et al., 2019). This association may therefore originate from an interaction
895 mediated by specific (immune) genetic features of *My. glareolus*, and not ecological conditions as
896 proposed by Eidelman et al. (2019). The antagonistic and host-specific nature of this association
897 lends further support to the interpretation that *Bartonella* spp. infections do not behave in similar
898 ways to other chronic infections in the community. However, few studies have investigated the
899 robustness of within-host interactions across different host species (e.g., (Lello et al., 2018)), and
900 this question deserves further investigation.

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901 Our results suggest that intrinsic ecological interactions could help shape the composition of the
902 pathogen community within hosts. However, this suggestion provides only a hypothesis that
903 requires further investigation. Interpretation of associations can be misleading, as they may arise
904 from unmeasured co-factors such as exposure to shared transmission routes, and may even run
905 counter to the underlying ecological process (Fenton et al., 2014). The associations we found here
906 were not visible (or even misleading, in the case of a 3-way interaction between hantavirus, Myco1
907 and Myco3), for instance, when ignoring extrinsic factors using the SCN analysis, despite the
908 increased statistical power it offered. Evidence for interactions between pathogens within hosts
909 initially came from laboratory studies (e.g., in the development of vaccines, reviewed in
910 (Casadevall & Pirofski, 2000)), and until recently, many studies conducted in the wild could not

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911 detect such interactions (e.g., (Behnke, 2008)). Developments in statistical approaches have
912 contributed to improve sampling designs and analyses, in particular by better controlling for
913 confounding factors, enabling the detection of associations resulting from these within-host
914 interactions (e.g., (Galen et al., 2019; Lello et al., 2004; Telfer et al., 2010)). However, it is unlikely
915 any statistical approach can ever solve the problem of an unmeasured explanatory variable. For
916 instance, our decision to screen only the spleen means we could have missed evidence of exposure
917 to pathogens that can only be found by screening the liver, kidney or brain (Mangombi et al., 2021).

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918 Experiments conducted in semi-controlled environments have been used to confirm the importance

927 of interactions suggested by the associations (e.g., (Knowles et al., 2013)). Both facilitation
928 mediated by immune responses (e.g., (Ezenwa et al., 2010)) and competition mediated by shared
929 resources (e.g., (Brown, 1986; Budischak et al., 2018)) have been emphasized.

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Deleted: (Brown, 1986; Budischak et al., 2018)

930 There remain additional important limits to the interpretation of snapshot observational studies
931 from wild populations such as ours. For instance, they cannot provide information about the
932 sequence or duration of infection, although these features strongly affect the outcome of within-host

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933 interactions (Eidelman et al., 2019). Moreover, both the 16S metabarcoding approach and
934 serological antibody tests can only be interpreted in terms of presence/absence of exposure to

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935 pathogens, although co-infection may rather impact parasite abundance (e.g., (Gorsich et al., 2014;
936 Thumbi et al., 2013)). Other extrinsic factors, such as seasonal variation in pathogen community

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937 composition, could also impact both interpretation and year-round generality of our results due to
938 adherence to autumn sampling dates (Maurice et al., 2015; Villette et al., 2020). Lastly, we also

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939 acknowledge several caveats to consider with our methods. We removed animals from which fewer
940 than 500 reads were amplified in one or both bacterial metabarcoding PCR replicates. While 16 of

941 these samples removed were due to random failure of PCR amplification from just one of the two
942 replicates, 12 of the animals had poor amplification in both PCR replicates. In the absence of an

943 internal positive control, e.g., a spike-in standard (Zemb et al., 2020), we were unable to verify
944 whether a lack of reads was due to poor DNA extraction or a true lack of infections. Although this

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945 has a risk of artificially inflating prevalence rates by selectively removing uninfected individuals, it
946 is unlikely to have had a qualitative effect on our results. Similarly, limiting our analyses to OTUs

947 with 500 reads or more in the entire dataset may select against detection of very rare or low-burden
948 infections. We also removed many OTUs corresponding to bacteria normally occurring in external

949 or internal microbiomes of healthy animals, some of which were represented by a high abundance
950 of reads in positive animals. This was due mainly to the fact that 16S data cannot often distinguish

951 between pathogenic and commensal taxa of many such genera. We know that, for instance,
952 *Helicobacter* species are naturally found in the digestive tract, but can also cause pathogenic

960 infections. Parasitism can affect host microbiome composition (Gaulke et al., 2019), and this in turn
961 can have impacts on host health and disease susceptibility (reviewed in (Murall et al., 2017;
962 Rosshart et al., 2017)). Thus, our choice to ignore OTUs corresponding to microbes typical of
963 healthy flora contributes to the problem of missing data, such as information on intestinal helminth
964 infections or other viruses, which may explain or alter the associations we were able to detect.
965 Furthermore, the evaluation of diversity measures (e.g., Shannon diversity index) based only on a
966 selection of taxa violates the assumption that all species are represented in the sample; thus, patterns
967 of diversity could also be influenced by missing data. These caveats are common problems for
968 disease surveillance and community ecology studies, irrespective of the diagnostic methods, and it
969 is difficult to speculate about their overall impacts on the present study. Finally, it is well-
970 understood that this bias towards detection of common pathogens and difficulty in interpreting
971 evidence for the absence of a pathogen in a given individual or population can make testing for
972 negative associations driven by antagonistic ecological interactions incredibly difficult, if not
973 impossible (Cougoul et al., 2019; Weiss et al., 2016).

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975 976 **Conclusions**

977 Our results add to a growing number of studies finding that (i) rodents host many important
978 zoonotic human pathogens and (ii) pathogen communities are shaped primarily by host species
979 identity. We also detected a number of previously undescribed associations among pathogens
980 within these rodent communities, and we also confirmed previously identified associations,
981 sometimes in other rodent species than those in which they were previously described. These
982 associations can be considered in the future as hypotheses for pathogen-pathogen interactions
983 within rodent hosts, and that participate in shaping the community of pathogens in rodent
984 communities. Long-term survey and experimental studies are now required to confirm these
985 interactions and understand the mechanisms underlying the patterns of co-infection detected. In
986 addition to these biological results, we have identified several methodological caveats, with regard

990 to both pathogen and association detection, that deserves further investigation to improve our ability

991 to make robust inference of pathogen interactions.

992

993

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998 EDENext. The manuscript is registered with the EDENext Steering Committee as EDENext409.

999 None of the rodent species investigated here has protected status (see list of the International Union
1000 for Conservation of Nature). All procedures and methods were carried out in accordance with
1001 relevant regulations and official guidelines from the American Society of Mammalogists. All
1002 protocols presented here were realized with prior explicit agreement from relevant institutional
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1008 preprint has been peer-reviewed and recommended by Peer Community In Ecology (PCI Ecology)
1009 (<https://doi.org/10.24072/pci.ecology.100071>).

1011 **Data Accessibility**

1012 Supplementary data deposited [in Zenodo \(https://doi.org/10.5281/zenodo.7092812\)](https://doi.org/10.5281/zenodo.7092812), include the
1013 following 16S metabarcoding data: (i) raw sequence reads (fastq format), (ii) raw output files
1014 generated by the mothur program (iii) raw abundance table and (iv) filtered occurrence table, as
1015 well as (v) scripts and data files for statistical analyses. Items iii-v are also provided in
1016 Supplemental Materials Appendix 2 to directly accompany this publication.

1018 **Conflict of Interest Disclosure**

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The authors of this article declare that they have no financial conflict of interest with the content of this article. JLA and NC are *PCI Ecology* recommenders.

Table 1. BLAST search results for OTUs suspected of belonging to pathogenic genera. [References are available in Supplemental Materials Appendix 3.](#)

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Infecting species identity	OTU Number	Number of Reads	Genbank Accession Number	BLAST results (% identity)	Pathogen Code	Reference
Pathogenic taxa, reliably detectable						
<i>Bartonella</i> spp.	Otu00001	6353372	MT027154	100% <i>Bartonella grahamii</i> (AB426637) from wild North America rodents; 99%-100% identity to many other pathogenic <i>Bartonella</i> species.	Bartonella	Deng et al., 2012
<i>Brevinema</i> spp.	Otu00123	5603	MT027155	97% <i>Brevinema andersonii</i> (NR_104855) type sequence, infectious spirochaete of short-tailed shrew and white-footed mouse in North America	Brevinema	Defosse et al., 1995
<i>Candidatus Neoehrlichia mikurensis</i>	Otu00039	18358	MT027156	100% <i>Candidatus Neoehrlichia mikurensis</i> (KF155504) tick-borne rodent disease, opportunistic in humans	Neoehrlichia	Andersson & Raberg, 2011
<i>Mycoplasma ravidpulmonis</i>	Otu00054	6086	MT027164	100% <i>Mycoplasma ravidpulmonis</i> (AF001173), grey lung disease agent in <i>Mus musculus</i> ; 88% <i>M. orale</i> from humans (LR214940)	Myc010	Pettersson et al., 2000
<i>Mycoplasma</i> spp.	Otu00004	845971	MT027157	99% identity to uncultured <i>Mycoplasma</i> species (KU697344) from small rodents in Senegal and uncultured eubacterium (AJ292461) from Ixodes ticks; 95% (KM538694) and 94% (MK353834) identity to uncultured hemotropic <i>Mycoplasma</i> species in European and South American bats	Myc01	Galan et al., 2016
<i>Mycoplasma</i> spp.	Otu00003	426034	MT027158	99% <i>Mycoplasma haemomuris</i> (AB758439) from <i>Rattus rattus</i>	Myc02	Conrado et al., 2016
<i>Mycoplasma</i> spp.	Otu00006	106443	MT027159	99% uncultured <i>Mycoplasma</i> spp. (KP843892) from leopards in South Korea; 99% identity to uncultured <i>Mycoplasma</i> (KT215637) from rodents in Brazil	Myc03	Goncalves et al., 2015
<i>Mycoplasma</i> spp.	Otu00010	72724	MT027160	99% <i>Mycoplasma coccoides</i> comb. nov. (AY171918); 97% <i>Candidatus Mycoplasma turicensis</i> (KJ530704) from Indian mongoose	Myc04	Conrado et al., 2015
<i>Mycoplasma</i> spp.	Otu00005	165095	MT027161	100% <i>Mycoplasma haemomuris</i> -like undescribed species (KJ739312) from <i>Rattus norvegicus</i>	Myc05	Alabi et al., 2020
<i>Mycoplasma</i> spp.	Otu00007	92237	MT027162	99% uncultured <i>Mycoplasma</i> spp. (KC863983) from <i>Micromys minutus</i> (eurasian harvest mouse) in Hungary; 98% <i>M. coccoides</i> comb. nov. (AY171918)	Myc06	Conrado et al., 2015
<i>Mycoplasma</i> spp.	Otu00012	39767	MT027163	93% uncultured <i>Mycoplasma</i> species (KU697341) of mice in Senegal; 92% <i>Mycoplasma</i> spp. (KP843892) from leopards in South Korea, 91% <i>Mycoplasma haemomuris</i> (AB820289) in rats	Myc07	Galan et al., 2016
<i>Mycoplasma</i> spp.	Otu00015	31528	MT027165	98% uncultured <i>Mycoplasma</i> spp. (KT215632) from wild rodent spleen in Brazil; 95% uncultured <i>Mycoplasma</i> spp. (KF713538) in little brown bats	Myc08	Ricardo Goncalves et al., 2015
<i>Mycoplasma</i> spp.	Otu00049	40125	MT027166	96% uncultured <i>Mycoplasma</i> spp. from Brazilian rodents (KT215638) and S. Korean leopard (KP843892)	Myc09	Ricardo Goncalves et al., 2015
<i>Orientia</i> spp.	Otu00111	876	MT027167	97% <i>Orientia tsutsugamushi</i> (KY583502) from humans in India, zoonotic Rickettsial pathogen (causes scrub typhus)	Orientia	Paris et al., 2013
<i>Rickettsia</i> spp.	Otu00008	72098	MT027168	98% <i>Rickettsia japonica</i> (MF496166) which causes Japanese spotted fever, <i>R. canadensis</i> (NR_029155) & <i>R. rhipicephali</i> (NR_074473) type strains	Rickettsia	Yamamoto et al., 1992
<i>Spiroplasma</i> spp.	Otu00093	4738	MT027169	95% uncultured <i>Spiroplasma</i> spp. (KT983901) from Ixodes tick on a dog; 94% identity to type strain of <i>Spiroplasma mirum</i> (NR_121794), the agent of suckling mouse cataract disease; <i>Spiroplasma ixodeti</i> causes similar disease in humans.	Spiroplasma	Cisak et al., 2015
Pathogenic taxa, not reliably detectable						
<i>Arcobacter cryaerophilus</i>	Otu00296	403	MT027170	100% <i>Arcobacter cryaerophilus</i> (CP032825) emerging enteropathogen in humans, zoonotic, pathogenic in rats	Arcobacter	Vandamme et al., 1992
<i>Borrelia miyamotoi</i>	Otu00318	419	MT027171	100% <i>Borrelia miyamotoi</i> (CP010308) in humans and Ixodes, zoonotic pathogen	Borrelia1	Krause et al., 2015
<i>Borrelia</i> spp.	Otu00514	206	MT027172	96% <i>Borrelia</i> sp. nov "Lake Gaillard" in <i>Peromyscus leucopus</i> (AYS36513), 95% <i>B. hermsii</i> (MF066892) from tick (<i>Ornithodoros hermsii</i>) bites in humans	Borrelia2	Bunikis et al., 2005
<i>Borrelia afzelii</i>	Otu00071	78	MT027173	100% <i>Borrelia afzelii</i> (CP009058) human pathogen closely related (98%) to <i>B. burgdorferii</i> (positive control sequence)	Borrelia3	Schuler et al., 2015
<i>Leptospira</i> spp.	Otu01015	257	MT027174	100% several pathogenic <i>Leptospira</i> from mammals, e.g., <i>L. interrogans</i> (LC474514) in humans	Leptospira	Vincent et al., 2019
<i>Mycoplasma pulmonis</i>	Otu00771	255	MT027175	100% <i>Mycoplasma pulmonis</i> (NR_041744) chronic respiratory pathogen of mice and rats	Myc0771	Piasecki et al., 2017
<i>Mycoplasma</i> spp.	Otu04125	164	MT027176	90% <i>Mycoplasma ravidpulmonis</i> (AF001173), grey lung disease agent in <i>Mus musculus</i> ; 80% <i>M. phocidae</i> from California sea lions (DQ521594)	Myc04125	Pettersson et al., 2000
Eukaryotic family Sarcocystidae	Otu00056	8684	Not submitted*	97% similar to plastid small ribosomal unit of <i>Hyaloklossia lieberkuehni</i> (AF297120), a parasitic protzoa of European green frog; 96% <i>Neospora caninum</i> (MK770339) & <i>Sarcocystis muris</i> (AF255924); 95% <i>Toxoplasma gondii</i> (TGU28056)	Sarcocystidae1	Calarco & Ellis, 2020
Eukaryotic family Sarcocystidae	Otu00191	3678	Not submitted*	92% <i>Neospora caninum</i> (MK770339) parasite; 90% <i>Toxoplasma gondii</i> (U87145) zoonotic pathogen	Sarcocystidae2	Calarco & Ellis, 2020
Eukaryotic family Sarcocystidae	Otu00254	1219	Not submitted*	98% <i>Sarcocystis muris</i> (AF255924) coccidian parasite first found in mice	Sarcocystidae3	Orosz, 2015

* No reference specimen for identification of Eukaryote

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Table 1 (continued). BLAST search results for OTUs suspected of belonging to pathogenic genera. References are available in Supplemental Materials Appendix 3.

Infesting species identity	OTU Number	Number of Reads	Genbank Accession Number	BLAST results (% identity)	Pathogen Code	Reference
Uncertain pathogenicity, reliably detectable						
<i>Corynebacterium xerosis</i>	Otu00050	1853	MT027177	100% <i>Corynebacterium xerosis</i> (MH141477), only opportunistic infections identified	Corynebacterium	Bernard, 2012
<i>Dietzia</i> spp.	Otu00102	2626	MT027178	100% <i>Dietzia</i> spp. e.g., <i>D. aurantiaca</i> (MK25331); common contaminant; opportunistic in humans; thought to out-compete <i>Trypanosomes</i>	Dietzia	Kampfer et al., 2012
<i>Helicobacter</i> spp.	Otu00013	34894	MT027179	96% homology to <i>Helicobacter suncus</i> (AB006147) isolated from shrews with chronic gastritis; 95% identity to type specimen for <i>H. mustelae</i> (NR_029169) which causes gastritis in ferrets; but could be normal gut flora	Helico1	Goto et al., 1998
<i>Helicobacter</i> spp.	Otu00025	8702	MT027180	97% similar to <i>Helicobacter trogantum</i> (AY686609) and <i>H. suncus</i> (AB006147), both enterohepatic <i>Helicobacter</i> spp. associated with intestinal diseases	Helico2	Goto et al., 1998
<i>Helicobacter</i> spp.	Otu00087	2303	MT027181	99% identical to <i>Helicobacter aurati</i> (NR_025124.1), a pathogen of Syrian hamsters; 98% identical to <i>H. fennelliae</i> (GQ867176), a human pathogen	Helico3	Patterson et al., 2000
<i>Helicobacter</i> spp.	Otu00128	1178	MT027182	99% <i>Helicobacter winghamensis</i> (AF363063), associated with gastroenteritis in humans; however, minor sequences were 100% identical to <i>H. rodentium</i> (AY631957) which is only associated to gastritis in rodents when coinfecting with other <i>Helicobacter</i> strains	Helico4	Melito et al., 2001
<i>Neisseria</i> spp.	Otu00612	780	MT027183	97% uncultured <i>Neisseria</i> spp. associated with human prostatitis (HM080767) and cataracts (MG696979), but indistinguishable from environmental samples and healthy flora (e.g., JF139578)	Neisseria1	Genbank, unpublished
Pasteurellaceae	Otu00129	1430	MT027184	100% uncultured bacterium (MN095269) of mouse oral flora; 99% <i>Muribacter muris</i> (KP278064) of unknown pathogenicity, water fowl pathogen <i>Avibacterium gallinarum</i> (AF487729), and cattle respiratory disease agent <i>Mannheimia haemolytica</i> (CP017491)	Pasteurella1	Nicklas et al., 2015
Pasteurellaceae	Otu00203	521	MT027185	99% <i>Aggregatibacter aphrophilus</i> (LR134327) and <i>Haemophilus parainfluenzae</i> (CP035368) opportunistic pathogens but otherwise part of normal flora	Pasteurella2	Genbank, unpublished
<i>Rickettsiella</i> spp.	Otu00187	592	MT027186	99%-100% identity to several endosymbionts of insects, eg. uncultured <i>Diplarickettsia</i> spp. in sand flies (KX363696), <i>Rickettsiella</i> spp. in Ixodes ticks (KP994859); 99% identity to <i>Rickettsiella agriatidis</i> (HQ640943) pathogen of wireworms	Rickettsiella	Duron et al., 2015
<i>Streptococcus</i> spp.	Otu00115	1681	MT027187	99% <i>Streptococcus hyointestinalis</i> from intestines of swine (KR819489)	Streptococcus	Genbank, unpublished
<i>Yersinia</i> spp.	Otu00041	7420	MT027188	A heterogeneous OTU some major sequences 100% <i>Yersinia</i> spp. and <i>Serratia</i> spp., including pathogenic zoonotic bacteria (e.g., <i>Y. pestis</i> NR_025160) and non-pathogenic endosymbionts of plants (NR_157762); some major sequences 100% <i>Pantoea agglomerans</i> (MNS15098) opportunists	Yersinia	Kim et al., 2003
Uncertain pathogenicity, not reliably detectable						
<i>Fusobacterium</i> spp.	Otu00791	108	MT027189	100% <i>Fusobacterium ulcerans</i> (CP028105) from tropical foot ulcers in humans; but also 100% identity with other fecal isolates of unknown pathogenicity in mammals (e.g., <i>F. varium</i> LR134390)	Fusobacterium	Genbank, unpublished
<i>Neisseria</i> spp.	Otu00454	148	MT027190	98% uncultured microbiota of bat mating organs (KY300287), 98% <i>Simonsiella muelleri</i> commensal from human saliva (AF328145); 97% <i>Kingella kingae</i> (MF073277) pathogen in humans	Neisseria2	Genbank, unpublished
<i>Treponema</i> spp.	Otu00235	348	MT027191	93% uncultured rumen <i>Treponema</i> spp. (AB537611)	Treponema	Bekele et al., 2011
<i>Williamsia</i> spp.	Otu00614	274	MT027192	100% <i>Williamsia phyllosphaerae</i> (MG205541) and <i>Williamsia maris</i> (NR_024671), closely related to opportunistic pathogens in humans	Williamsia	Genbank, unpublished

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Infesting species identity	OTU Number	Number of Reads	Genl Acces: Nur
Pathogenic taxa, reliably detectable			
<i>Bartonella</i> spp.	Otu00001	6353372	MT02
<i>Brevinema</i> spp.	Otu00123	5603	MT02
<i>Candidatus Neoehrlichia mikurensis</i>	Otu00039	18358	MT02
<i>Mycoplasma ravipulmonis</i>	Otu00054	6086	MT02
<i>Mycoplasma</i> spp.	Otu00004	845971	MT02
<i>Mycoplasma</i> spp.	Otu00003	426034	MT02
<i>Mycoplasma</i> spp.	Otu00006	106443	MT02
<i>Mycoplasma</i> spp.	Otu00010	72724	MT02
<i>Mycoplasma</i> spp.	Otu00005	165095	MT02
<i>Mycoplasma</i> spp.	Otu00007	92237	MT02
<i>Mycoplasma</i> spp.	Otu00012	39767	MT02
<i>Mycoplasma</i> spp.	Otu00015	31528	MT02
<i>Mycoplasma</i> spp.	Otu00049	40125	MT02
<i>Orientia</i> spp.	Otu00111	876	MT02
<i>Rickettsia</i> spp.	Otu00008	72098	MT02
<i>Spiroplasma</i> spp.	Otu00093	4738	MT02
Pathogenic taxa, not reliably detectable			
<i>Arcobacter cryaerophilus</i>	Otu00296	403	MT02
<i>Borrelia miyamotoi</i>	Otu00318	419	MT02
<i>Borrelia</i> spp.	Otu00514	206	MT02
<i>Borrelia afzelii</i>	Otu00071	78	MT02
<i>Leptospira</i> spp.	Otu01015	257	MT02
<i>Mycoplasma pulmonis</i>	Otu00771	255	MT02
<i>Mycoplasma</i> spp.	Otu04125	164	MT02
Eukaryotic family Sarcocystidae	Otu00056	8684	XXX
Eukaryotic family Sarcocystidae	Otu00191	3678	XXX
Eukaryotic family Sarcocystidae	Otu00254	1219	XXX

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Infesting species identity	OTU Number	Number of Reads	Genl Acces: Nur
Uncertain pathogenicity, reliably detectable			
<i>Corynebacterium xerosis</i>	Otu00050	1853	MT02
<i>Dietzia</i> spp.	Otu00102	2626	MT02
<i>Helicobacter</i> spp.	Otu00013	34894	MT02
<i>Helicobacter</i> spp.	Otu00025	8702	MT02
<i>Helicobacter</i> spp.	Otu00087	2303	MT02
<i>Helicobacter</i> spp.	Otu00128	1178	MT02
<i>Neisseria</i> spp.	Otu00612	780	MT02
Pasteurellaceae	Otu00129	1430	MT02

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Pathogen community composition and co-infection patterns in a wild community of rodents

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ABSTRACT

Rodents are major reservoirs of pathogens that can cause disease in humans and livestock. It is therefore important to know what pathogens naturally circulate in rodent populations, and to understand the factors that may influence their distribution in the wild. Here, we describe the occurrence and distribution patterns of a range of endemic and zoonotic pathogens circulating among rodent communities in northern France. The community sample consisted of 713 rodents, including 11 host species from diverse habitats. Rodents were screened for virus exposure (hantaviruses, cowpox virus, Lymphocytic choriomeningitis virus, Tick-borne encephalitis virus) using antibody assays. Bacterial communities were characterized using 16S rRNA amplicon sequencing of splenic samples. Multiple correspondence (MCA), multiple regression and association screening (SCN) analyses were used to determine the degree to which extrinsic factors (study year and site; host habitat, species, sex and age class) contributed to pathogen community structure, and to identify patterns of associations between pathogens within hosts. We found a rich diversity of bacterial genera, with 36 known or suspected to be pathogenic. We revealed that host species is the most important determinant of pathogen community composition, and that hosts that share habitats can have very different pathogen communities. Pathogen diversity and co-infection rates also vary among host species. Aggregation of pathogens responsible for zoonotic diseases suggests that some rodent species may be more important for transmission risk than others. Moreover, we detected positive associations between several pathogens, including *Bartonella*, *Mycoplasma* species, Cowpox

44 virus (CPXV) and hantaviruses, and these patterns were generally specific to particular host
45 species. Altogether, our results suggest that host and pathogen specificity is the most
46 important driver of pathogen community structure, and that interspecific pathogen-pathogen
47 associations also depend on host species.

48
49 **Keywords:** 16S rRNA amplicon high throughput sequencing, Disease Ecology, Microbial
50 Interactions, Pathobiome, Rodent reservoirs, Zoonoses

1. Introduction

Infectious diseases are among the most important global threats to biodiversity, wildlife and human health, and are associated with potential severe socioeconomic consequences (Daszak et al., 2000; Jones et al., 2008; K. F. Smith et al., 2006). Although combatting these risks is a main worldwide priority, our understanding of the processes underlying disease emergence still remains too limited for developing efficient prediction, prevention and management strategies. In humans, the majority of emerging pathogens originate as zoonoses from animal host populations in which they naturally circulate (Jones et al., 2008; Taylor et al., 2001). Thus, identifying the epidemiological features (e.g., prevalence, diversity, host specificity, geographic distribution) of zoonotic pathogen communities in their wild hosts, and the factors that influence pathogen occurrence in those communities, is as important to human health as it is to understanding the fundamentals of disease ecology (Garchitorena et al., 2017).

Both extrinsic and intrinsic factors can contribute to the composition of natural pathogen communities within and between wild animal species, populations and individuals. Factors extrinsic to the hosts include geographic location, climate, periodicity of epidemic cycles and abiotic features influencing inter-specific transmission opportunities (e.g., (Burthe et al., 2006; Harvell et al., 2002; Poulin et al., 2012). Factors extrinsic to the pathogens such as host species identity, sex, age, and body condition as well as genetic and immunogenetic features have also been intensively studied (e.g., (Beldomenico et al., 2008; Bordes et al., 2017; Charbonnel et al., 2014; Salvador et al., 2011; Streicker et al., 2010, 2013)). Although less investigated, inter-specific ecological interactions (e.g., competition, facilitation) among pathogens within animal hosts are also likely to be an important intrinsic force in determining the composition of pathogen communities. Ecological interactions between free-living species are well-known to play a part in the distribution, abundance, and many other qualitative and quantitative features of populations; the application of this basic tenant of community ecology to pathogen incidence and expression of disease has become recognized as imperative for assessing both risks and potential benefits posed to human health, agriculture, wildlife, and conservation (Pedersen & Fenton, 2007). Simultaneous infection by multiple parasite species is ubiquitous in nature (Cox, 2001; Moutailler et al., 2016; Petney & Andrews, 1998), and prior infections can have lasting effects on future susceptibility via e.g., changes to host condition and behavior or through immune-mediated processes (Karvonen et al., 2019; Kumar et al., 2018; Quiñones-Parra et al., 2016; Singer, 2010). Interactions among co-circulating parasites may have important consequences for disease severity, pathogen transmission, host and pathogen evolution or co-evolution, and community-level responses to perturbations (Abbate et al., 2018; Alizon et al., 2013; Jolles et al., 2008; Seppälä & Jokela, 2016; Telfer et al., 2010). Consequences of interaction may be life-long, as exposure to pathogens circulating among juveniles have been found to be strongly associated with those experienced by adults (Fountain-Jones et al., 2019). Such interactions can also play a role in the consequences of pathogen emergence (e.g., emerging bacterial infection increasing susceptibility to an endemic virus (Beechler et al., 2015)). Henceforth, and through the advent of sequencing technologies in particular, it is now possible and essential to investigate disease emergence from a multi-host / multi-pathogen perspective (Galan et al., 2016), considering the potential influence of pathogen interactions on current and future disease distributions (Abbate et al., 2018; Budischak et al., 2015; Cattadori et al., 2008; Jolles et al., 2008).

Rodent communities are relevant models for developing this community ecology approach to disease distribution and emergence. They harbor a wide variety of pathogenic taxa (Bordes et al., 2013; Diagne et al., 2017; Koskela et al., 2016; Pulosof, Morand, Krasnov, & Nunn, 2015) and are important reservoir hosts of agents of zoonoses that have severe implications for human health. Han et al. (2015) have revealed that about 10% of the 2277 extant rodent species are reservoirs of 66 agents of zoonoses, including viruses, bacteria, fungi, helminths, and protozoa. They also described 79 hyper-reservoir rodent species that could be infected by multiple zoonotic agents. Strong ecological interactions, such as facilitation and competition, have been shown in wild rodent populations among some of these zoonotic agents (Telfer et al., 2010), as well as between non-zoonotic agents and zoonotic agents (e.g., helminthes and bacteria (Carvalho-Pereira et al., 2019); helminthes and viruses (Guivier et al., 2014; Sweeny et al., 2020); helminthes and protozoa (Knowles et al., 2013)). In addition, rodents share a number of habitats with humans, including urban settings, agricultural lands, and forests, providing opportunities for human-rodent contact and pathogen transmission (Davis et al., 2005). Describing the distribution and composition of natural pathogen

103 communities in rodent populations, and determining the drivers behind pathogen associations, is imperative
104 for understanding the risks they may pose for public health.

105 In this study, we analyzed the pathogen communities carried by rodent communities in a rural area of
106 northern France, a region known to be endemic for several rodent-borne diseases including nephropathia
107 epidemica (Puumala orthohantavirus (Sauvage et al., 2002)) and borreliosis (Razzauti et al., 2015). We
108 investigated exposure histories (via the presence of antiviral antibodies) for several viruses (hantaviruses,
109 cowpox virus, lymphocytic choriomeningitis virus, Tick-borne encephalitis virus) and current or recent
110 exposure to bacterial pathogens (using high-throughput 16S metabarcoding of host splenic tissue). We
111 described the pathogens detected, their prevalence in the community and their individual distributions
112 among host populations. We then tested the role of extrinsic factors (e.g., habitat, host species, host age) in
113 explaining variation in pathogen distributions, and for associations (non-random co-infection frequencies)
114 between pathogens that might indicate intrinsic drivers (e.g., competition, facilitation) of pathogen
115 community composition. We expected that host species and habitat would be the most important factors
116 structuring pathogen community composition because most pathogens are largely host-specific, but those
117 sharing habitats should also share opportunities for transmission (Davies & Pedersen, 2008). After
118 accounting for extrinsic factors, we expected to retrieve several pathogen-pathogen associations previously
119 identified in the literature. This included i) positive associations between cowpox virus and *Bartonella*
120 infections (*Microtus agrestis*, (Telfer et al., 2010)); ii) positive associations between distinct *Mycoplasma*
121 species in mammalian hosts (Fettweis et al., 2014; Sykes et al., 2008; Tagawa et al., 2012; Volokhov et al.,
122 2017)); iii) associations between *Bartonella* and hemotropic *Mycoplasma* species (both positive and negative
123 associations, as well as experimental demonstration of dynamic interactions, have been described in
124 *Gerbillus andersonii* (Eidelman et al., 2019)). Lastly, we also expected to find previously-undescribed
125 associations due to the large bacteria and rodent dataset included in our study. All these associations were
126 likely to differ between host species, as differences in host specificity are also likely to be accompanied by
127 differences in transmission dynamics and host responses to infection (Dallas et al., 2019; Davies & Pedersen,
128 2008; Singer, 2010).

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2. Methods

131 2.1. Study area and host sampling

132 Rodent sampling was conducted over two years (Autumn 2010 & 2011) in rural habitats surrounding two
133 villages (Boult-aux-Bois and Briquenay) in the Ardennes region of northern France (previously described in
134 (Gotteland et al., 2014)). Sex and age class (based on specific body measurements and classed as 'adult' for
135 sexually mature animals and 'juvenile' for both juveniles and sexually immature sub-adults) were recorded
136 for each animal, a blood sample was taken for serological analyses, and animals were then euthanized using
137 isoflurane inhalation. Spleens were taken and stored in RNA_{later} Stabilizing Solution (Invitrogen) at -20°C.
138 Species captured from the two sites included (family: Cricetidae) 195 *Arvicola scherman* (montane water
139 vole), 10 *Microtus agrestis* (field vole), 66 *Microtus arvalis* (common vole), 203 *Myodes glareolus* (bank vole);
140 and (family: Muridae) 43 *Apodemus flavicollis* (yellow-necked mouse), 156 *Apodemus sylvaticus* (wood
141 mouse), 32 *Rattus norvegicus* (brown rat). These seven focal host species were collected from traps placed in
142 distinct landscapes (henceforth referred to as host 'habitats') (Supplemental Materials Figure S1): *R.*
143 *norvegicus* were found uniquely on farms, *Ar. scherman* and *Mi. arvalis* were found almost entirely in
144 meadows, and the five remaining species occupied both forests and hedgerows. Demographic differences
145 between host species were observed for sex (e.g., male bias in *Ap. sylvaticus*; Figure S2A) and age classes
146 (e.g., relative abundance of juveniles in *Mi. arvalis* and *Ap. sylvaticus* hosts; Figure S2B). Five *Microtus*
147 *subterraneus* (European pine vole) and one each of three additional host species (one cricetid one echimyid
148 and one murid) were also found in these communities, but excluded from analyses due to their rarity; these
149 rare (non-focal) hosts and their pathogens are described in Supplemental Materials Appendix 1.

150

151 2.2. Detecting virus exposure and bacterial infection

152 Among the 713 rodents sampled for this study, indirect fluorescent antibody tests (IFATs; see for details
153 (Kallio-Kokko et al., 2006)) were successfully performed on 677 animals to detect immunoglobulin G (IgG)

154 specific to or cross-reacting with cowpox virus (CPXV, *Orthopoxvirus*), Puumala or Dobrava-Belgrade virus
155 (respectively PUUV and DOBV, *Orthohantavirus*, collectively referred to henceforth as “hantavirus”),
156 lymphocytic choriomeningitis virus (LCMV, *Mammarenavirus*), and Tick-borne encephalitis virus (TBEV,
157 *Flavivirus*). We refer to these antiviral antibody tests as indicating a history of past exposure, but antibodies
158 against hantavirus and LCMV also likely indicate continued chronic infection. In contrast, current or very
159 recent exposure to bacterial infection was tested via 16S rRNA gene amplicon sequencing of splenic tissue,
160 giving no indication of past exposure history. The spleen was chosen because this organ is known to filter
161 microbial cells in mammals, allowing the detection of a wide array of pathogenic and zoonotic agents.
162 Funding was available to test for bacteria in just half of the animals, chosen haphazardly to equally represent
163 all host species, study sites and years, resulting in successful analysis for 332 rodents (see Figure 1 for a
164 breakdown of number of individuals sampled per focal host species). For each individual animal, the DNA
165 from splenic tissue was extracted using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer
166 recommendations. Each DNA extraction was analyzed twice independently. We followed the method
167 described in Galan *et al.* (2016) to perform PCR amplification, indexing, pooling, multiplexing, de-
168 multiplexing, taxonomic identification using the SILVA SSU Ref NR 119 database as a reference
169 (<http://www.arb-silva.de/projects/ssu-ref-nr/>). Briefly, DNA samples were amplified by PCR using universal
170 primers targeting the hyper variable region V4 of the 16S rRNA gene (251 bp) and sequencing via Illumina
171 MiSeq. The V4 region has been proven to have reasonable taxonomic resolution at the genus level (Claesson
172 *et al.*, 2010). A multiplexing strategy enabled the identification of bacterial genera in each individual sample
173 (Kozich *et al.*, 2013). Data filtering was performed as described in Galan *et al.* (2016) to determine
174 presence/absence of bacterial infections (summarized in Figure S3). Briefly, we discarded all bacterial OTUs
175 containing fewer than 50 reads in the entire dataset and animals for which one or both individual PCR
176 samples produced fewer than 500 reads. A bacterial OTU was considered present in an animal if the two
177 independent PCR samples were both above a threshold number of reads, defined as the greater of either
178 0.012% of the total number of reads in the run for that OTU (i.e., filtering using the rate of indexing leak) or
179 the maximum number of reads for that OTU in any negative control sample (i.e., filtering using the presence
180 of reads in the negative controls due to contaminations) (Galan *et al.*, 2016). We removed chimera (Ashelford
181 *et al.*, 2006), using the *Uchime* program implemented in *mothur*, and manually checked OTUs representing
182 suspected chimera not identified by the program. For each OTU suspected as pathogenic, Basic Local
183 Alignment Search Tool (BLAST) searches of the most common sequences were conducted to infer species
184 identity where possible. Given that rare, low abundance taxa tend to show high dissimilarity between sample
185 replicates (D. P. Smith & Peay, 2014) we assumed that only OTUs with at least 500 reads across all animals in
186 the dataset were considered reliably detectable, allowing us to assign absent status to these OTUs in animals
187 failing to meet the criteria for OTU presence. Thus, only OTUs for which there were at least 500 reads across
188 all animals in the dataset (for which present and absent statuses could be assigned), and where reasonable
189 certainty of pathogenicity could be established from the literature (see Table 1), were considered in analyses
190 of the pathogen community.

191

192 2.3. Statistical Methods

193 All statistical analyses were implemented in R version 3.2.2 (R Core Team, 2015). Throughout our
194 analyses, we refer to simultaneous bacterial infections as “co-infection”, while analyses involving
195 simultaneous presence of antiviral antibodies and bacterial infection are referred to as evaluating “co-
196 exposure”. While we can only be sure that antiviral antibodies represent past exposure, we cannot rule out
197 simultaneous viral and bacterial “co-infection”, particularly for viruses known to cause chronic infections.
198 Likewise, detection of current (or very recent) bacterial infection, particularly for taxa known to cause
199 chronic infections, cannot tell us how long the animal has carried the infection. Thus, we refrain from
200 assuming sequence of infection for statistical tests unless specified by an *a priori* hypothesis from the
201 literature.

202

203 2.3.1. Testing for extrinsic drivers of pathogen community composition across the rodent community

204 We analyzed pathogen community composition across the whole rodent community. We use the term
205 “pathogen community” to refer to the group of viruses and pathogenic bacteria for which we had the means
206 to include, which was not exhaustive; thus measures of diversity are to be considered relative and not
207 absolute. We first estimated pathogen community richness using the Shannon diversity index (alpha

208 diversity) considering pathogenic bacterial OTUs and antiviral antibodies found in each study year, study site,
209 habitat, host species, host sex and host age group (default options (natural logarithm Shannon index) in
210 'diversity' function from the *vegan* package). We evaluated a linear regression model using analysis of
211 deviance ('lm' and 'drop1' functions from the basic *stats* package) to test for significance of differences in
212 pathogen diversity due to the fixed factors listed above after first correcting for all other factors in the model
213 (marginal error tested against the *F*-distribution). Post-hoc comparisons and correction for multiple tests
214 were performed using function 'TukeyHSD' from package *stats* and 'HSD.test' from package *agricolae* to
215 group factor levels that were not significantly different. An additional Akaike Information Criterion (AIC)-
216 based model selection analysis was performed to assess any qualitative influence of spurious predictors on
217 these comparisons using the 'glmulti' and 'weightable' function from package *glmulti*. The impact of host
218 species diversity (Shannon diversity with Chao's estimator correction using 'Shannon' in package *entropart*)
219 on pathogen species diversity was tested for by correlation ('cor.test', package *stats*) across each year x site x
220 habitat community.

221 We next tested for differences in pathogen community composition (beta diversity) between host
222 species, habitats, study sites, years, age classes, and sexes by applying a permutational multivariate analysis
223 of variance (PERMANOVA) on a Bray-Curtis dissimilarity matrix ('adonis2' function in the *vegan* package). To
224 explore how intrinsic factors (pathogen-pathogen associations) contributed to the structure of the pathogen
225 community, we used multiple correspondence analysis (MCA) to reduce variance in presence/absence of
226 each bacterial pathogen species and antiviral antibody, implemented with the function 'MCA' in the
227 *FactoMinR* package and visualization tools found in the *factoextra* package. This produces a set of
228 quantitative and orthogonal descriptors (dimensions) describing the pathogen community composition,
229 revealing correlated variables. With each MCA dimension as a continuous dependent response variable, we
230 then evaluated linear regression models using analysis of deviance with post-hoc comparisons (as detailed
231 above) to understand how the variation described by each MCA dimension was influenced by the extrinsic
232 factors.

233

234 2.3.2. Testing for associations between co-circulating pathogens

235 Because we identified a large number of pathogens, the number of potential association combinations to
236 consider was excessively high, especially with regard to the relatively small number of rodents sampled. We
237 therefore decided to test the significance only of those associations (i) clearly suggested by the community-
238 wide MCA or (ii) previously described in the literature: positive association between *Bartonella* spp. and
239 CPXV (Telfer *et al.* 2010), positive associations between *Mycoplasma* species (Fettweis *et al.*, 2014; Sykes *et al.*,
240 2008; Tagawa *et al.*, 2012; Volokhov *et al.*, 2017), and both positive (Eidelman *et al.*, 2019; Kedem *et al.*,
241 2014) and negative (Cohen *et al.*, 2015) associations between *Bartonella* spp. and hemotropic *Mycoplasma*
242 species. Given the *a priori* assumption that associations would differ between host species, we analyzed each
243 host species separately; where evidence suggested no significant differences between host species (non-
244 significant variation in the MCA dimension among host species or non-significant host species identity x
245 explanatory pathogen term in logistic regressions), we pooled individuals into a single analysis to gain
246 statistical power.

247 We tested the significance of each association using both association screening (SCN) analysis (Vaumourin
248 *et al.*, 2014) and multiple logistic regression analysis (GLMs, modeling the binomial 'presence/absence' status
249 of each pathogen as a function of the occurrence of other pathogens) on the subset of host species in which
250 the pathogens were found to circulate. We first performed SCN analysis, as this approach is among the most
251 suitable for detecting pathogen associations in cross-sectional studies (Vaumourin *et al.* 2014). Briefly, given
252 the prevalence of each pathogen species in the study population, SCN analysis generates a simulation-based
253 95% confidence envelope around the expected frequency of each possible combination of concurrent
254 infection status (a total of 2^{NP} combinations, where NP = the number of pathogen species) under the null
255 hypothesis of random pathogen associations. Observed frequencies of co-infection combinations falling
256 above or below this envelope are considered to occur more or less frequently, respectively, than in 95% of
257 the random simulations. Significance of the association is given as a *p*-value, calculated as the number of
258 instances in which the simulated co-infection frequency differed (above or below the upper or lower
259 threshold, respectively) from the observed frequency divided by the total number of simulations (Vaumourin
260 *et al.*, 2014).

261 The benefit of the SCN approach is a relatively high level of statistical power and the ability to identify
262 precisely which combinations of pathogens occur outside the random expectations (Vaumourin *et al.* (2014)).
263 However, the SCN is sensitive to heterogeneity in the data due to extrinsic factors (e.g., host specificity, or
264 structuring in space, time, age or sex), which can both create and mask true associations. A multiple logistic
265 regression (GLM) approach was thus also systematically employed, as it has the benefit of explicitly taking
266 into account potentially confounding extrinsic factors. Binomial exposure (presence/absence of either
267 bacterial infection or antiviral antibodies) to a single pathogen was set as the dependent variable with
268 exposure to the hypothetically associated pathogen(s) treated as independent explanatory variable(s) and
269 extrinsic factors (host sex, host age, study year, study site, and where appropriate, habitat) were specified as
270 covariates using function 'glm' in the *stats* package with a binomial logit link. When the multiple host species
271 were involved, we tested an interaction term (host species identity x explanatory pathogen), and either (if p
272 < 0.05) performed separate analyses for each host species or (if $p \geq 0.05$) simply added host species identity
273 as another covariate in the model. When there was no *a priori* assumption concerning timing of exposure
274 (e.g., antiviral antibody presence is more likely to affect current acute bacterial infection than the reverse),
275 the occurrence of each pathogen involved in a given association was set as the dependent variable in
276 reciprocal GLMs. In contrast to the MCA dimensions above, a model selection step was first performed using
277 the 'glmulti' and 'weightable' function from package *glmulti* to find the best model among top-ranking
278 models (small-sample size corrected Akaike Information Criterion (AICc) score less than 2 + lowest AICc) that
279 retained all explanatory pathogens of interest, in an effort to limit the appearance of associations due to the
280 inclusion of spurious predictors. Statistical significance of the association was then assessed after first
281 correcting for all remaining covariates in the best model using the 'drop1' function (-2 log likelihood ratio
282 tests via single-term deletions compared to the full model). Despite a large number of *a priori* hypotheses,
283 we regarded a p-value of < 0.05 as significant due to the very low number of individuals of each host species
284 sampled. Though conceivably important, we also did not have sufficient power to test for additional
285 interaction terms.

286

287 2.3.3. Evaluating false discovery

288 Given the large number of significance tests performed on this single dataset, we compiled all relevant p-
289 values (N=77) and applied a Benjamini-Hochberg correction procedure to estimate how many of the
290 significant results may fall within the false discovery zone (using function 'p.adjust' in the *stats* package).
291 Where model selection was performed, p-values were taken from full models prior to model selection.
292 Among tests of positively correlated hypotheses (e.g., pairwise tests of intrinsic pathogen-pathogen
293 associations N=20), only one of the p-values from the pair was included in calculating the false discovery rate
294 (selected randomly). SCN analysis results were not included, because they were also expected to be
295 positively correlated with the logistic regressions, and because the method inherently performs correction
296 for multiple tests. Between hypotheses, the data were often composed of different non-overlapping subsets
297 of varying sizes, and sample sizes varied widely with some being very small. Thus, application of this
298 procedure likely indicates a conservative (low) estimate for how many null hypotheses should truly be
299 rejected.

300

3. Results

301 3.1. Taxonomic identification and prevalence of pathogens

302

303 3.1.1. Viral exposure

304 The most abundant virus detected was CPXV, with 222 (32.8%) positive sera of the 677 animals tested for
305 anti-CPXV antibodies. It was detected in all focal host species. However, significant variation in prevalence
306 was observed between focal host species (highly prevalent (43-70%) in *Ar. scherman*, *Mi. agrestis*, and *My.*
307 *glareolus*; Figure 1; $\chi^2 = 119.5$, $df = 6$, $p < 10^{-15}$). Anti-hantavirus antibodies were detected in 16 animals
308 (2.4%), and were significantly structured among host species (with exposure highest in *Mi. arvalis* (9.7%), *R.*
309 *norvegicus* (3.3%) and *My. glareolus* (3.1%); Figure 1; $\chi^2 = 19.4$, $df = 6$, $p = 0.0036$). Anti-LCMV antibodies
310 were detected in two *Mi. arvalis* individuals (Figure 1). No animals were positive for anti-TBE antibodies.

311

312 3.1.2. Bacterial pathogens

313 Out of 952 bacterial OTUs represented by at least 50 reads in the dataset, 498 were considered positive in
314 at least one animal after data filtering (presented in Supplemental Materials Table S1). Two OTUs (00024 &
315 00037) identified as *Bartonella* with low bootstrap values (74 and 92 respectively) appeared to represent
316 chimeric sequences between the two highly amplified genera (*Bartonella* and *Mycoplasma*) in co-infected
317 samples. Two OTUs (00009 & 00117) which were unclassified but which had a large number of reads in
318 positive animals were also found to represent chimeric sequences between the two genera, despite high
319 bootstrap values (100). Three additional chimeric *Mycoplasma* OTUs with under 500 reads were also
320 excluded (OTUs 00076, 00159, and 00316). Two OTUs (00002 & 00059) were found to be redundant with
321 OTUs Myco1 and Myco3, respectively, and two more (00134 & 00220) were chimera between Myco OTUs.
322 These 11 OTUs were manually removed from the database, and are not included in Table S1.

323 We identified 43 OTUs belonging to bacterial genera with members known or thought to be pathogenic in
324 mammals (Table 1). After BLAST queries, we found 16 of these OTUs (representing 7 distinct genera) which
325 could be considered as reliably detectable pathogens in the focal host species (Figure 1). An additional 24
326 OTUs were considered potentially pathogenic but excluded from analyses because they were only observed
327 in rare host species, because presence-absence could not be reliably established due to a low total number
328 of reads (<500 in the dataset, e.g., *Borrelia* spp. and *Leptospira* spp.), because we could not rule out
329 contamination by natural sources of non-pathogenic flora during dissection (e.g., *Helicobacter* spp.,
330 *Streptococcus* spp.) or by known contaminants of sequencing reagents (e.g., *Williamsia* spp.; (Salter et al.,
331 2014)), or because their identity to a pathogenic species was uncertain due to insufficient genetic variation at
332 the 16S rRNA locus (e.g., *Yersinia* spp.) (Table 1). We also identified three OTUs belonging to the eukaryotic
333 family Sarcocystidae (98% sequence similarity to the coccidian parasite *Sarcocystis muris*); though each OTU
334 was represented by >500 reads, there are currently no data on the reliability of this method for detection
335 (Table 1). Individual infection status for each of these OTUs is given in Table S2.

336 The 16 reliably detectable pathogenic OTUs included *Bartonella* spp., 10 *Mycoplasma* spp. OTUs,
337 *Rickettsia canadensis*, “*Candidatus* Neoehrlichia mikurensis”, *Orientia* spp., *Brevinema andersonii*, and
338 *Spiroplasma* spp. Phylogenetic analysis including published sequences from BLAST queries revealed that the
339 10 *Mycoplasma* spp. OTUs belonged to three distinct species: *Myco. haemomuris* (Myco1-3,5,7-9), *Myco.*
340 *coccoides* (Myco4 and Mco6), and “*Candidatus Myco. ravigulmonis*” (Myco10) (Figure S4). In general, these
341 bacterial infections were present in all but 30 of the 332 animals tested (91.0% prevalent), and were not
342 concentrated in any particular focal host species ($\chi^2 = 9.7$, $df = 6$, $p = 0.139$). Prevalence of each pathogen in
343 each focal host species is presented in Figure 1.

344

345 **3.2. Extrinsic drivers of pathogen community diversity and composition within rodent community**

346

347 *3.2.1. Analyses of pathogen diversity*

348 We found evidence for the co-circulation of between 3 (in *Mi. agrestis*) and 12 (in *My. glareolus*)
349 pathogen taxa per host species across the rodent community (Figure 1). Using multiple regression analysis on
350 the Shannon diversity index, we found that marginal mean pathogen diversity differed significantly between
351 host species ($F_{5,69} = 6.86$, $p < 10^{-4}$) and habitats ($F_{2,69} = 4.97$, $p = 0.0096$), and it was significantly higher in
352 adults than in juveniles ($F_{1,69} = 21.43$, $p < 10^{-4}$). Pathogen diversity did not, however, differ between study
353 sites, years, or host sexes (Figure 2, Table S3). Post-hoc Tukey tests showed that after correcting for all other
354 factors in the model, meadow habitats had higher diversity of pathogen exposure than forest habitats, and
355 the diversity of

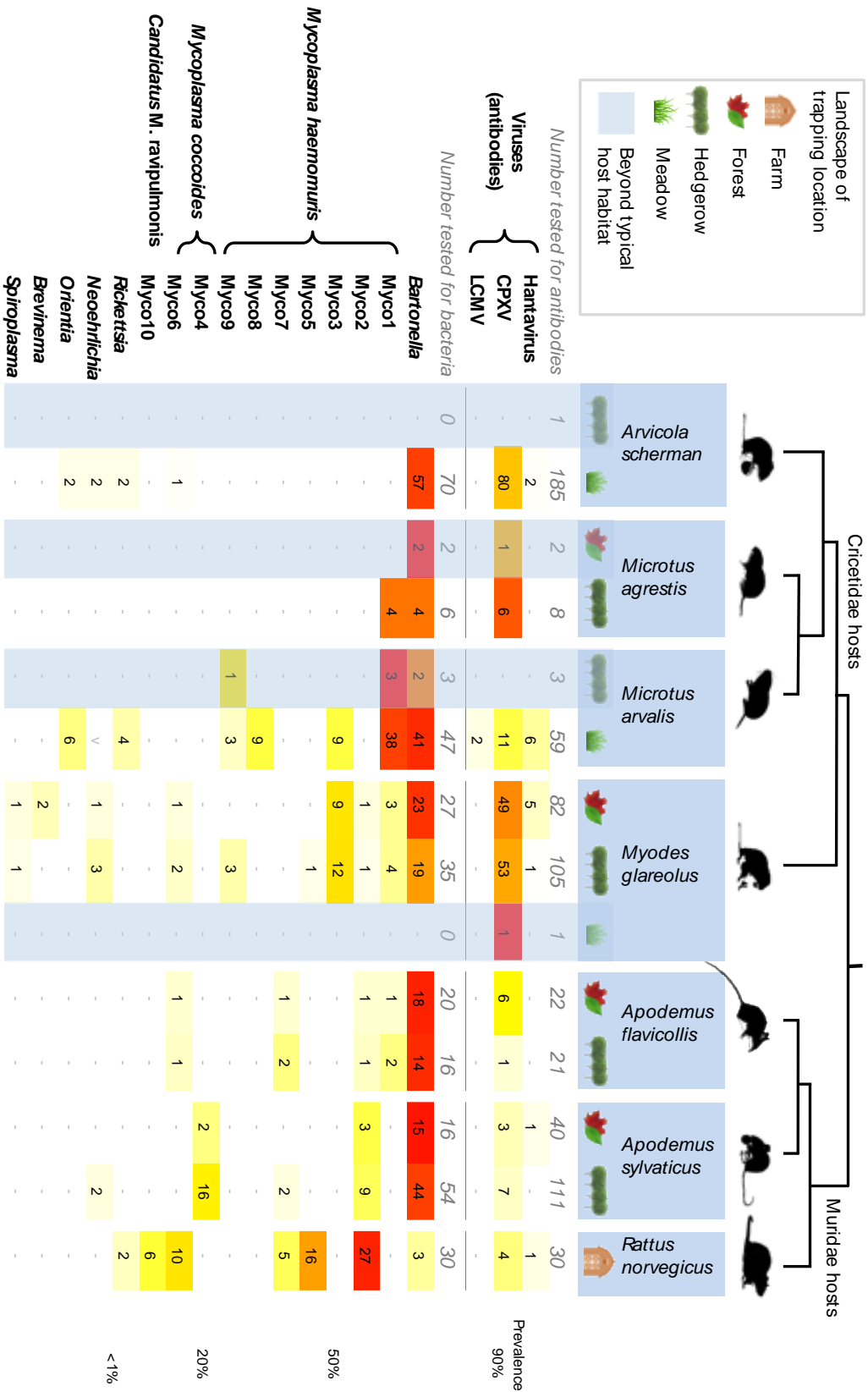
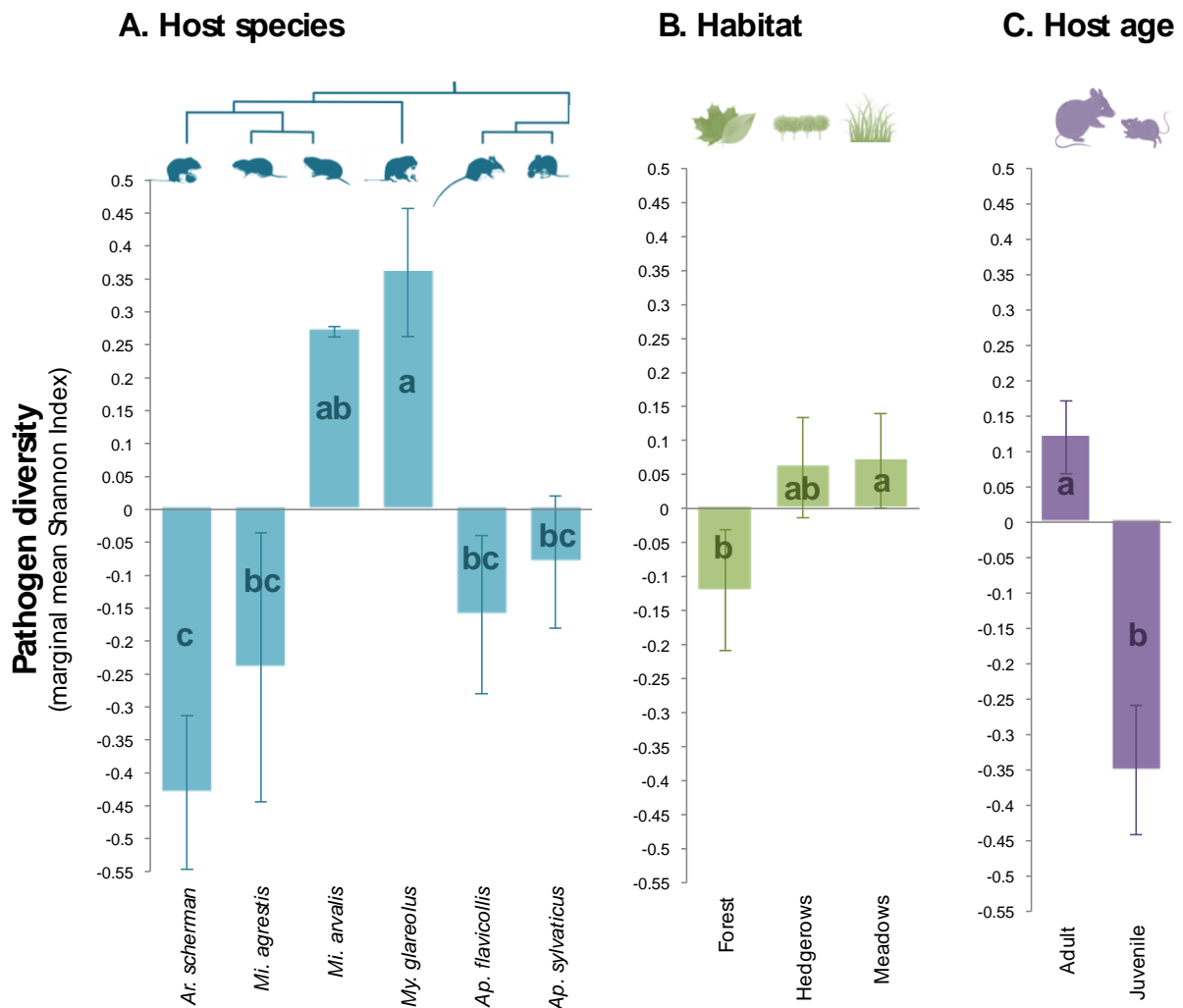


Figure 1 - Pathogen occurrence across the rodent species community.

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357
358

359 pathogen communities in host species fell along a continuum between *My. glareolus* (high) and *Ar. scherman*
 360 (low) extremes (Figure 2, Table S3). The results were qualitatively identical when non-significant predictors
 361 were removed from the model following model selection (Figure S5). Host species diversity in each
 362 community (year x site x habitat) was positively correlated with pathogen diversity ($r = 0.62$, $t = 2.5$, $df = 10$, p
 363 $= 0.032$).

364 To understand the relative pathogen diversity of *R. norvegicus* hosts, not included in the above analysis
 365 because they were entirely confounded with farm habitats, we analyzed a modified model excluding host
 366 habitat and including all seven focal host species. Post-hoc Tukey tests from this model showed that *R.*
 367 *norvegicus* hosts had the second most diverse pathogen community (Table S4).



368 **Figure 2** - Extrinsic drivers of pathogen diversity in a rodent species community. Differences in Shannon
 369 diversity index was tested on marginal means for each factor in the multiple regression model. Different
 370 letters signify statistically significant differences at $p < 0.05$, with post-hoc Tukey adjustments for multi-
 371 level factors.
 372

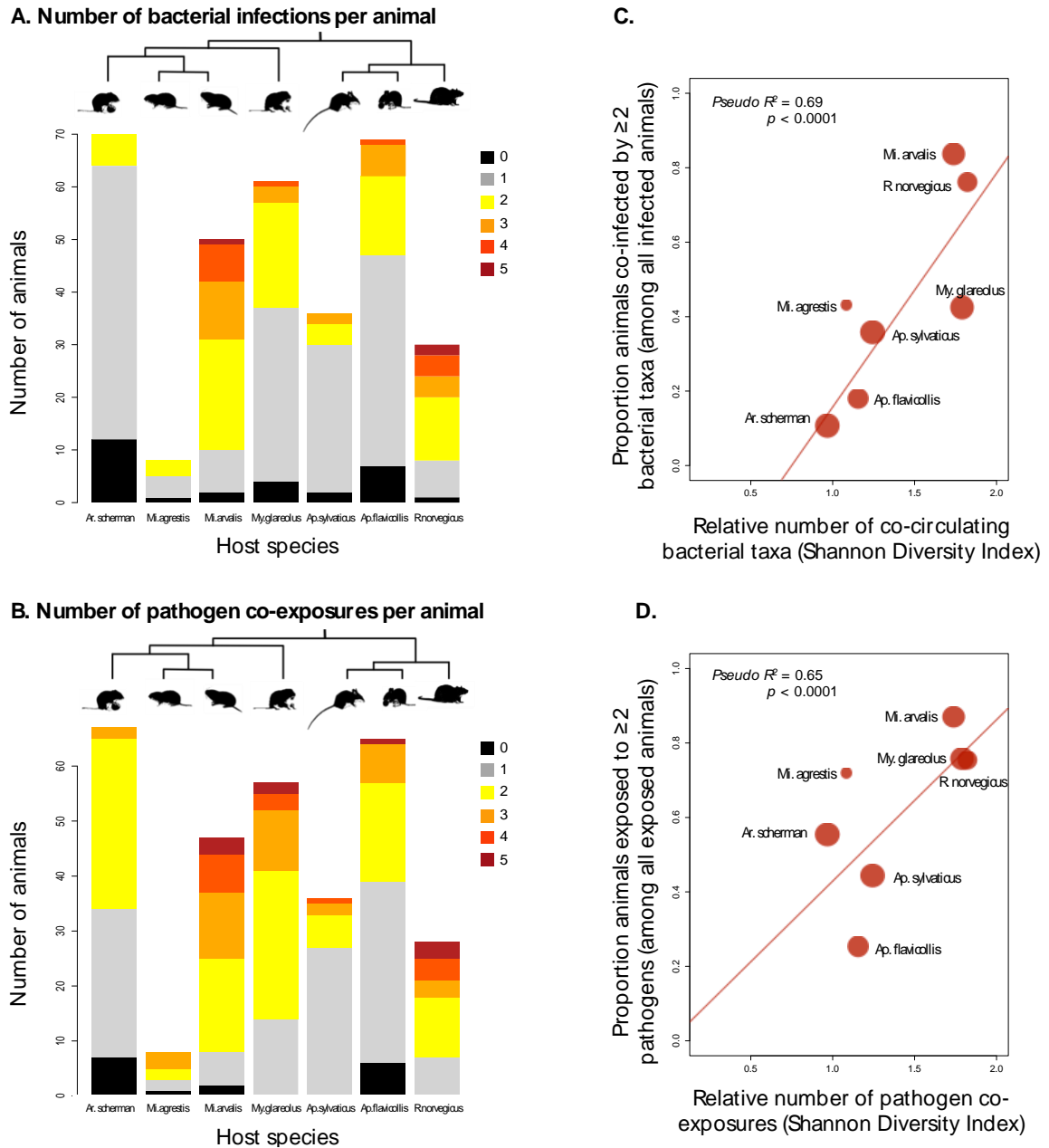


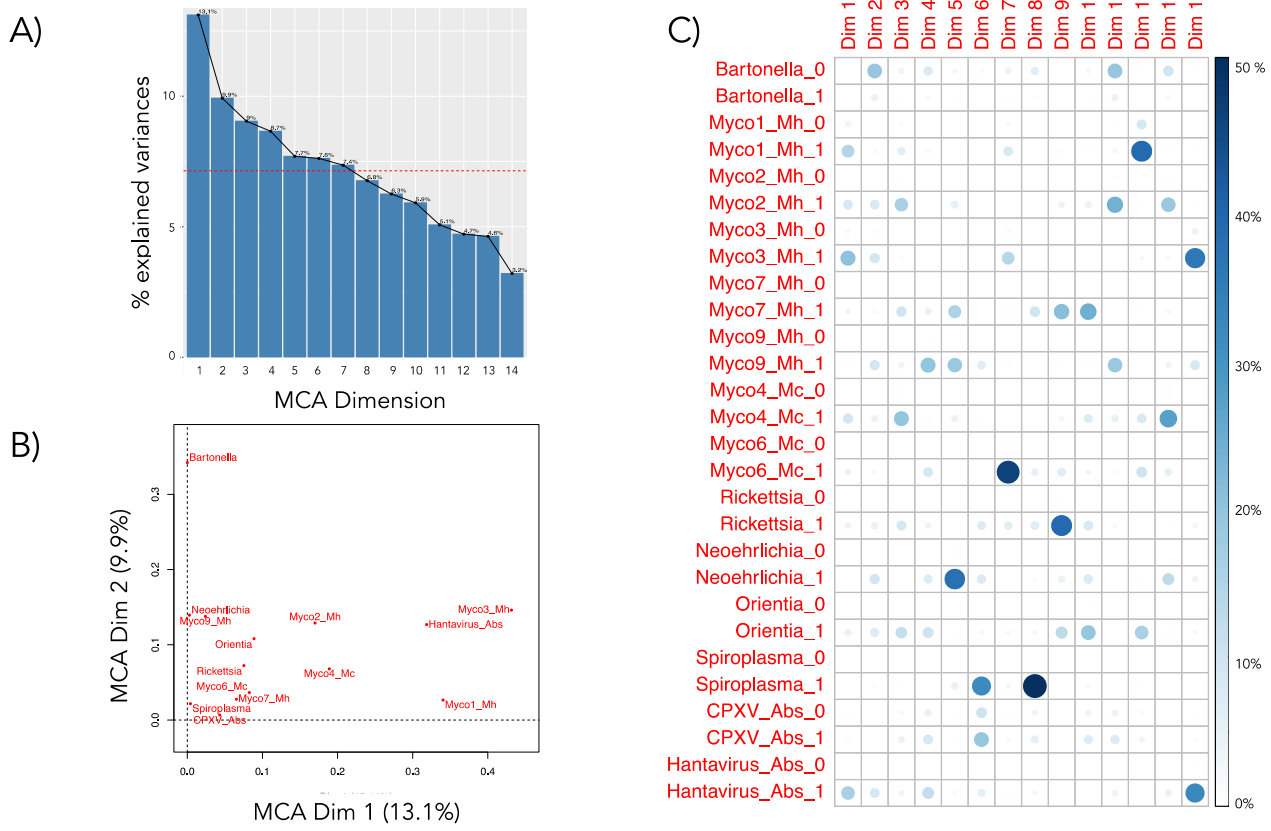
Figure 3 - Bacterial co-infection and co-exposure patterns across host species.

373
 374
 375
 376
 377 We also found an enormous amount of both bacterial co-infections and concurrent history of viral
 378 exposures (Figures 3A, 3B). The percentage of animals co-infected with two or more reliably detectable
 379 pathogenic bacterial OTUs among all those infected in each host species ranged between 84.4% (in *Mi.*
 380 *arvalis*) and 10.5% (in *Ar. scherman*). This co-infection frequency was significantly correlated with the
 381 diversity (Shannon Index) of bacteria circulating in each rodent species (Figure 3C; analysis of deviance
 382 $Pseudo-R^2 = 0.69$, $p < 10^{-11}$, calculated using logistic regression weighted by the number of infected animals
 383 per species). Bacterial co-infections were more frequent than expected in *Mi. arvalis*, and less frequent than
 384 expected in *My. glareolus* (according to Cook's Distance, Figure S6A). Results were similar when co-
 385 occurrence of antiviral antibodies was considered along with bacterial OTU exposure (Figure 3D; $Pseudo-R^2 =$
 386 0.65 , $p < 10^{-7}$). While *Mi. arvalis* had both more bacterial co-infections and slightly more pathogen co-
 387 exposures than expected based on pathogen diversity, other outliers differed between the two measures
 388 (Figure S6B): *My. glareolus* co-exposure frequencies were not lower than expected, and both *Apodemus*
 389 species had lower than expected co-exposures. Host species diversity in each community did not correlate

390 with bacterial co-infection ($r = 0.33$, $t = 1.11$, $df = 10$, $p = 0.29$) or pathogen co-exposure ($r = 0.022$, $t = 0.071$,
 391 $df = 10$, $p = 0.95$) frequencies.

392
 393 **3.2.2. Analyses of pathogen community composition**

394 Many pathogen taxa were found only in a single host species (*Mycoplasma haemomuris* OTU Myco8,
 395 “*Candidatus Mycoplasma ravipulmonis*” (Myco10), *Brevinema* spp., *Spiroplasma* spp., LCMV), and each host
 396 species had a unique combination of co-circulating pathogens (Figure 1). In order to best identify extrinsic
 397 and intrinsic factors potentially driving the composition of pathogen communities within the rodent
 398 community, we reduced the dataset to limit biases. We excluded *R. norvegicus* individuals due to competing
 399 *a priori* hypotheses that host species and habitat would be important factors (as this host species was the
 400 only one found in farm habitats, confounding these two variables; but see MCA results when *R. norvegicus*
 401 was included in Figures S7, S8). Likewise, we excluded pathogens that occurred only in one habitat type of
 402 one host species (not including *R. norvegicus*). Two additional individuals were excluded due to missing sex
 403 and age information. Analyses were performed on the remaining 280 individuals from six host species and
 404 their 14 pathogens (*Bartonella*, Myco1, Myco2, Myco3, Myco4, Myco6, Myco7, Myco9, *Rickettsia*,
 405 *Neoehrlichia*, *Orientia*, *Spiroplasma*, and antibodies against CPXV and hantaviruses) (Figure 4).



406
 407
 408 **Figure 4** - Results of multiple correspondence analysis (MCA) for pathogen community composition in
 409 rodents (excluding *R. norvegicus*) are described by (A) the contribution of each dimension to the
 410 overall variance in the data, (B) variable correlations with the first two dimensions of the MCA, and (C)
 411 variable contributions to each orthogonal MCA dimension. Horizontal line in (A) represents the percent
 412 variance expected due to chance ($100/14 = 7.14\%$).
 413

414 Overall, pathogen species composition was significantly structured by host species identity ($F_{5,252} = 16.23$,
 415 $p = 0.001$) and habitat type ($F_{2,252} = 2.51$, $p = 0.024$; Table S5). Out of 14 orthogonal dimensions returned by
 416 the MCA, the first two captured 23.0% of the variation in pathogen and antibody occurrence, and the first
 417 seven explained a cumulative 63.4% of the total variance (Figure 4A). Further dimensions captured less
 418 variance than would be expected if all dimensions contributed equally to overall inertia in the data.
 419 Dimension 1 (MCA Dim1; explaining 13.1% of the variation in pathogen community and loading heavily with
 420 the presence of Myco1, Myco3 and anti-hantavirus antibodies) differed significantly between host species

421 ($F_{5,268} = 23.83, p < 0.0001$) and age classes ($F_{1,268} = 6.27, p = 0.013$; Table S6). Dimension 2 (MCA Dim2;
422 explaining 9.9% of the variation in pathogen community and primarily describing the occurrence of
423 *Bartonella*) was also structured significantly by host species ($F_{5,268} = 3.89, p = 0.002$; Table S6). While these
424 first two dimensions varied by host species (Figure S10A) and host age class (Figure S10B), variance in host
425 habitats (Figure S10C) was not significant after accounting for the other factors. Host species was the most
426 consistently important extrinsic driver of pathogen community composition, significantly explaining variation
427 captured in six of the first seven dimensions, MCA Dim1 – MCA Dim7 (except for MCA Dim 5; Table S6).
428

429 3.3. Associations between pathogens

431 3.3.1. Validation of the associations detected by MCA

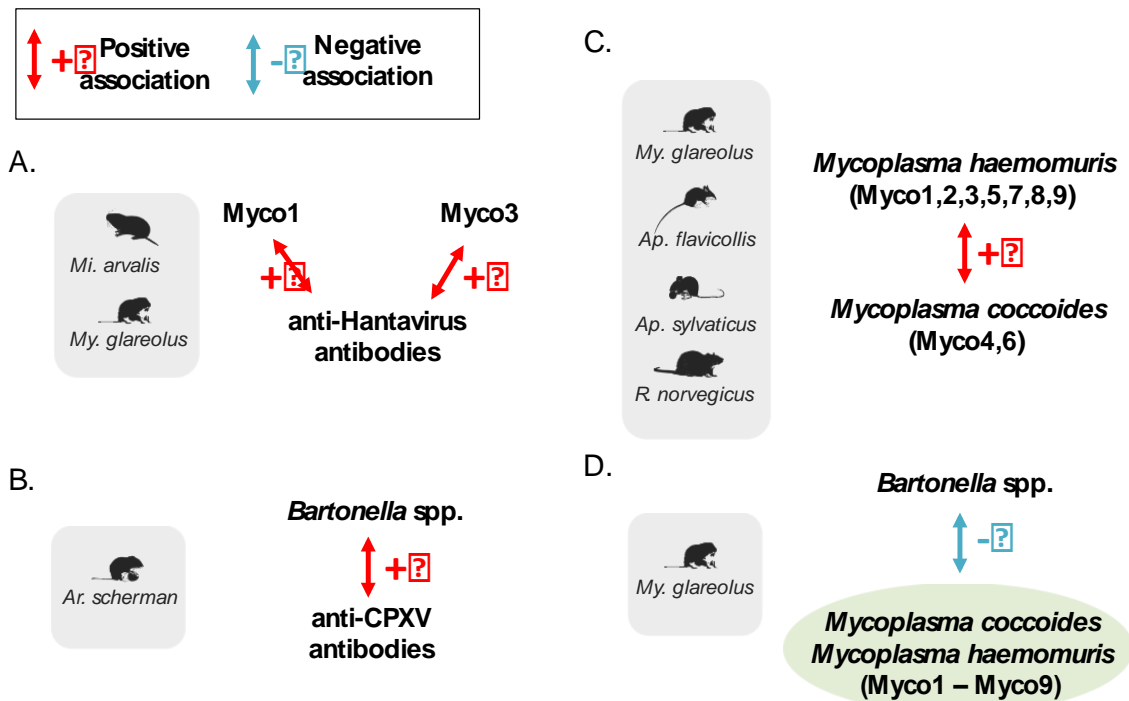
432 We applied SCN and GLM analyses to further characterize patterns detected using MCA. Strong and
433 relatively equal loading of MCA Dim1 with Myco1, Myco3, and anti-hantavirus antibody presence indicated
434 that these three pathogens were positively associated with one-another. Indeed, the six animals with anti-
435 hantavirus antibodies were found exclusively in animals infected with Myco3, and Myco1 was found in 2/3 of
436 hantavirus-exposed animals but in just 1/3 of those without hantavirus exposure. The MCA also revealed
437 significant differences among host species and host age classes for Dim 1; hantavirus and Myco3 only
438 circulated in two host species (*Mi. arvalis* and *My. glareolus*) and 34 of those 35 occurrences were in adults.
439 To exclude positive associations arising from mutual host specificity and age-related accumulation of
440 exposure probability, we focused our analyses on the dataset restricted to adults of the two host species in
441 which all three pathogens co-circulated (*Mi. arvalis* and *My. glareolus*). Individual SCN analyses performed on
442 adults of each host species revealed no associations (Table S7A). However, since values of MCA Dim 1 did not
443 differ between the two host species (according to post-hoc tests given in Table S6), we also ran a single SCN
444 analysis on the pooled data from adults of both species to improve statistical power (Table S7A). According
445 to this pooled SCN analysis, the three-way co-occurrence of Myco1, Myco3 and anti-hantavirus antibodies
446 was significantly more frequent than would be expected by random chance ($p = 0.008$), with a trend for anti-
447 hantavirus antibodies occurring by themselves more rarely than expected (sitting on the lower bound at
448 zero; $p = 0.13$; Table S7A, Figure S11). We also investigated this association using GLM. However, given the
449 small number of hantavirus exposures and perfect association with Myco3 infection, there was insufficient
450 statistical power to explicitly test for an association between all three pathogens and extrinsic factors. We
451 therefore ran three reciprocal GLM models on the restricted dataset, one for each pathogen as a function of
452 extrinsic factors to control for heterogeneous host groups (host species, host sex, study site, habitat, and
453 year sampled) and exposure to the two other pathogens (Table S7B). The number of extrinsic variables in
454 each model was reduced using model selection (results in Figure S12). These models showed that there
455 remained significant unexplained positive associations between hantavirus exposure and Myco1 infection
456 (anti-hantavirus antibodies \sim Myco1: $\chi^2 = 5.80, p = 0.016$) and between hantavirus exposure and Myco3
457 infection (Myco3 \sim anti-hantavirus antibodies: $\chi^2 = 13.66, p < 0.001$), but that there was no evidence of direct
458 association between Myco1 and Myco3 infections (Myco1 \sim Myco3: $\chi^2 = 0.01, p = 0.94$; Myco3 \sim Myco1: $\chi^2 <$
459 $0.37, p = 0.54$; Figure 5A).

460 The third MCA dimension also presented a clear hypothesis with sufficient statistical power to be tested.
461 MCA Dim3 was characterized by co-variation in Myco2 (*Myco. haemomuris*) and Myco4 (*Myco. coccoides*)
462 infections suggesting a positive association between members of these two *Mycoplasma* species. Myco2 and
463 Myco4 OTUs co-circulated only in *Ap. sylvaticus* hosts, thus we limited our analysis to this host species. There
464 was no significant association between the two OTUs detected by SCN analysis (Table S8A), and after
465 correcting for extrinsic factors remaining in the models after model selection (Figure S13), there remained
466 only a non-significant trend (Myco2 \sim Myco4: $\chi^2 = 2.79, p = 0.12$; Myco4 \sim Myco2: $\chi^2 = 2.34, p = 0.13$; Table
467 S8B) for a positive association between the two OTUs. No additional associations with sufficient variance for
468 statistical tests were clearly suggested by the MCA analysis.

470 3.3.2. Validation of associations described in the literature

471 We tested the *a priori* hypothesis that seropositivity to CPXV would be positively associated with
472 *Bartonella* infection, previously detected in *Mi. agrestis* (Telfer et al., 2010). The whole dataset was
473 considered as these two pathogens co-circulated in all host species (Figure 1). SCN analyses performed
474 independently for each host species revealed no associations (Table S9A), and the MCA results suggested

475 that pooling data across host species would be inappropriate. After correcting for extrinsic factors using
 476 GLM, we found reciprocal evidence for a positive association in *Ar. scherman* hosts (*Bartonella* ~ anti-CPXV
 477 antibodies: $\chi^2 = 5.07$, $p = 0.024$; anti-CPXV antibodies ~ *Bartonella*: $\chi^2 = 5.07$, $p = 0.024$; Figure 5B), but not in
 478 any other host species (Figure S14; Table S9B). It is of note that there were only eight *Mi. agrestis* individuals,
 479 rendering statistical power to test for the association while controlling for extrinsic factors insufficient in this
 480 host species where the association was previously described. While prevalence of both pathogens in *Mi.*
 481 *agrestis* was relatively high compared to other host species, one of the two animals without *Bartonella*
 482 infection was positive for anti-CPXV antibodies, also precluding evidence for a within-species trend.



483 **Figure 5:** Associations between pathogens in a community of rodents. Association hypotheses were
 484 generated by multiple correspondence analysis (A) or previously noted in the literature (B, C, D). Only
 485 associations supported by significant statistical tests ($p < 0.05$) are illustrated. Red arrows represent
 486 positive associations, blue arrows represent negative associations.
 487

488 We next focused on the potential associations between OTUs identified as belonging to two different
 489 species of hemotropic *Mycoplasma*, *Myco. haemomuris* (HM) and *Myco. coccoides* (HC), within the four host
 490 species in which they both circulated (*My. glareolus*, *Ap. flavicollis*, *Ap. sylvaticus*, *R. norvegicus*; Figure 1;
 491 Figure S4). We found no significant associations using independent SCN analyses for each host species (Table
 492 S10A). However, after controlling for extrinsic factors using GLM, a significant positive association was
 493 detected (HM ~ HC: $\chi^2 = 9.5$, $p = 0.0021$; HC ~ HM: $\chi^2 = 9.59$, $p = 0.002$), and did not differ between host
 494 species (non-significant interaction term between host species by explanatory pathogen occurrence in each
 495 reciprocal model, Figure S15; Table S10B; Figure 5C). We note that only one *R. norvegicus* animal was
 496 uninfected with *Myco. haemomuris*, and that animal also had no *Myco. coccoides* infection; thus the trend
 497 for the association in this host species was also positive but lacked sufficient variance for independent
 498 statistical analysis.

499 Finally, we tested for associations between *Bartonella* spp. and hemotropic *Mycoplasma* species,
 500 grouping the occurrence of different OTUs of the latter (Myco1 – Myco9) into a single presence-absence
 501 variable. There was no association detected by SCN analyses (Table S11A), and marginal evidence that any
 502 association may differ by host species after correcting for extrinsic factors using GLM with model selection
 503 (Figure S16; Table S11B). After controlling for extrinsic factors using independent GLMs for each host species
 504 (where possible), we found a negative association between the two pathogen groups only in *My. glareolus*
 505 hosts (*Bartonella* ~ *Mycoplasma*: $\chi^2 = 4.14$, $p = 0.042$; *Mycoplasma* ~ *Bartonella*: $\chi^2 = 6.59$, $p = 0.010$, Table
 506 S11B, Figure 5D).

507
508 **3.4. Evaluating false discovery**
509 Benjamini-Hochberg correction of p-values from hypothesis tests throughout the study suggested that
510 those above ~ 0.01 may lie above the false discovery cutoff for statistical significance (Figure S17), and that
511 null hypotheses rejected with smaller p-values have been rejected with confidence.
512

513 Discussion

514 Rodents have long been recognized as important reservoirs of infectious agents, with a high transmission
515 potential to humans and domestic animals (Kruse et al., 2004). Europe is identified as a hotspot of rodent
516 reservoir diversity and one third of rodent species are considered hyper-reservoirs, carrying up to 11
517 zoonotic agents (Han et al., 2015). Nevertheless, associations between these pathogens have still only rarely
518 been investigated (but see, for example, studies from field voles in the UK (Telfer et al., 2010) and in Poland
519 (Pawelczyk et al., 2004), gerbils in Israel (Cohen et al., 2015), across a rodent community in North America
520 (Dallas et al., 2019), and co-infection frequencies of zoonotic pathogens from rodents in Croatia (Tadin et al.,
521 2012)).

522 In this study, we confirmed that rodent communities in northern France may harbor a large diversity of
523 potential zoonotic pathogens, with at least 10 bacterial genera and antibodies against at least four genera of
524 viruses. Some of these pathogens have already been reported in the study region or in geographic proximity,
525 including viruses (*Orthohantavirus*, *Orthopoxvirus*, *Mammarenavirus* (Charbonnel et al., 2008; Salvador et al.,
526 2011)) and bacteria (e.g., *Bartonella*, *Mycoplasma*, *Rickettsia*, "*Candidatus Neoehrlichia*", *Orientia*,
527 *Spiroplasma*, *Treponema*, *Leptospira*, *Borrelia*, *Neisseria*, *Pasteurella*; see (Razzauti et al., 2015; Vayssier-
528 Taussat et al., 2012)). A previously undetected relative of the putatively pathogenic spirochaete *Brevinema*
529 *andersonii* that infects short-tailed shrews and white-footed mice in North America (Defosse et al., 1995) was
530 among our findings, and TBEV is not known to circulate this far east (Lindquist & Vapalahti, 2008). The high
531 prevalence of anti-hantavirus antibodies in *Mi. arvalis* is likely explained by cross-reactivity between the anti-
532 PUUV antibodies used in our assay and those elicited against the related *Tula orthohantavirus* (TULA) virus
533 common to European voles (Deter et al., 2007; Tegshduuren et al., 2010).

534 Three zoonotic pathogens were particularly prevalent: *Orthopoxvirus*, *Bartonella* spp., and *Mycoplasma*
535 spp. The wide range of hosts with anti-*Orthopoxvirus* antibodies corroborates prior evidence that cowpox
536 virus could be widespread in European rodents, particularly voles (Bennett et al., 1997; Essbauer et al., 2010;
537 Forbes et al., 2014). An astounding 77% of all individuals in the study were infected by *Bartonella* spp., a
538 diverse group of hemotrophs known to commonly infect rodents and other mammals (Bai et al., 2009;
539 Breitschwerdt & Kordick, 2000) and which have also been implicated in both zoonotic and human-specific
540 disease (Breitschwerdt, 2014; Iralu et al., 2006; Vayssier-Taussat et al., 2016). We could not assess the
541 specific diversity of *Bartonella* spp. circulating in these rodent communities because accurate resolution in
542 this genus requires additional genetic markers (L. Guy et al., 2013; Matar et al., 1999). Hemotropic and
543 pneumotropic *Mycoplasma* spp. were also highly prevalent across all host species, though surprisingly lower
544 than expected in *Ar. scherman* (Villette et al., 2017). These *Mycoplasma* species are also known pathogens of
545 humans and rodents (Baker, 1998; Harwick et al., 1972). Here, we found two distinct hemotropic
546 *Mycoplasma* species (*Myco. haemomuris* and *Myco. coccoides*) and the pneumotropic *Mycoplasma* species
547 *Myco. pulmonis* and "*Candidatus Myco. ravigulmonis*". The former two are both hemotropic mycoplasmas
548 responsible for vector-transmitted infectious anaemia of wild mice, rats, and other rodent species (Messick,
549 2004; Neimark et al., 2001, 2005). In contrast, *Myco. pulmonis* and "*Candidatus Myco. ravigulmonis*" cause
550 respiratory infections, are more closely related to other pneumotropic mycoplasmas, and "*Candidatus Myco.*
551 *ravigulmonis*" has only ever before been described in laboratory mice (formerly termed Grey Lung virus
552 (Andrews & Glover, 1945; Graham & Schoeb, 2011; Neimark et al., 1998; Piasecki et al., 2017)).

553 Our results also corroborated the status of hyper-reservoir (more than two zoonotic pathogens carried by
554 a reservoir species) for all seven of the focal rodent species studied here (Han et al., 2015). Even the rare
555 host species *Mi. subterraneous* also carried two potentially zoonotic pathogens (*Bartonella* spp. and
556 *Brevinema* spp.; Appendix 1). Overall, we found a high variability in the number of pathogens circulating in
557 each species despite correction for sampling effort, with low levels observed in *Apodemus* species and
558 *Arvicola scherman*, and high levels detected in *Mi. arvalis*, *My. glareolus*, and *R. norvegicus*. While

559 physiology, genetics, and behavior can contribute to the number of pathogen species able to infect a given
560 host species, larger geographic range size is highly correlated with higher pathogen species diversity
561 (Morand, 2015); this explanation matches the pattern among hosts in the communities sampled here (i.e.,
562 *Ar. scherman* and *Apodemus* spp. have small geographic ranges compared to those of *Mi. arvalis*, *My.*
563 *glareolus*, and *R. norvegicus*).

564 Several studies have emphasized the influence of host habitat specialization on parasite species richness,
565 low habitat specialization being associated with both high species richness of macro- and micro-parasites
566 (e.g., (Morand & Bordes, 2015)). Our results did not fully corroborate this association; while the grassland-
567 specific *Ar. scherman* had the lowest pathogen diversity and the multi-habitat spanning *My. glareolus* had
568 the highest pathogen diversity, entirely farm-dwelling *R. norvegicus* had high pathogen diversity nearly equal
569 to that of *My. glareolus*, and the two *Apodemus* hosts (neither with significantly higher pathogen diversity
570 than *Ar. scherman*) were found across both meadows and hedgerows. Instead, we found that more diverse
571 host species communities hosted more diverse pathogen communities. However, the implications of that
572 result are complex because while exposure to diverse (i.e., potentially novel) pathogens is a risk for disease
573 emergence, diverse host species communities are thought to keep individual pathogen prevalence low due
574 to the dilution effect – which should limit risk of zoonoses (Keesing et al., 2010).

575 The search for factors that drive parasite species richness, diversity and community composition has been
576 at the core of numerous studies (Krasnov et al., 2010; Mouillot et al., 2005; Nunn et al., 2003; Poulin, 1995;
577 Poulin & Morand, 2000; Sallinen et al., 2020). Here, we emphasized that both pathogen diversity and
578 community composition was mainly structured by host species identity, despite both shared habitats and
579 shared pathogen taxa. Pathogen beta diversity was also structured by habitat, which could result from
580 particular environmental suitability (e.g., for vectors) or opportunities for cross-species transmission. We
581 found no evidence that any specific pathogen-pathogen associations were likely to be as important as host
582 species identity in determining pathogen distributions across the community of rodents. The strong influence
583 of host characteristics (Cohen et al., 2015) and host species identity (Dallas et al., 2019) on pathogen
584 community composition has recently been described in comparison to intrinsic pathogen-pathogen
585 associations in other rodents. Moreover, the pathogen community composition provided a unique signature
586 for each rodent species, even among those most closely related (e.g., *Ap. flavicollis* and *Ap. sylvaticus*). This
587 result is in line with the conclusions of meta-analyses showing that phylogeny, over other host traits, has a
588 minimal impact on pathogen diversity in rodent species (C. Guy et al., 2019; Luis et al., 2013).

589 The importance of host species identity in shaping pathogen community composition may not stem from
590 strict host-pathogen specificity, as most pathogens were found to infect multiple host species – a broad
591 result echoed across animal communities (Cleaveland et al., 2001; Pedersen et al., 2005; Streicker et al.,
592 2013; Taylor et al., 2001; Woolhouse et al., 2001). However, we might be cautious as more precise molecular
593 analyses are necessary to test whether different species of a bacteria genus or divergent populations of the
594 same bacteria species may circulate independently in different rodent host species, with little or no
595 transmission. For example, two genera seemed to be largely shared among the rodent species studied here,
596 *Bartonella* and *Mycoplasma*. But previous studies have shown strong host-specificity when considering the
597 genetic variants of *Bartonella* (Brook et al., 2017; Buffet et al., 2013; Withenshaw et al., 2016). Evidence in
598 the literature for host specificity of *Mycoplasma* species has led to a mix of conclusions (Pitcher & Nicholas,
599 2005), as cases of cross-species transmission are commonly reported – particularly in humans – despite a
600 general consensus that most species are highly host-specific. We found that some *Mycoplasma* taxa were
601 dominant contributors to prevalence in a single host species, and that when shared, they were shared with
602 just a few other specific host species. Rare infections in unexpected host species (e.g., Myco6 in *Ar. scherman*
603 and Myco1 in *Ap. flavicollis*) were represented by fewer sequence reads compared to positive samples in
604 host species where they were more prevalent, suggesting a low potential for amplification and sustained
605 transmission from these occasional hosts (Figure S4). On the other hand, while the Cricetidae appeared to be
606 susceptible only – with rare exception – to taxa within the *Myco. haemomuris* group, host species in the
607 Muridae family were susceptible to all three distinct *Mycoplasma* species detected. The biggest exception to
608 this pattern was that three of 62 *Myodes glareolus* (sister to all other sampled Cricetidae in the study)
609 animals were found to be infected by both hemotropic *Mycoplasma* species. These results both support the
610 observation that cross-species transmission naturally occurs among wild rodents and suggest that the degree
611 of host specificity may be driven by both host and pathogen factors.

612 Concurrent exposure to multiple pathogens within individuals was also frequent, as high as 89 % (in *Mi.*
613 *arvalis* hosts), in line with recent studies that have shown that co-infections by multiple pathogens are
614 common in natural populations (e.g., in mammals, birds, amphibians, ticks, humans (Clark et al., 2016;
615 Griffiths et al., 2011; Moutailler et al., 2016; Stutz et al., 2018; Telfer et al., 2010)). Variation in the frequency
616 of pathogen co-exposure was highly correlated to the diversity of pathogens circulating in each host species,
617 suggesting the dominance of a random process of pathogen exposure for each individual. However, there
618 were a few intriguing outliers: *My. glareolus* hosts were less co-infected than expected based on diversity of
619 bacterial taxa, but not when viral antibodies were included; conversely, *Ar. scherman* hosts were more co-
620 exposed when viruses were considered, but not when only bacteria were considered; and *Mi. arvalis* hosts
621 had consistently higher proportions of co-exposures whether viruses were or were not considered along with
622 bacteria. The non-random grouping of pathogen exposures within individuals (as in *Mi. arvalis*) may result
623 from heterogeneity in extrinsic transmission, environmental, or susceptibility factors (Beldomenico et al.,
624 2008; Beldomenico & Begon, 2010; Cattadori et al., 2006; Fenton et al., 2010; Swanson et al., 2006) or from
625 intrinsic interactions between pathogens (e.g., facilitation mediated by hosts immune response). Differences
626 in the pattern of co-exposure frequencies when including or excluding antiviral antibodies (as with *My.*
627 *glareolus* and *Ar. scherman*) could result from different mechanisms (e.g., bacterial manipulation of innate
628 immunity (Diacovich & Gorvel, 2010)) affecting pathogen community assemblage. However, a lack of
629 deviance from the expected co-exposure frequency does not exclude the possibility that both extrinsic and
630 intrinsic processes may be occurring.

631 We found evidence in support of three previously identified pathogen-pathogen associations (positive
632 association between *Myco. haemomuris* and *Myco. coccoides* infections; positive association between
633 *Bartonella* spp. infection and the presence of anti-CPXV antibodies; negative association between *Bartonella*
634 spp. and hemotropic *Mycoplasma* spp. infections) and characterized one set of associations not previously
635 described (positive associations between the presence of anti-hantavirus antibodies and infections by two
636 specific *Myco. haemomuris* OTUs) – each in a unique subset of host species. *Mycoplasma* spp. blood
637 infections are likely transmitted through bites of blood-sucking arthropod vectors (Volokhov et al., 2017),
638 meaning vectors could prefer some individuals over others (Malmqvist et al., 2004). Positive associations
639 detected between *Myco. haemomuris* and *Myco. coccoides* could also result from similarities in rodent
640 susceptibility. Indeed *Mycoplasma* spp. infection can lead to acute or chronic infection, and the
641 establishment of chronic bacteremia seems to occur in immunosuppressed or immunocompromised
642 individuals (Cohen et al., 2018). Co-infections with multiple *Mycoplasma* spp. might therefore be more likely
643 to be detected in these immunocompromised rodents with chronic infections. The existence of chronic
644 infections might also lead to additional co-infections and positive associations as a result of disease-induced
645 changes in population dynamics, immune system function, or through direct pathogen-pathogen interactions
646 (Aivelo & Norberg, 2018; Fenton, 2008; Fountain-Jones et al., 2019).

647 Whether through the accumulation of exposure probabilities or increased susceptibility, the previously-
648 undocumented positive association we found here between *Myco. haemomuris* OTUs (*Myco1* and *Myco3*)
649 and anti-hantavirus antibodies may similarly be explained by the chronic nature of both *Mycoplasma* spp.
650 and hantavirus infections in rodents (e.g., for Puumala hantavirus in bank voles (Meyer & Schmaljohn, 2000;
651 Vaheri et al., 2013; Yanagihara et al., 1985)). This positive association was found in both host species where
652 the majority of hantavirus exposures occurred (*Microtus arvalis* and *Myodes glareolus*), consistent with the
653 generality of association between *Mycoplasma* species across host taxa detailed above, suggesting the
654 intrinsic ecology of these pathogens contributes to shaping variation in the pathogen community. Curiously,
655 we found no evidence for direct associations between OTUs of the same *Mycoplasma* species, thus
656 facilitation interactions are unlikely to explain the high diversity of *Mycoplasma* taxa both within and
657 between host species.

658 Infections by *Bartonella* species are also known to often result in subclinical and persistent bacteremia in
659 mammals, including rodents (Birtles et al., 2001; Kosoy et al., 2004). The positive association detected in *Ar.*
660 *scherman* between *Bartonella* spp. and anti-CPXV antibodies might therefore be explained by, for example,
661 joint accumulation of both chronic bacterial infections and long-lived antiviral antibodies used to test for
662 prior exposure to relatively short-lived CPXV infections. However, if the same processes governing
663 association of the chronic infections described above were at play here, we would have expected to find
664 both pathogens implicated in positive associations (i) with other chronic infections, and (ii) across host
665 species given their ubiquitous prevalence. While the failure to recover the association in *Mi. agrestis*

666 (previously described (Telfer et al., 2010)) was likely due to low statistical power, the lack of a general
667 pattern across other host species despite adequate sampling suggests a more specific, and potentially
668 immune-mediated, ecological process between these two pathogens. Indeed, pox virus infections, including
669 CPXV, have been shown to induce immunomodulation that increases host susceptibility to other parasites
670 (Johnston & McFadden, 2003). These interactions could be of variable intensities according to the rodent
671 species considered, due to potential differences in impacts of CPXV infection on immunity across host
672 species, or to the influence of other infections not examined here on host immune responses during pox
673 infections (e.g., helminths (Cattadori et al., 2007), protozoa (Telfer et al., 2010)). Furthermore, *Bartonella*
674 spp. infection was negatively associated with *Mycoplasma* spp. infections in *My. glareolus*, corroborating
675 negative interactions reported in co-infection experiments in gerbils (Eidelman et al., 2019). This association
676 may therefore originate from an interaction mediated by specific (immune) genetic features of *My. glareolus*,
677 and not ecological conditions as proposed by Eidelman et al. (2019). The antagonistic and host-specific
678 nature of this association lends further support to the interpretation that *Bartonella* spp. infections do not
679 behave in similar ways to other chronic infections in the community. However, few studies have investigated
680 the robustness of within-host interactions across different host species (e.g., (Lello et al., 2018)), and this
681 question deserves further investigation.

682 Our results suggest that intrinsic ecological interactions could help shape the composition of the
683 pathogen community within hosts. However, this suggestion provides only a hypothesis that requires further
684 investigation. Interpretation of associations can be misleading, as they may arise from unmeasured co-
685 factors such as exposure to shared transmission routes, and may even run counter to the underlying
686 ecological process (Fenton et al., 2014). The associations we found here were not visible (or even misleading,
687 in the case of a 3-way interaction between hantavirus, Myco1 and Myco3), for instance, when ignoring
688 extrinsic factors using the SCN analysis, despite the increased statistical power it offered. Evidence for
689 interactions between pathogens within hosts initially came from laboratory studies (e.g., in the development
690 of vaccines, reviewed in (Casadevall & Pirofski, 2000)), and until recently, many studies conducted in the wild
691 could not detect such interactions (e.g., (Behnke, 2008)). Developments in statistical approaches have
692 contributed to improve sampling designs and analyses, in particular by better controlling for confounding
693 factors, enabling the detection of associations resulting from these within-host interactions (e.g., (Galen et
694 al., 2019; Lello et al., 2004; Telfer et al., 2010)). However, it is unlikely any statistical approach can ever solve
695 the problem of an unmeasured explanatory variable. For instance, our decision to screen only the spleen
696 means we could have missed evidence of exposure to pathogens that can only be found by screening the
697 liver, kidney or brain (Mangombi et al., 2021). Experiments conducted in semi-controlled environments have
698 been used to confirm the importance of interactions suggested by the associations (e.g., (Knowles et al.,
699 2013)). Both facilitation mediated by immune responses (e.g., (Ezenwa et al., 2010)) and competition
700 mediated by shared resources (e.g., (Brown, 1986; Budischak et al., 2018)) have been emphasized.

701 There remain additional important limits to the interpretation of snapshot observational studies from
702 wild populations such as ours. For instance, they cannot provide information about the sequence or duration
703 of infection, although these features strongly affect the outcome of within-host interactions (Eidelman et al.,
704 2019). Moreover, both the 16S metabarcoding approach and serological antibody tests can only be
705 interpreted in terms of presence/absence of exposure to pathogens, although co-infection may rather
706 impact parasite abundance (e.g., (Gorsich et al., 2014; Thumbi et al., 2013)). Other extrinsic factors, such as
707 seasonal variation in pathogen community composition, could also impact both interpretation and year-
708 round generality of our results due to adherence to autumn sampling dates (Maurice et al., 2015; Villette et
709 al., 2020). Lastly, we also acknowledge several caveats to consider with our methods. We removed animals
710 from which fewer than 500 reads were amplified in one or both bacterial metabarcoding PCR replicates.
711 While 16 of these samples removed were due to random failure of PCR amplification from just one of the
712 two replicates, 12 of the animals had poor amplification in both PCR replicates. In the absence of an internal
713 positive control, e.g., a spike-in standard (Zemb et al., 2020), we were unable to verify whether a lack of
714 reads was due to poor DNA extraction or a true lack of infections. Although this has a risk of artificially
715 inflating prevalence rates by selectively removing uninfected individuals, it is unlikely to have had a
716 qualitative effect on our results. Similarly, limiting our analyses to OTUs with 500 reads or more in the entire
717 dataset may select against detection of very rare or low-burden infections. We also removed many OTUs
718 corresponding to bacteria normally occurring in external or internal microbiomes of healthy animals, some of
719 which were represented by a high abundance of reads in positive animals. This was due mainly to the fact

720 that 16S data cannot often distinguish between pathogenic and commensal taxa of many such genera. We
721 know that, for instance, *Helicobacter* species are naturally found in the digestive tract, but can also cause
722 pathogenic infections. Parasitism can affect host microbiome composition (Gaulke et al., 2019), and this in
723 turn can have impacts on host health and disease susceptibility (reviewed in (Murall et al., 2017; Rosshart et
724 al., 2017)). Thus, our choice to ignore OTUs corresponding to microbes typical of healthy flora contributes to
725 the problem of missing data, such as information on intestinal helminth infections or other viruses, which
726 may explain or alter the associations we were able to detect. Furthermore, the evaluation of diversity
727 measures (e.g., Shannon diversity index) based only on a selection of taxa violates the assumption that all
728 species are represented in the sample; thus, patterns of diversity could also be influenced by missing data.
729 These caveats are common problems for disease surveillance and community ecology studies, irrespective of
730 the diagnostic methods, and it is difficult to speculate about their overall impacts on the present study.
731 Finally, it is well-understood that this bias towards detection of common pathogens and difficulty in
732 interpreting evidence for the absence of a pathogen in a given individual or population can make testing for
733 negative associations driven by antagonistic ecological interactions incredibly difficult, if not impossible
734 (Cougoul et al., 2019; Weiss et al., 2016).
735

736

Conclusions

737 Our results add to a growing number of studies finding that (i) rodents host many important zoonotic
738 human pathogens and (ii) pathogen communities are shaped primarily by host species identity. We also
739 detected a number of previously undescribed associations among pathogens within these rodent
740 communities, and we also confirmed previously identified associations, sometimes in other rodent species
741 than those in which they were previously described. These associations can be considered in the future as
742 hypotheses for pathogen-pathogen interactions within rodent hosts, and that participate in shaping the
743 community of pathogens in rodent communities. Long-term survey and experimental studies are now
744 required to confirm these interactions and understand the mechanisms underlying the patterns of co-
745 infection detected. In addition to these biological results, we have identified several methodological caveats,
746 with regard to both pathogen and association detection, that deserves further investigation to improve our
747 ability to make robust inference of pathogen interactions.
748
749

750

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762

763

Data, scripts, code, and supplementary information availability

764

765 Supplementary data deposited in Zenodo (<https://doi.org/10.5281/zenodo.7092812>) include the
766 following 16S metabarcoding data: (i) raw sequence reads (fastq format), (ii) raw output files generated by
767 the mothur program (iii) raw abundance table and (iv) filtered occurrence table, as well as (v) scripts and
768 data files for statistical analyses. Items iii-v are also provided in Supplemental Materials Appendix 2 to
769 directly accompany this publication.
770

771

Conflict of interest disclosure

772 The authors of this article declare that they have no financial conflict of interest with the content of this
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774

775

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779

Table 1. BLAST search results for OTUs suspected of belonging to pathogenic genera. References are available in Supplemental Materials Appendix 3.

Infecting species identity	OTU Number	Number of Reads	Genbank		BLAST results (% identity)	Pathogen Code	Reference
			Accession Number	Number			
Pathogenic taxa, reliably detectable							
<i>Bartonella</i> spp.	Otu00001	6353372	MT027154		100% <i>Bartonella grahamii</i> (AB426637) from wild North America rodents; 99%-100% identity to many other pathogenic <i>Bartonella</i> species.	Bartonella	Deng et al., 2012
<i>Brevinema</i> spp.	Otu00123	5603	MT027155		97% <i>Brevinema andersonii</i> (NR_104855) type sequence, infectious spirochaete of short-tailed shrew and white-footed mouse in North America	Brevinema	Defosse et al., 1995
<i>Candidatus Neoehrlichia mikurensis</i>	Otu00039	18358	MT027156		100% <i>Candidatus Neoehrlichia mikurensis</i> (KF155504) tick-borne rodent disease, opportunistic in humans	Neoehrlichia	Andersson & Raberg, 2011
<i>Mycoplasma ravidpulmonis</i>	Otu00054	6086	MT027164		100% <i>Mycoplasma ravidpulmonis</i> (AF001173), grey lung disease agent in <i>Mus musculus</i> ; 88% <i>M. orale</i> from humans (LR214940)	Myco10	Petterson et al., 2000
<i>Mycoplasma</i> spp.	Otu00004	845971	MT027157		99% identity to uncultured <i>Mycoplasma</i> species (KU697344) from small rodents in Senegal and uncultured eubacterium (AJ292461) from Ixodes ticks; 95% (KM538694) and 94% (MK353834) identity to uncultured hemotropic <i>Mycoplasma</i> species in European and South American bats	Myco1	Galan et al., 2016
<i>Mycoplasma</i> spp.	Otu00003	426034	MT027158		99% <i>Mycoplasma haemomuris</i> (AB758439) from <i>Rattus rattus</i>	Myco2	Conrado et al., 2016
<i>Mycoplasma</i> spp.	Otu00006	106443	MT027159		99% uncultured <i>Mycoplasma</i> spp. (KP843892) from leopards in South Korea; 99% identity to uncultured <i>Mycoplasma</i> (KT215637) from rodents in Brazil	Myco3	Goncalves et al., 2015
<i>Mycoplasma</i> spp.	Otu00010	72724	MT027160		99% <i>Mycoplasma coccoides</i> comb. nov. (AY171918); 97% <i>Candidatus Mycoplasma turicensis</i> (KJ530704) from Indian mongoose	Myco4	Conrado et al., 2015
<i>Mycoplasma</i> spp.	Otu00005	165095	MT027161		100% <i>Mycoplasma haemomuris</i> -like undescribed species (KJ739312) from <i>Rattus norvegicus</i>	Myco5	Alabi et al., 2020
<i>Mycoplasma</i> spp.	Otu00007	92237	MT027162		99% uncultured <i>Mycoplasma</i> spp. (KC863983) from <i>Micromys minutus</i> (eurasian harvest mouse) in Hungary; 98% <i>M. coccoides</i> comb. nov. (AY171918)	Myco6	Conrado et al., 2015
<i>Mycoplasma</i> spp.	Otu00012	39767	MT027163		93% uncultured <i>Mycoplasma</i> species (KU697341) of mice in Senegal; 92% <i>Mycoplasma</i> spp. (KP843892) from leopards in South Korea, 91% <i>Mycoplasma haemomuris</i> (AB820289) in rats	Myco7	Galan et al., 2016
<i>Mycoplasma</i> spp.	Otu00015	31528	MT027165		98% uncultured <i>Mycoplasma</i> spp. (KT215632) from wild rodent spleen in Brazil; 95% uncultured <i>Mycoplasma</i> spp. (KF713538) in little brown bats	Myco8	Ricardo Goncalves et al., 2015
<i>Mycoplasma</i> spp.	Otu00049	40125	MT027166		96% uncultured <i>Mycoplasma</i> spp. from Brazilian rodents (KT215638) and S. Korean leopard (KP843892)	Myco9	Ricardo Goncalves et al., 2015
<i>Orientia</i> spp.	Otu00111	876	MT027167		97% <i>Orientia tsutsugamushi</i> (KY583502) from humans in India, zoonotic Rickettsial pathogen (causes scrub typhus)	Orientia	Paris et al., 2013
<i>Rickettsia</i> spp.	Otu00008	72098	MT027168		98% <i>Rickettsia japonica</i> (MF496166) which causes Japanese spotted fever, <i>R. canadensis</i> (NR_029155) & <i>R. rhipicephali</i> (NR_074473) type strains	Rickettsia	Yamamoto et al., 1992
<i>Spiroplasma</i> spp.	Otu00093	4738	MT027169		95% uncultured <i>Spiroplasma</i> spp. (KT983901) from Ixodes tick on a dog; 94% identity to type strain of <i>Spiroplasma mirum</i> (NR_121794), the agent of suckling mouse cataract disease; <i>Spiroplasma ixodetis</i> causes similar disease in humans.	Spiroplasma	Cisak et al., 2015
Pathogenic taxa, not reliably detectable							
<i>Arcobacter cryaerophilus</i>	Otu00296	403	MT027170		100% <i>Arcobacter cryaerophilus</i> (CP032825) emerging enteropathogen in humans, zoonotic, pathogenic in rats	Arcobacter	Vandamme et al., 1992
<i>Borrelia miyamotoi</i>	Otu00318	419	MT027171		100% <i>Borrelia miyamotoi</i> (CP010308) in humans and Ixodes, zoonotic pathogen	Borrelia1	Krause et al., 2015
<i>Borrelia</i> spp.	Otu00514	206	MT027172		96% <i>Borrelia</i> sp. nov "Lake Gaillard" in <i>Peromyscus leucopus</i> (AY536513), 95% <i>B. hermsii</i> (MF066892) from tick (<i>Ornithodoros hermsii</i>) bites in humans	Borrelia2	Bunikis et al., 2005
<i>Borrelia afzelii</i>	Otu00071	78	MT027173		100% <i>Borrelia afzelii</i> (CP009058) human pathogen closely related (98%) to <i>B. burgdorferii</i> (positive control sequence)	Borrelia3	Schuler et al., 2015
<i>Leptospira</i> spp.	Otu01015	257	MT027174		100% several pathogenic <i>Leptospira</i> species from mammals, e.g., <i>L. interrogans</i> (LC474514) in humans	Leptospira	Vincent et al., 2019
<i>Mycoplasma pulmonis</i>	Otu00771	255	MT027175		100% <i>Mycoplasma pulmonis</i> (NR_041744) chronic respiratory pathogen of mice and rats	Myco0771	Piasecki et al., 2017
<i>Mycoplasma</i> spp.	Otu04125	164	MT027176		90% <i>Mycoplasma ravidpulmonis</i> (AF001173), grey lung disease agent in <i>Mus musculus</i> ; 80% <i>M. phocidae</i> from California sea lions (DQ521594)	Myco4125	Petterson et al., 2000
Eukaryotic family Sarcocystidae	Otu00056	8684	Not submitted*		97% similar to plastid small ribosomal unit of <i>Hyaloklossia lieberkuehni</i> (AF297120), a parasitic protozoa of European green frog; 96% <i>Neospora caninum</i> (MK770339) & <i>Sarcocystis muris</i> (AF255924); 95% <i>Toxoplasma gondii</i> (TGU28056)	Sarcocystidae1	Calarco & Ellis, 2020
Eukaryotic family Sarcocystidae	Otu00191	3678	Not submitted*		92% <i>Neospora caninum</i> (MK770339) parasite; 90% <i>Toxoplasma gondii</i> (U87145) zoonotic pathogen	Sarcocystidae2	Calarco & Ellis, 2020
Eukaryotic family Sarcocystidae	Otu00254	1219	Not submitted*		98% <i>Sarcocystis muris</i> (AF255924) coccidian parasite first found in mice	Sarcocystidae3	Orosz, 2015

* No reference specimen for identification of Eukaryote

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Table 1 (continued). BLAST search results for OTUs suspected of belonging to pathogenic genera. References are available in Supplemental Materials Appendix 3.

Infecting species identity	OTU Number	Number of Reads	Genbank		Pathogen Code	Reference
			Accession Number	BLAST results (% identity)		
Uncertain pathogenicity, reliably detectable						
<i>Corynebacterium xerosis</i>	Otu00050	1853	MT027177	100% <i>Corynebacterium xerosis</i> (MH141477), only opportunistic infections identified	Corynebacterium	Bernard, 2012
<i>Dietzia</i> spp.	Otu00102	2626	MT027178	100% <i>Dietzia</i> spp. e.g., <i>D. aurantiaca</i> (MK25331); common contaminant; opportunistic in humans; thought to out-compete <i>Trypanosomes</i>	Dietzia	Kampfer et al., 2012
<i>Helicobacter</i> spp.	Otu00013	34894	MT027179	96% homology to <i>Helicobacter suncus</i> (AB006147) isolated from shrews with chronic gastritis; 95% identity to type specimen for <i>H. mustelae</i> (NR_029169) which causes gastritis in ferrets; but could be normal gut flora	Helico1	Goto et al., 1998
<i>Helicobacter</i> spp.	Otu00025	8702	MT027180	97% similar to <i>Helicobacter trogontum</i> (AY686609) and <i>H. suncus</i> (AB006147), both enterohepatic <i>Helicobacter</i> spp. associated with intestinal diseases	Helico2	Goto et al., 1998
<i>Helicobacter</i> spp.	Otu00087	2303	MT027181	99% identical to <i>Helicobacter aurati</i> (NR_025124.1), a pathogen of Syrian hamsters; 98% identical to <i>H. fennelliae</i> (GQ867176), a human pathogen	Helico3	Patterson et al., 2000
<i>Helicobacter</i> spp.	Otu00128	1178	MT027182	99% <i>Helicobacter winghamensis</i> (AF363063), associated with gastroenteritis in humans; however, minor sequences were 100% identical to <i>H. rodentium</i> (AY631957) which is only associated to gastritis in rodents when coinfecting with other <i>Helicobacter</i> strains	Helico4	Melito et al., 2001
<i>Neisseria</i> spp.	Otu00612	780	MT027183	97% uncultured <i>Neisseria</i> spp. associated with human prostatitis (HM080767) and cataracts (MG696979), but undistinguishable from environmental samples and healthy flora (e.g., JF139578)	Neisseria1	Genbank, unpublished
Pasteurellaceae	Otu00129	1430	MT027184	100% uncultured bacterium (MN095269) of mouse oral flora; 99% <i>Muribacter muris</i> (KP278064) of unknown pathogenicity, water fowl pathogen <i>Avibacterium gallinarum</i> (AF487729), and cattle respiratory disease agent <i>Mannheimia haemolytica</i> (CP017491)	Pasteurella1	Nicklas et al., 2015
Pasteurellaceae	Otu00203	521	MT027185	99% <i>Aggregatibacter aphrophilus</i> (LR134327) and <i>Haemophilus parainfluenzae</i> (CP035368) opportunistic pathogens but otherwise part of normal flora	Pasteurella2	Genbank, unpublished
<i>Rickettsiella</i> spp.	Otu00187	592	MT027186	99%-100% identity to several endosymbionts of insects, eg. uncultured <i>Diplorickettsia</i> spp. in sand flies (KX363696), <i>Rickettsiella</i> spp. in Ixodes ticks (KP994859); 99% identity to <i>Rickettsiella agriotidis</i> (HQ640943) pathogen of wireworms	Rickettsiella	Duron et al., 2015
<i>Streptococcus</i> spp.	Otu00115	1681	MT027187	99% <i>Streptococcus hyointestinalis</i> from intestines of swine (KR819489)	Streptococcus	Genbank, unpublished
<i>Yersinia</i> spp.	Otu00041	7420	MT027188	A heterogeneous OTU some major sequences 100% <i>Yersinia</i> spp. and <i>Serratia</i> spp., including pathogenic zoonotic bacteria (e.g., <i>Y. pestis</i> NR_025160) and non-pathogenic endosymbionts of plants (NR_157762); some major sequences 100% <i>Pantoea agglomerans</i> (MN515098) opportunists	Yersinia	Kim et al., 2003
Uncertain pathogenicity, not reliably detectable						
<i>Fusobacterium</i> spp.	Otu00791	108	MT027189	100% <i>Fusobacterium ulcerans</i> (CP028105) from tropical foot ulcers in humans; but also 100% identity with other fecal isolates of unknown pathogenicity in mammals (e.g., <i>F. varium</i> LR134390)	Fusobacterium	Genbank, unpublished
<i>Neisseria</i> spp.	Otu00454	148	MT027190	98% uncultured microbiota of bat mating organs (KY300287), 98% <i>Simonsiella muelleri</i> commensal from human saliva (AF328145); 97% <i>Kingella kingae</i> (MF073277) pathogen in humans	Neisseria2	Genbank, unpublished
<i>Treponema</i> spp.	Otu00235	348	MT027191	93% uncultured rumen <i>Treponema</i> spp. (AB537611)	Treponema	Bekele et al., 2011
<i>Williamsia</i> spp.	Otu00614	274	MT027192	100% <i>Williamsia phyllosphaerae</i> (MG205541) and <i>Williamsia maris</i> (NR_024671), closely related to opportunistic pathogens in humans	Williamsia	Genbank, unpublished

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