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), regression and association screening (SCN) analyses were used to determine the degree to which extrinsic factors 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 virus (CPXV) and hantaviruses, and these patterns were generally specific to particular host species. Altogether, our results suggest that host and pathogen specificity is the most important driver of pathogen community structure, and that interspecific pathogen-pathogen associations also depend on host species.

Keywords (6max)

16S rRNA amplicon high throughput sequencing; Disease Ecology; Microbial 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, Cunningham & Hyatt 2000; Smith, Sax & Lafferty 2006; Jones *et al.* 2008). 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 (Taylor, Latham & Woolhouse 2001; Jones *et al.* 2008). 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., (Harvell *et al.* 2002; Burthe *et al.* 2006; 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; Streicker *et al.* 2010; Streicker, Fenton & Pedersen 2013; Salvador *et al.* 2011; Charbonnel *et al.* 2014; Bordes *et al.* 2017)). 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

73 human health, agriculture, wildlife, and conservation (Pedersen & Fenton 2007). Simultaneous
74 infection by multiple parasite species is ubiquitous in nature (Petney & Andrews 1998; Cox 2001;
75 Moutailler *et al.* 2016), and prior infections can have lasting effects on future susceptibility via e.g.,
76 changes to host condition and behavior or through immune-mediated processes (Singer 2010;
77 Quiñones-Parra *et al.* 2016; Kumar *et al.* 2018). Interactions among co-circulating parasites may
78 have important consequences for disease severity, transmission and community-level responses to
79 perturbations (Jolles *et al.* 2008; Telfer *et al.* 2010). Consequences of interaction may be life-
80 long, as exposure to pathogens circulating among juveniles have been found to be strongly
81 associated with those experienced by adults (e.g., Fountain-Jones *et al*. 2019). Such interactions
82 can also play a role in the consequences of pathogen emergence (e.g., emerging bacterial
83 infection increasing susceptibility to an endemic virus (Beechler *et al*. 2015)). Henceforth, and
84 through the advent of sequencing technologies in particular, it is now possible and essential to
85 investigate disease emergence from a multi-host / multi-pathogen perspective (Galan *et al.* 2016),
86 considering the potential influence of pathogen interactions on current and future disease
87 distributions (Cattadori, Boag & Hudson 2008; Jolles *et al.* 2008; Budischak *et al.* 2015; Abbate *et*
88 *al.* 2018).

89 Rodent communities are relevant models for developing this community ecology approach to
90 disease distribution and emergence. They harbor a wide variety of pathogenic taxa (e.g., Bordes *et*
91 *al.* 2013; Pilosof *et al.* 2015; Koskela *et al.* 2016; Diagne *et al.* 2017) and are important reservoir
92 hosts of agents of zoonoses that have severe implications for human health. Han et al. (2015) have
93 revealed that about 10% of the 2277 extant rodent species are reservoirs of 66 agents of zoonoses,
94 including viruses, bacteria, fungi, helminths, and protozoa. They also described 79 hyper-reservoir
95 rodent species that could be infected by multiple zoonotic agents. Strong ecological interactions
96 have been shown in wild rodent populations among some of these zoonotic agents (Telfer *et al.*
97 2010), as well as between non-zoonotic agents and zoonotic agents (e.g., helminthes and bacteria
98 (Carvalho-Pereira *et al.* 2019); helminthes and viruses (Guivier *et al.* 2014; Sweeny *et al.* 2020);

99 helminthes and protozoa (Knowles *et al.* 2013)). In addition, rodents share a number of habitats
100 with humans, including urban settings, agricultural lands, and forests, providing opportunities for
101 human-rodent contact and pathogen transmission (Davis, Calvet & Leirs, 2005). Describing the
102 distribution and composition of natural pathogen communities in rodent populations, and
103 determining the drivers behind pathogen associations, is imperative for understanding the risks they
104 may pose for public health.

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

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

2. Materials and methods

2.1. Study area and host sampling

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

151 three additional host species (one cricetid one echimyid and one murid) were also found in these
152 communities, but excluded from analyses due to their rarity; these rare (non-focal) hosts and their
153 pathogens are described in Supplemental Materials Appendix 1.
154
155 *2.2. Detecting virus exposure and bacterial infection*
156 Among the 713 rodents sampled for this study, indirect fluorescent antibody tests (IFATs; see for
157 details (Kallio-Kokko *et al.* 2006)) were successfully performed on 677 animals to detect
158 immunoglobulin G (IgG) specific to or cross-reacting with cowpox virus (CPXV, *Orthopoxvirus*),
159 Puumala or Dobrava-Belgrade virus (respectively PUUV and DOBV, *Orthohantavirus*, collectively
160 referred to henceforth as "hantavirus"), lymphocytic choriomeningitis virus (LCMV,
161 *Mammarenavirus*), and Tick-borne encephalitis virus (TBEV, *Flavivirus*). We refer to these
162 antiviral antibody tests as indicating a history of past exposure, but antibodies against hantavirus
163 and LCMV also likely indicate continued chronic infection. In contrast, current or very recent
164 exposure to bacterial infection was tested via 16S rRNA gene amplicon sequencing of splenic
165 tissue, giving no indication of past exposure history. The spleen was chosen because this organ is
166 known to filter microbial cells in mammals, allowing the detection of a wide array of pathogenic
167 and zoonotic agents. Funding was available to test for bacteria in just half of the animals, chosen
168 haphazardly to equally represent all host species, study sites and years, resulting in successful
169 analysis for 332 rodents (see Figure 1 for a breakdown of number of individuals sampled per focal
170 host species). For each individual animal, the DNA from splenic tissue was extracted using the
171 DNeasy Blood & Tissue kit (Qiagen) following the manufacturer recommendations. Each DNA
172 extraction was analyzed twice independently. We followed the method described in Galan *et al.*
173 (2016) to perform PCR amplification, indexing, pooling, multiplexing, de-multiplexing, taxonomic
174 identification using the SILVA SSU Ref NR 119 database as a reference (http://www.arb-
175 silva.de/projects/ssu-ref-nr/). Briefly, DNA samples were amplified by PCR using universal primers
176 targeting the hyper variable region V4 of the 16S rRNA gene (251 bp) and sequencing via Illumina

177 MiSeq. The V4 region has been proven to have reasonable taxonomic resolution at the genus level
178 (Claesson *et al.* 2010). A multiplexing strategy enabled the identification of bacterial genera in each
179 individual sample (Kozich *et al.* 2013). Data filtering was performed as described in Galan *et al*.
180 (2016) to determine presence/absence of bacterial infections (summarized in Figure S3). Briefly, we
181 discarded all bacterial OTUs containing fewer than 50 reads in the entire dataset and animals for
182 which one or both individual PCR samples produced fewer than 500 reads. A bacterial OTU was
183 considered present in an animal if the two independent PCR samples were both above a threshold
184 number of reads, defined as the greater of either 0.012% of the total number of reads in the run for
185 that OTU (i.e., filtering using the rate of indexing leak) or the maximum number of reads for that
186 OTU in any negative control sample (i.e., filtering using the presence of reads in the negative
187 controls due to contaminations) (Galan *et al.* 2016). We removed chimera using the *Uchime*
188 program implemented in *mothur*, and manually checked OTUs representing suspected chimera not
189 identified by the program. For each OTU suspected as pathogenic, Basic Local Alignment Search
190 Tool (BLAST) searches of the most common sequences were conducted to infer species identity
191 where possible. OTUs with at least 500 reads across all animals in the dataset were considered
192 reliably detectable, allowing us to assign absent status to these OTUs in animals failing to meet the
193 criteria for OTU presence. Only OTUs for which there were at least 500 reads across all animals in
194 the dataset (for which present and absent statuses could be assigned), and where reasonable
195 certainty of pathogenicity could be established from the literature, were considered in analyses of
196 the pathogen community.
197
198 *2.3. Statistical Methods*
199 All statistical analyses were implemented in R version 3.2.2 (R Core Team 2015). Throughout
200 our analyses, we refer to simultaneous bacterial infections as "co-infection", while analyses
201 involving simultaneous presence of antiviral antibodies and bacterial infection are referred to as
202 evaluating "co-exposure". While we can only be sure that antiviral antibodies represent past

203 exposure, we cannot rule out simultaneous viral and bacterial "co-infection", particularly for viruses
204 known to cause chronic infections. Likewise, detection of current (or very recent) bacterial
205 infection, particularly for taxa known to cause chronic infections, cannot tell us how long the
206 animal has carried the infection. Thus, we refrain from assuming sequence of infection for statistical
207 tests unless specified by an *a priori* hypothesis from the literature.

208

209 *2.3.1. Testing for extrinsic drivers of pathogen community composition across the rodent*
210 *community*

211 We analyzed pathogen community composition across the whole rodent community. We use the
212 term "pathogen community" to refer to the group of viruses and pathogenic bacteria for which we
213 had the means to include, which was not exhaustive; thus measures of diversity are to be considered
214 relative and not absolute. We first estimated pathogen community richness using the Shannon
215 diversity index (alpha diversity) considering pathogenic bacterial OTUs and antiviral antibodies
216 found in each study year, study site, habitat, host species, host sex and host age group (default
217 options (natural logarithm Shannon index) in 'diversity' function from the *vegan* package). We
218 evaluated a linear regression model using analysis of deviance ('lm' and 'drop1' functions from the
219 basic *stats* package) to test for significance of differences in pathogen diversity due to the fixed
220 factors listed above after first correcting for all other factors in the model (marginal error tested
221 against the *F*-distribution). Post-hoc comparisons and correction for multiple tests were performed
222 using function 'TukeyHSD' from package *stats* and 'HSD.test' from package *agricolae* to group
223 factor levels that were not significantly different. The impact of host species diversity (Shannon
224 diversity with Chao's estimator correction using 'Shannon' in package *entropart*) on pathogen
225 species diversity was tested for by correlation ('cor.test', package *stats*) across each year x site x
226 habitat community.

227 We next tested for differences in pathogen community composition (beta diversity) between host
228 species, habitats, study sites, years, age groups, and sexes by applying a permutational multivariate
229 analysis of variance (PERMANOVA) on a Bray-Curtis dissimilarity matrix ('adonis2' function in

230 the *vegan* package). To explore how intrinsic factors (pathogen-pathogen associations) contributed
231 to the structure of the pathogen community, we used multiple correspondence analysis (MCA) to
232 reduce variance in presence/absence of each bacterial pathogen species and antiviral antibody,
233 implemented with the function 'MCA' in the *FactoMinR* package and visualization tools found in
234 the *factoextra* package. This produces a set of quantitative and orthogonal descriptors (dimensions)
235 describing the pathogen community composition, revealing correlated variables. With each MCA
236 dimension as a continuous dependent response variable, we then evaluated linear regression models
237 using analysis of deviance with post-hoc comparisons (as detailed above) to understand how the
238 variation described by each MCA dimension was influenced by the extrinsic factors.
239
240 *2.3.2. Testing for associations between co-circulating pathogens*
241 Because we identified a large number of pathogens, the number of potential association
242 combinations to consider was excessively high, especially with regard to the relatively small
243 number of rodents sampled. We therefore decided to test the significance only of those
244 associations (i) clearly suggested by the community-wide MCA or (ii) previously described in
245 the literature: positive association between *Bartonella* spp. and CPXV (Telfer *et al.* 2010), positive
246 associations between *Mycoplasma* species (Sykes *et al.* 2008; Tagawa *et al.* 2012; Fettweis *et al.*
247 2014; Volokhov *et al.* 2017), and both positive (Kedem *et al.* 2014; Eidelman *et al.* 2019) and
248 negative (Cohen, Einav & Hawlena 2015) associations between *Bartonella* spp. and hemotropic
249 *Mycoplasma* species. Given the *a priori* assumption that associations would differ between host
250 species, we analyzed each host species separately; where evidence suggested no significant
251 differences between host species (non-significant variation in the MCA dimension among host
252 species or non-significant host species identity x explanatory pathogen term in logistic regressions),
253 we pooled individuals into a single analysis to gain statistical power.
254 We tested the significance of each association using both association screening (SCN) analysis
255 (Vaumourin *et al.* 2014) and multiple logistic regression analysis (GLMs, modeling the binomial

256 ‘presence/absence’ status of each pathogen as a function of the occurrence of other pathogens) on
257 the subset of host species in which the pathogens were found to circulate. We first performed SCN
258 analysis, as this approach is among the most suitable for detecting pathogen associations in cross-
259 sectional studies (Vaumourin *et al*. 2014). Briefly, given the prevalence of each pathogen species in
260 the study population, SCN analysis generates a simulation-based 95% confidence envelope around
261 the expected frequency of each possible combination of concurrent infection status (a total of 2^{NP}
262 combinations, where NP = the number of pathogen species) under the null hypothesis of random
263 pathogen associations. Observed frequencies of co-infection combinations falling above or below
264 this envelope are considered to occur more or less frequently, respectively, than in 95% of the
265 random simulations. Significance of the association is given as a *p*-value, calculated as the number
266 of instances in which the simulated co-infection frequency differed (above or below the upper or
267 lower threshold, respectively) from the observed frequency divided by the total number of
268 simulations (Vaumourin *et al.* 2014).

269 The benefit of the SCN approach is a relatively high level of statistical power and the ability to
270 identify precisely which combinations of pathogens occur outside the random expectations
271 (Vaumourin *et al*. (2014)). However, the SCN is sensitive to heterogeneity in the data due to
272 extrinsic factors (e.g., host specificity, or structuring in space, time, age or sex), which can both
273 create and mask true associations. A multiple logistic regression (GLM) approach was thus also
274 systematically employed, as it has the benefit of explicitly taking into account potentially
275 confounding extrinsic factors. Binomial exposure (presence/absence of either bacterial infection or
276 antiviral antibodies) to a single pathogen was set as the dependent variable with exposure to the
277 hypothetically associated pathogen(s) treated as independent explanatory variable(s) and extrinsic
278 factors (host sex, host age, study year, study site, and where appropriate, habitat) were specified as
279 covariates using function ‘glm’ in the *stats* package with a binomial logit link. When the multiple
280 host species were involved, we tested an interaction term (host species identity x explanatory
281 pathogen), and either (if $p < 0.05$) performed separate analyses for each host species or (if $p \geq 0.05$)

282 simply added host species identity as another covariate in the model. When there was no *a priori*
283 assumption concerning timing of exposure (e.g., antiviral antibody presence is more likely to affect
284 current acute bacterial infection than the reverse), the occurrence of each pathogen involved in a
285 given association was set as the dependent variable in reciprocal GLMs. As for the MCA
286 dimensions above, statistical significance of the association was assessed after first correcting for all
287 covariates in the model using the 'drop1' function (-2 log likelihood ratio tests via single-term
288 deletions compared to the full model). Despite a large number of *a priori* hypotheses, we regarded a
289 p-value of < 0.05 as significant due to the very low number of individuals of each host species
290 sampled. Though conceivably important, we also did not have sufficient power to test for additional
291 interaction terms.
292
293 *2.3.3. Evaluating false discovery*
294 Given the large number of significance tests performed on this single dataset, we compiled all
295 relevant p-values (N=77) and applied a Benjamini-Hochberg correction procedure to estimate how
296 many of the significant results may fall within the false discovery zone (using function 'p.adjust' in
297 the *stats* package). Among tests of positively correlated hypotheses (e.g., pairwise tests of intrinsic
298 pathogen-pathogen associations N=20), only one of the p-values from the pair was included in
299 calculating the false discovery rate (selected randomly). SCN analysis results were not included,
300 because they were also expected to be positively correlated with the logistic regressions, and
301 because the method inherently performs correction for multiple tests. Between hypotheses, the data
302 were often composed of different non-overlapping subsets of varying sizes, and sample sizes varied
303 widely with some being very small. Thus, application of this procedure likely indicates a
304 conservative (low) estimate for how many null hypotheses should truly be rejected.
305
306 **3. Results**
307 *3.1. Taxonomic identification and prevalence of pathogens*

308 *3.1.1. Viral exposure*

309 The most abundant virus detected was CPXV, with 222 (32.8%) positive sera of the 677 animals
310 tested for anti-CPXV antibodies. It was detected in all focal host species. However, significant
311 variation in prevalence was observed between focal host species (highly prevalent (43-70%) in *Ar.*
312 *scherman*, *Mi. agrestis*, and *My. glareolus*; Figure 1; $\chi^2 = 119.5$, $df = 6$, $p < 10^{-15}$). Anti-hantavirus
313 antibodies were detected in 16 animals (2.4%), and were significantly structured among host
314 species (with exposure highest in *Mi. arvalis* (9.7%), *R. norvegicus* (3.3%) and *My. glareolus*
315 (3.1%); Figure 1; $\chi^2 = 19.4$, $df = 6$, $p = 0.0036$). Anti-LCMV antibodies were detected in two *Mi.*
316 *arvalis* individuals (Figure 1). No animals were positive for anti-TBE antibodies.

317

318 *3.1.2. Bacterial pathogens*

319 Out of 952 bacterial OTUs represented by at least 50 reads in the dataset, 498 were considered
320 positive in at least one animal after data filtering (presented in Supplemental Materials Table S1).
321 Two OTUs (00024 & 00037) identified as *Bartonella* with low bootstrap values (74 and 92
322 respectively) appeared to represent chimeric sequences between the two highly amplified genera
323 (*Bartonella* and *Mycoplasma*) in co-infected samples. Two OTUs (00009 & 00117) which were
324 unclassified but which had a large number of reads in positive animals were also found to represent
325 chimeric sequences between the two genera, despite high bootstrap values (100). Three additional
326 chimeric *Mycoplasma* OTUs with under 500 reads were also excluded (OTUs 00076, 00159, and
327 00316). Two OTUs (00002 & 00059) were found to be redundant with OTUs Myco1 and Myco3,
328 respectively, and two more (00134 & 00220) were chimera between Myco OTUs. These 11 OTUs
329 were manually removed from the database, and are not included in Table S1.

330 We identified 43 OTUs belonging to bacterial genera with members known or thought to be
331 pathogenic in mammals (Table 1). After BLAST queries, we found 16 of these OTUs (representing
332 7 distinct genera) which could be considered as reliably detectable pathogens in the focal host
333 species (Figure 1). An additional 24 OTUs were considered potentially pathogenic but excluded

334 from analyses because they were only observed in rare host species, because presence-absence
335 could not be reliably established due to a low total number of reads (<500 in the dataset, e.g.,
336 *Borrelia* spp. and *Leptospira* spp.), because we could not rule out contamination by natural sources
337 of non-pathogenic flora during dissection (e.g., *Helicobacter* spp., *Streptococcus* spp.) or by known
338 contaminants of sequencing reagents (e.g., *Williamsia* spp.; (Salter *et al.* 2014)), or because their
339 identity to a pathogenic species was uncertain due to insufficient genetic variation at the 16S rRNA
340 locus (e.g., *Yersinia* spp.) (Table 1). We also identified three OTUs belonging to the eukaryotic
341 family Sarcocystidae (98% sequence similarity to the coccidian parasite *Sarcocystis muris*); though
342 each OTU was represented by >500 reads, there are currently no data on the reliability of this
343 method for detection (Table 1). Individual infection status for each of these OTUs is given in Table
344 S2.

345 The 16 reliably detectable pathogenic OTUs included *Bartonella* spp., 10 *Mycoplasma* spp.
346 OTUs, *Rickettsia canadensis*, "*Candidatus* Neoehrlichia mikurensis", *Orientia* spp., *Brevinema*
347 *andersonii*, and *Spiroplasma* spp. Phylogenetic analysis including published sequences from
348 BLAST queries revealed that the 10 *Mycoplasma* spp. OTUs belonged to three distinct species:
349 *Myco. haemomuris* (Myco1-3,5,7-9), *Myco. coccoides* (Myco4 and Mco6), and "*Candidatus* Myco.
350 ravipulmonis" (Myco10) (Figure S4). In general, these bacterial infections were present in all but 30
351 of the 332 animals tested (91.0% prevalent), and were not concentrated in any particular focal host
352 species (χ^2 = 9.7, df = 6, p = 0.139). Prevalence of each pathogen in each focal host species is
353 presented in Figure 1.

354

355 *3.2. Extrinsic drivers of pathogen community diversity and composition within rodent community*

356 *3.2.1. Analyses of pathogen diversity*

357 We found evidence for the co-circulation of between 3 (in *Mi. agrestis*) and 12 (in *My.*
358 *glareolus*) pathogen taxa per host species across the rodent community (Figure 1). Using multiple
359 regression analysis on the Shannon diversity index, we found that marginal mean pathogen diversity

differed significantly between 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 adults than in juveniles ($F_{1,69} = 21.43$, $p < 10^{-4}$). Pathogen diversity did not, however, differ between study sites, years, or host sexes (Figure 2, Table S3). Post-hoc Tukey tests showed that after correcting for all other factors in the model, meadow habitats had higher diversity of pathogen exposure than forest habitats, and the diversity of

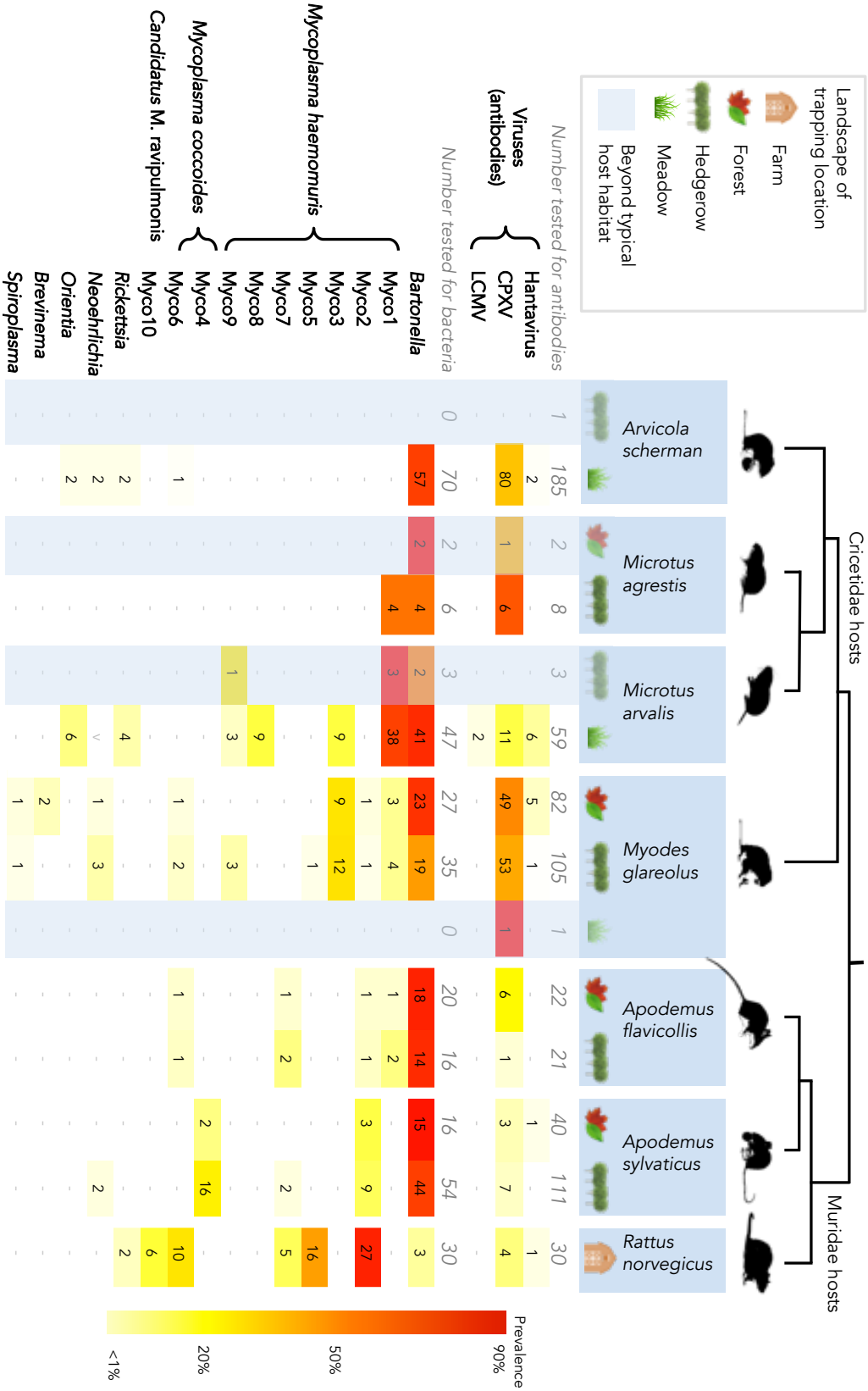
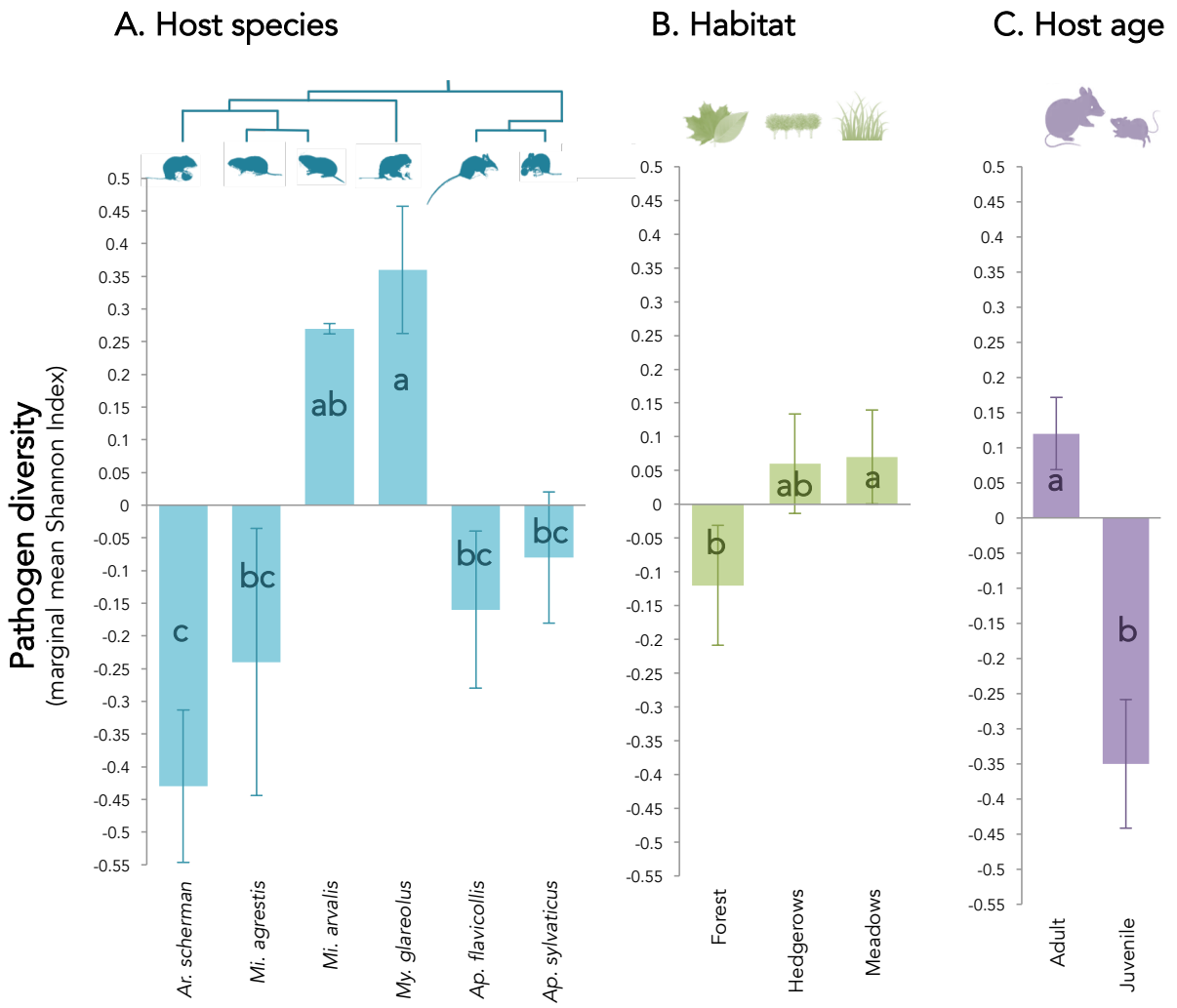


Figure 1. Pathogen occurrence across the rodent species community.

366 pathogen communities in host species fell along a continuum between *My. glareolus* (high) and *Ar.*

367 *scherman* (low) extremes (Figure 2, Table S3). Host species diversity in each community (year x

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.



368 site x habitat) was positively correlated with pathogen diversity ($r = 0.62$, $t = 2.5$, df = 10, $p =$

369 0.032).

370 To understand the relative pathogen diversity of *R. norvegicus* hosts, excluded from the model

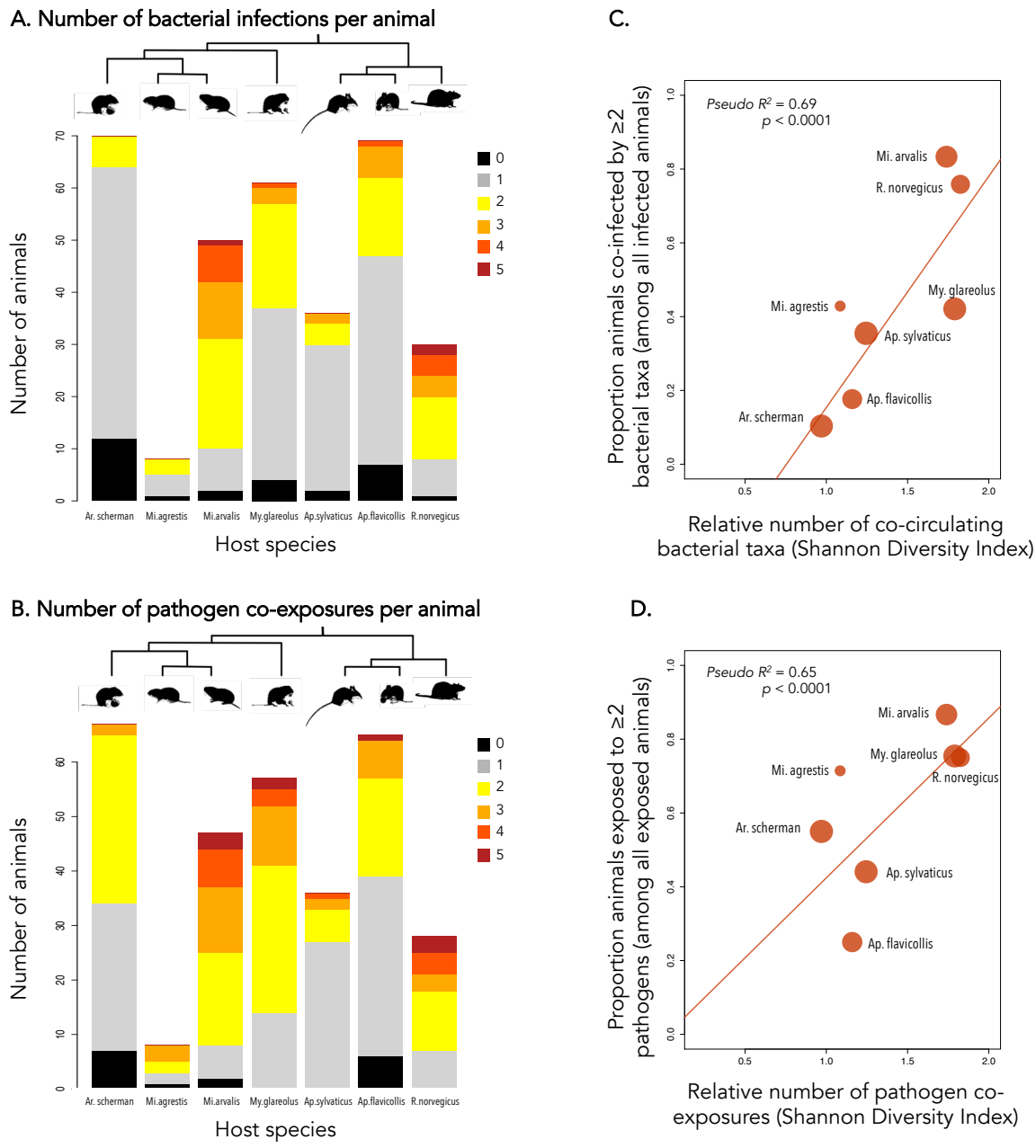
371 because they were entirely confounded with farm habitats, we analyzed two additional modified

372 models: one excluding host habitat and the other excluding host species. Post-hoc Tukey tests from

373 these models respectively showed that *R. norvegicus* hosts had the second most diverse pathogen

community, and that farm habitats were thus in the same high-diversity category as meadow habitats (Table S4).

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



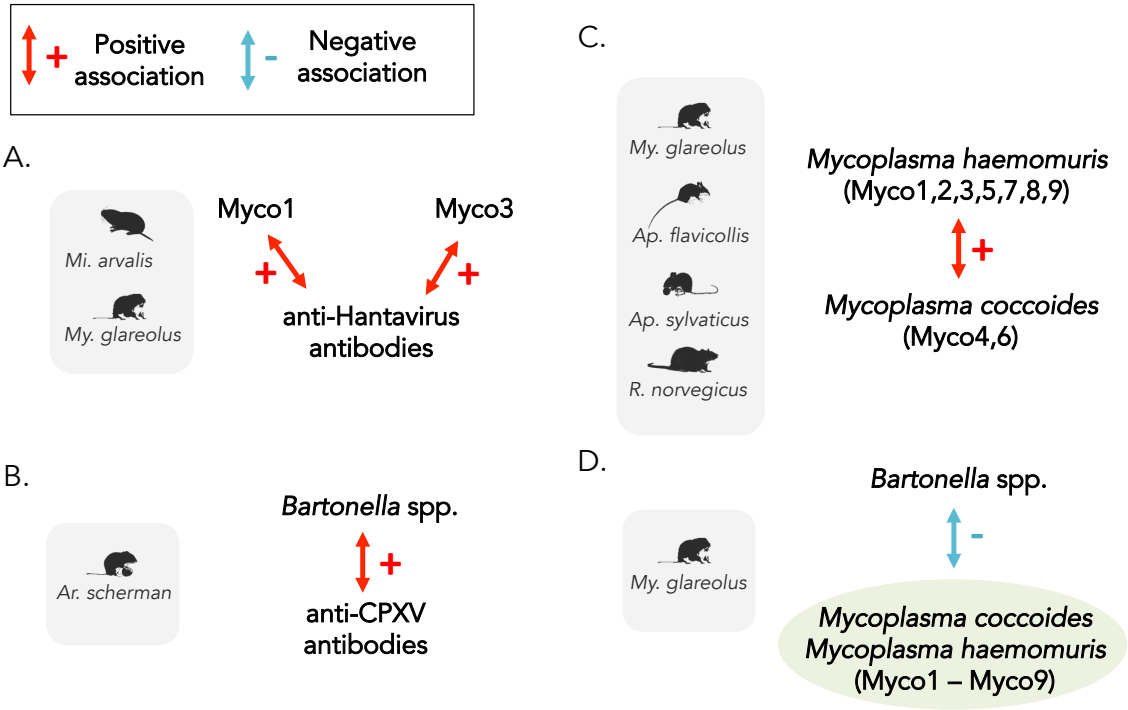
We also found an enormous amount of both bacterial co-infections and concurrent history of viral exposures (Figures 3A, 3B). The percentage of animals co-infected with two or more reliably detectable pathogenic bacterial OTUs among all those infected in each host species ranged between 84.4% (in *Mi.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

382 3C; analysis of deviance *Pseudo-*R^2 = 0.69, $p < 10^{-11}$, calculated using logistic regression weighted
383 by the number of infected animals per species). Bacterial co-infections were more frequent than
384 expected in *Mi. agrestis*, and less frequent than expected in *My. glareolus* (according to Cook's
385 Distance, Figure S5A). Results were similar when co-occurrence of antiviral antibodies was
386 considered along with bacterial OTU exposure (Figure 3D; *Pseudo-*R^2 = 0.65, $p < 10^{-7}$). While *Mi.*
387 *arvalis* had both more bacterial co-infections and slightly more pathogen co-exposures than
388 expected based on pathogen diversity, other outliers differed between the two measures (Figure
389 S5B): *My. glareolus* co-exposure frequencies were not lower than expected, and both *Apodemus*
390 species had lower than expected co-exposures. Host species diversity in each community did not
391 correlate with bacterial co-infection (r = 0.33, t = 1.11, df = 10, p = 0.29) or pathogen co-exposure
392 (r = 0.022, t = 0.071, df = 10, p = 0.94) frequencies.
393
394 *3.2.2. Analyses of pathogen community composition*
395 Many pathogen taxa were found only in a single host species (*Mycoplasma haemomuris* OTU
396 Myco8, "*Candidatus* Mycoplasma ravipulmonis" (Myco10), *Brevinema* spp., *Spiroplasma* spp.,
397 LCMV), and each host species had a unique combination of co-circulating pathogens (Figure 1). In
398 order to best identify extrinsic and intrinsic factors potentially driving the composition of pathogen
399 communities within the rodent community, we reduced the dataset to limit biases. We excluded *R.*
400 *norvegicus* individuals due to competing *a priori* hypotheses that host species and habitat would be
401 important factors (as this host species was the only one found in farm habitats, confounding these
402 two variables; but see MCA results when *R. norvegicus* was included in Figures S6, S7). Likewise,
403 we excluded pathogens that occurred only in one habitat type of one host species (not including *R.*
404 *norvegicus*). Two additional individuals were excluded due to missing sex and age information.
405 Analyses were performed on the remaining 280 individuals from six host species and their 14
406 pathogens (*Bartonella*, Myco1, Myco2, Myco3, Myco4, Myco6, Myco7, Myco9, *Rickettsia*,
407 *Neoehrlichia*, *Orientia*, *Spiroplasma*, and antibodies against CPXV and hantaviruses) (Figure S8).

408 Overall, pathogen species composition was significantly structured by host species identity
409 ($F_{5,252}$ = 16.23, p = 0.001) and habitat type ($F_{2,252}$ = 2.51, p = 0.024; Table S5). Out of 14
410 orthogonal dimensions returned by the MCA, the first two captured 23.0% of the variation in
411 pathogen and antibody occurrence, and the first seven explained a cumulative 63.4% of the total
412 variance (Figure S9). Further dimensions captured less variance than would be expected if all
413 dimensions contributed equally to overall inertia in the data. Dimension 1 (MCA Dim1; explaining
414 13.1% of the variation in pathogen community and loading heavily with the presence of Myco1,
415 Myco3 and anti-hantavirus antibodies) differed significantly between host species ($F_{5,268}$ = 23.83, p
416 < 0.0001) and age classes ($F_{1,268}$ = 6.27, p = 0.013; Table S6). Dimension 2 (MCA Dim2;
417 explaining 9.9% of the variation in pathogen community and primarily describing the occurrence of
418 *Bartonella*) was also structured significantly by host species ($F_{5,268}$ = 3.89, p = 0.002; Table S6).
419 While these first two dimensions varied by host species (Figure S10A) and host age class (Figure
420 S10B), variance in host habitats (Figure S10C) was not significant after accounting for the other
421 factors. Host species was the most consistently important extrinsic driver of pathogen community
422 composition, significantly explaining variation captured in six of the first seven dimensions, MCA
423 Dim1 – MCA Dim7 (except for MCA Dim 5; Table S6).
424
425 *3.3. Associations between pathogens*
426 *3.3.1. Validation of the associations detected by MCA*
427 We applied SCN and GLM analyses to further characterize patterns detected using MCA. Strong
428 and relatively equal loading of MCA Dim1 with Myco1, Myco3, and anti-hantavirus antibody
429 presence indicated that these three pathogens were positively associated with one-another. Indeed,
430 the six animals with anti-hantavirus antibodies were found exclusively in animals infected with
431 Myco3, and Myco1 was found in 2/3 of hantavirus-exposed animals but in just 1/3 of those without
432 hantavirus exposure. The MCA also revealed significant differences among host species and host
433 age classes for Dim 1; hantavirus and Myco3 only circulated in two host species (*Mi. arvalis* and

434 *My. glareolus*) and 34 of those 35 occurrences were in adults. To exclude positive associations
435 arising from mutual host specificity and age-related accumulation of exposure probability, we
436 focused our analyses on the dataset restricted to adults of the two host species in which all three
437 pathogens co-circulated (*Mi. arvalis* and *My. glareolus*). Individual SCN analyses performed on
438 adults of each host species revealed no associations (Table S6A). However, since values of MCA
439 Dim 1 did not differ between the two host species (according to post-hoc tests given in Table S6),
440 we also ran a single SCN analysis on the pooled data from adults of both species to improve
441 statistical power (Table S7A). According to this pooled SCN analysis, the three-way co-occurrence
442 of Myco1, Myco3 and anti-hantavirus antibodies was significantly more frequent than would be
443 expected by random chance (p = 0.008), with a trend for anti-hantavirus antibodies occurring by
444 themselves more rarely than expected (sitting on the lower bound at zero; p = 0.13; Table S7A,
445 Figure S11). We also investigated this association using GLM. However, given the small number of
446 hantavirus exposures and perfect association with Myco3 infection, there was insufficient statistical
447 power to explicitly test for an association between all three pathogens and extrinsic factors. We
448 therefore ran three reciprocal GLM models on the restricted dataset, one for each pathogen as a
449 function of extrinsic factors to control for heterogeneous host groups (host species, host sex, study
450 site, habitat, and year sampled) and exposure to the two other pathogens (Table S7B). These models
451 showed that there remained significant unexplained positive associations between hantavirus
452 exposure and Myco1 infection (anti-hantavirus antibodies ~ Myco1: χ^2 = 5.67, p = 0.017; Myco1 ~
453 anti-hantavirus antibodies: χ^2 = 2.89, p = 0.09) and between hantavirus exposure and Myco3
454 infection (Myco3 ~ anti-hantavirus antibodies: χ^2 = 12.11, p < 0.001), but that there was no
455 evidence of direct association between Myco1 and Myco3 infections (Myco1 ~ Myco3: χ^2 = 0.07, p
456 = 0.8; Myco3 ~ Myco1: χ^2 < 0.01, p = 0.96; Figure 4A).

Figure 4: 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.



The third MCA dimension also presented a clear hypothesis with sufficient statistical power to be tested. MCA Dim3 was characterized by co-variation in Myco2 (*Myco. haemomuris*) and Myco4 (*Myco. coccoides*) infections suggesting a positive association between members of these two *Mycoplasma* species. Myco2 and Myco4 OTUs co-circulated only in *Ap. sylvaticus* hosts, thus we limited our analysis to this host species. There was no significant association between the two OTUs detected by SCN analysis (Table S8A), and after correcting for all extrinsic factors, there remained only a non-significant trend (Myco2 ~ Myco4: $\chi^2 = 2.47$, p = 0.12; Myco4 ~ Myco2: $\chi^2 = 2.47$, p = 0.12; Table S8B) for a positive association between the two OTUs. No additional associations with sufficient variance for statistical tests were clearly suggested by the MCA analysis.

471 *3.3.2. Validation of associations described in the literature*

472 We tested the *a priori* hypothesis that seropositivity to CPXV would be positively associated
473 with *Bartonella* infection, previously detected in *Mi. agrestis* (Telfer *et al.* 2010). The whole dataset
474 was considered as these two pathogens co-circulated in all host species (Figure 1). SCN analyses
475 performed independently for each host species revealed no associations (Table S9A), and the MCA
476 results suggested that pooling data across host species would be inappropriate. After correcting for
477 extrinsic factors using GLM, we found reciprocal evidence for a positive association in *Ar.*
478 *scherman* hosts (*Bartonella* ~ anti-CPXV antibodies: $\chi^2 = 5.37$, $p = 0.020$; anti-CPXV antibodies ~
479 *Bartonella*: $\chi^2 = 5.21$, $p = 0.022$; Figure 4B), but not in any other host species (Table S9B). It is of
480 note that there were only eight *Mi. agrestis* individuals, rendering statistical power to test for the
481 association while controlling for extrinsic factors insufficient in this host species where the
482 association was previously described. While prevalence of both pathogens in *Mi. agrestis* was
483 relatively high compared to other host species, one of the two animals without *Bartonella* infection
484 was positive for anti-CPXV antibodies, also precluding evidence for a within-species trend.

485 We next focused on the potential associations between OTUs identified as belonging to two
486 different species of hemotropic *Mycoplasma, Myco. haemomuris* (HM) and *Myco. coccoides* (HC),
487 within the four host species in which they both circulated (*My. glareolus*, *Ap. flavicollis*, *Ap.*
488 *sylvaticus*, *R. norvegicus*; Figure 1; Figure S4). We found no significant associations using
489 independent SCN analyses for each host species (Table S10A). However, after controlling for
490 extrinsic factors using GLM, a significant positive association was detected (HM ~ HC: $\chi^2 = 9.5$, p
491 $= 0.0021$; HC ~ HM: $\chi^2 = 9.59$, $p = 0.002$), and did not differ between host species (non-significant
492 interaction term between host species by explanatory pathogen occurrence in each reciprocal model,
493 Table S10B; Figure 4C). We note that only one *R. norvegicus* animal was uninfected with *Myco.*
494 *haemomuris*, and that animal also had no *Myco. coccoides* infection; thus the trend for the
495 association in this host species was also positive but lacked sufficient variance for independent
496 statistical analysis.

497 Finally, we tested for associations between *Bartonella* spp. and hemotropic *Mycoplasma* species,
498 grouping the occurrence of different OTUs of the latter (Myco1 – Myco9) into a single presence-
499 absence variable. There was no association detected by SCN analyses (Table S11A), and marginal
500 evidence that any association may differ by host species after correcting for extrinsic factors using
501 GLM (Table S11B). After controlling for extrinsic factors using independent GLMs for each host
502 (where possible), we found a negative association between the two pathogen groups only in *My.*
503 *glareolus* hosts (*Bartonella* ~ *Mycoplasma*: $\chi^2 = 5.73$, $p = 0.017$; *Mycoplasma* ~ *Bartonella*: $\chi^2 =$
504 5.65, $p = 0.017$, Table S11B, Figure 4D).
505
506 *3.4. Evaluating false discovery*
507 Benjamini-Hochberg correction of p-values from hypothesis tests throughout the study suggested
508 that those above ~0.01 may lie above the false discovery cutoff for statistical significance (Figure
509 S12), and that null hypotheses rejected with smaller p-values have been rejected with confidence.
510

511 **4. Discussion**

512 Rodents have long been recognized as important reservoirs of infectious agents, with a high
513 transmission potential to humans and domestic animals (Kruse, Kirkemo & Handeland 2004).
514 Europe is identified as a hotspot of rodent reservoir diversity and one third of rodent species are
515 considered hyper-reservoirs, carrying up to 11 zoonotic agents (Han *et al.* 2015). Nevertheless,
516 associations between these pathogens have still only rarely been investigated (but see, for example,
517 studies from field voles in the UK (Telfer *et al.* 2010) and in Poland (Pawelczyk *et al.* 2004),
518 gerbils in Israel (Cohen *et al.* 2015), across a rodent community in North America (Dallas *et al.*
519 2019), and co-infection frequencies of zoonotic pathogens from rodents in Croatia (Tadin *et al.*
520 2012)).
521 In this study, we confirmed that rodent communities in northern France may harbor a large
522 diversity of potential zoonotic pathogens, with at least 10 bacterial genera and antibodies against at

523 least four genera of viruses. Some of these pathogens have already been reported in the study region
524 or in geographic proximity, including viruses (*Orthohantavirus*, *Orthopoxvirus*, *Mammarenavirus*
525 (Charbonnel *et al.* 2008; Salvador *et al.* 2011)) and bacteria (e.g., *Bartonella, Mycoplasma,*
526 *Rickettsia, "Candidatus* Neoehrlichia*", Orientia, Spiroplasma, Treponema, Leptospira, Borrelia,*
527 *Neisseria, Pasteurella*; see (Vayssier-Taussat *et al.* 2012; Razzauti *et al.* 2015)). A previously
528 undetected relative of the putatively pathogenic spirochaete *Brevinema andersonii* that infects
529 short-tailed shrews and white-footed mice in North America (Defosse *et al.* 1995) was among our
530 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
532 between the anti-PUUV antibodies used in our assay and those elicited against the related *Tula*
533 *orthohantavirus* (TULA) virus common to European voles (Deter *et al.* 2007; Tegshduuren *et al.*
534 2010).

535 Three zoonotic pathogens were particularly prevalent: *Orthopoxvirus*, *Bartonella* spp., and
536 *Mycoplasma* spp. The wide range of hosts with anti-*Orthopoxvirus* antibodies corroborates prior
537 evidence that cowpox virus could be widespread in European rodents, particularly voles (Bennett *et*
538 *al.* 1997; Essbauer, Pfeffer & Meyer 2010; Forbes *et al.* 2014). An astounding 77% of all
539 individuals in the study were infected by *Bartonella* spp., a diverse group of hemotrophs known to
540 commonly infect rodents and other mammals (Breitschwerdt & Kordick 2000; Bai *et al.* 2009) and
541 which have also been implicated in both zoonotic and human-specific disease (Iralu *et al.* 2006;
542 Breitschwerdt 2014; Vayssier-Taussat *et al.* 2016). We could not assess the specific diversity of
543 *Bartonella* spp. circulating in these rodent communities because accurate resolution in this genus
544 requires additional genetic markers (Matar *et al.* 1999; Guy *et al.* 2013). Hemotropic and
545 pneumotropic *Mycoplasma* spp. were also highly prevalent across all host species, though
546 surprisingly lower than expected in *Ar. scherman* (Villette *et al.* 2017). These *Mycoplasma* species
547 are also known pathogens of humans and rodents (Harwick, Kalmanson & Guze 1972; Baker 1998).
548 Here, we found to two distinct hemotropic *Mycoplasma* species (*Myco. haemomuris* and *Myco.*

549 *coccoides*) and the pneumotropic *Mycoplasma* species *Myco. pulmonis* and "*Candidatus* Myco.
550 ravipulmonis". The former two are both hemotropic mycoplasmas responsible for vector-
551 transmitted infectious anaemia of wild mice, rats, and other rodent species (Neimark *et al.* 2001,
552 2005; Messick 2004). In contrast, *Myco. pulmonis* and "*Candidatus* Myco. ravipulmonis" cause
553 respiratory infections, are more closely related to other pneumotropic mycoplasmas, and
554 "*Candidatus* Myco. ravipulmonis" has only ever before been described in laboratory mice (formerly
555 termed Grey Lung virus (Andrews & Glover 1945; Neimark, Mitchelmore & Leach 1998; Graham
556 & Schoeb 2011; Piasecki, Chrzastek & Kasprzykowska 2017)).

557 Our results also corroborated the status of hyper-reservoir (more than two zoonotic pathogens
558 carried by a reservoir species) for all seven of the focal rodent species studied here (Han *et al.*
559 2015). Even the rare host species *Mi. subterraneous* also carried two potentially zoonotic pathogens
560 (*Bartonella* spp. and *Brevinema* spp.; Appendix 1). Overall, we found a high variability in the
561 number of pathogens circulating in each species despite correction for sampling effort, with low
562 levels observed in *Apodemus* species and *Arvicola scherman*, and high levels detected in *Mi.*
563 *arvalis, My. glareolus*, and *R. norvegicus*. While physiology, genetics, and behavior can contribute
564 to the number of pathogen species able to infect a given host species, larger geographic range size is
565 highly correlated with higher pathogen species diversity (Morand 2015); this explanation matches
566 the pattern among hosts in the communities sampled here (i.e., *Ar. scherman* and *Apodemus* spp.
567 have small geographic ranges compared to those of *Mi. arvalis*, My. *glareolus*, and *R. norvegicus*).

568 Several studies have emphasized the influence of host habitat specialization on parasite species
569 richness, low habitat specialization being associated with both high species richness of macro- and
570 micro-parasites (e.g., (Morand & Bordes 2015)). Our results did not fully corroborate this
571 association; while the grassland-specific *Ar. scherman* had the lowest pathogen diversity and the
572 multi-habitat spanning *My. glareolus* had the highest pathogen diversity, entirely farm-dwelling *R.*
573 *norvegicus* had high pathogen diversity nearly equal to that of *My. glareolus*, and the two
574 *Apodemus* hosts (neither with significantly higher pathogen diversity than *Ar. scherman*) were

575 found across both meadows and hedgerows. Instead, we found that more diverse host species
576 communities hosted more diverse pathogen communities. However, the implications of that result
577 are complex because while exposure to diverse (i.e., potentially novel) pathogens is a risk for
578 disease emergence, diverse host species communities are thought to keep individual pathogen
579 prevalence low due to the dilution effect – which should limit risk of zoonoses (Keesing *et al.*
580 2010).

581 The search for factors that drive parasite species richness, diversity and community composition
582 has been at the core of numerous studies (Poulin 1995; Poulin & Morand 2000; Nunn *et al.* 2003;
583 Mouillot *et al.* 2005; Krasnov *et al.* 2010; Sallinen *et al.* 2020). Here, we emphasized that both
584 pathogen diversity and community composition was mainly structured by host species identity,
585 despite both shared habitats and shared pathogen taxa. Pathogen beta diversity was also structured
586 by habitat, which could result from particular environmental suitability (e.g., for vectors) or
587 opportunities for cross-species transmission. We found no evidence that any specific pathogen-
588 pathogen associations were likely to be as important as host species identity in determining
589 pathogen distributions across the community of rodents. The strong influence of host characteristics
590 (Cohen *et al.* 2015) and host species identity (Dallas *et al.* 2019) on pathogen community
591 composition has recently been described in comparison to intrinsic pathogen-pathogen associations
592 in other rodents. Moreover, the pathogen community composition provided a unique signature for
593 each rodent species, even among those most closely related (e.g., *Ap. flavicolis* and *Ap. sylvaticus*).
594 This result is in line with the conclusions of meta-analyses showing that phylogeny, over other host
595 traits, has a minimal impact on pathogen diversity in rodent species (Luis *et al.* 2013; Guy *et al.*
596 2019).

597 The importance of host species identity in shaping pathogen community composition may not
598 stem from strict host-pathogen specificity, as most pathogens were found to infect multiple host
599 species – a broad result echoed across animal communities (Taylor *et al.* 2001; Woolhouse, Taylor
600 & Haydon 2001; Cleaveland, Laurenson & Taylor 2001; Pedersen *et al.* 2005; Streicker *et al.*

601 2013). However, we might be cautious as more precise molecular analyses are necessary to test
602 whether different species of a bacteria genus or divergent populations of the same bacteria species
603 may circulate independently in different rodent host species, with little or no transmission. For
604 example, two genera seemed to be largely shared among the rodent species studied here, *Bartonella*
605 and *Mycoplasma*. But previous studies have shown strong host-specificity when considering the
606 genetic variants of *Bartonella* (Buffet *et al.* 2013; Withenshaw *et al.* 2016; Brook *et al.* 2017)
607 Evidence in the literature for host specificity of *Mycoplasma* species has led to a mix of conclusions
608 (Pitcher & Nicholas 2005), as cases of cross-species transmission are commonly reported –
609 particularly in humans – despite a general consensus that most species are highly host-specific. We
610 found that some *Mycoplasma* taxa were dominant contributors to prevalence in a single host
611 species, and that when shared, they were shared with just a few other specific host species. Rare
612 infections in unexpected host species (e.g., Myco6 in *Ar. scherman* and Myco1 in *Ap. flavicollis*)
613 were represented by fewer sequence reads compared to positive samples in host species where they
614 were more prevalent, suggesting a low potential for amplification and sustained transmission from
615 these occasional hosts (Figure S4). On the other hand, while the Cricetidae appeared to be
616 susceptible only – with rare exception – to taxa within the *Myco. haemomuris* group, host species in
617 the Muridae family were susceptible to all three distinct *Mycoplasma* species detected. The biggest
618 exception to this pattern was that three of 62 *Myodes glareolus* (sister to all other sampled
619 Cricetidae in the study) animals were found to be infected by both hemotropic *Mycoplasma* species.
620 These results both support the observation that cross-species transmission naturally occurs among
621 wild rodents and suggest that the degree of host specificity may be driven by both host and
622 pathogen factors.

623 Concurrent exposure to multiple pathogens within individuals was also frequent, as high as 89 %
624 (in *Mi. arvalis* hosts), in line with recent studies that have shown that co-infections by multiple
625 pathogens are common in natural populations (e.g., in mammals, birds, amphibians, ticks, humans
626 (Telfer *et al.* 2010; Griffiths *et al.* 2011; Moutailler *et al.* 2016; Clark *et al.* 2016; Stutz *et al.*

627 2018)). Variation in the frequency of pathogen co-exposure was highly correlated to the diversity of
628 pathogens circulating in each host species, suggesting the dominance of a random process of
629 pathogen exposure for each individual. However, there were a few intriguing outliers: *My. glareolus*
630 hosts were less co-infected than expected based on diversity of bacterial taxa, but not when viral
631 antibodies were included; conversely, *Ar. scherman* hosts were more co-exposed when viruses were
632 considered, but not when only bacteria were considered; and *Mi. arvalis* hosts had consistently
633 higher proportions of co-exposures whether viruses were or were not considered along with
634 bacteria. The non-random grouping of pathogen exposures within individuals (as in *Mi. arvalis*)
635 may result from heterogeneity in extrinsic transmission, environmental, or susceptibility factors
636 (Cattadori *et al.* 2006; Swanson *et al.* 2006; Beldomenico *et al.* 2008; Fenton, Viney & Lello 2010;
637 Beldomenico & Begon 2010) or from intrinsic interactions between pathogens (e.g., facilitation
638 mediated by hosts immune response). Differences in the pattern of co-exposure frequencies when
639 including or excluding antiviral antibodies (as with *My. glareolus* and *Ar. scherman*) could result
640 from different mechanisms (e.g., bacterial manipulation of innate immunity (Diacovich & Gorvel
641 2010)) affecting pathogen community assemblage. However, a lack of deviance from the expected
642 co-exposure frequency does not exclude the possibility that both extrinsic and intrinsic processes
643 may be occurring.

644 We found evidence in support of three previously identified pathogen-pathogen associations
645 (positive association between *Myco. haemomuris* and *Myco. coccoides* infections; positive
646 association between *Bartonella* spp. infection and the presence of anti-CPXV antibodies; negative
647 association between *Bartonella* spp. and hemotropic *Mycoplasma* spp. infections), and
648 characterized one set of associations not previously described (positive associations between the
649 presence of anti-hantavirus antibodies and infections by two specific *Myco. haemomuris* OTUs) –
650 each in a unique subset of host species. *Mycoplasma* spp. blood infections are likely transmitted
651 through bites of blood-sucking arthropod vectors (Volokhov *et al.* 2017), meaning vectors could
652 prefer some individuals over others. Positive associations detected between *Myco. haemomuris* and

653 *Myco. coccoides* could also result from similarities in rodent susceptibility. Indeed *Mycoplasma*
654 spp. infection can lead to acute or chronic infection, and the establishment of chronic bacteremia
655 seems to occur in immunosuppressed or immunocompromised individuals (Cohen *et al.* 2018). Co-
656 infections with multiple *Mycoplasma* spp. might therefore be more likely to be detected in these
657 immunocompromised rodents with chronic infections. The existence of chronic infections might
658 also lead to additional co-infections and positive associations as a result of disease-induced changes
659 in population dynamics, immune system function, or through direct pathogen-pathogen interactions
660 (Fenton 2008; Aivelo & Norberg 2018; Fountain-Jones *et al.* 2019).

661 Whether through the accumulation of exposure probabilities or increased susceptibility, the
662 previously-undocumented positive association we found here between *Myco. haemomuris* OTUs
663 (Myco1 and Myco3) and anti-hantavirus antibodies may similarly be explained by the chronic
664 nature of both *Mycoplasma* spp. and hantavirus infections in rodents (e.g., for Puumala hantavirus
665 in bank voles (Yanagihara, Amyx & Gajdusek 1985; Meyer & Schmaljohn 2000; Vaheri *et al.*
666 2013)). This positive association was found in both host species where the majority of hantavirus
667 exposures occurred (*Microtus arvalis* and *Myodes glareolus*), consistent with the generality of
668 association between *Mycoplasma* species across host taxa detailed above, suggesting the intrinsic
669 ecology of these pathogens contributes to shaping variation in the pathogen community. Curiously,
670 we found no evidence for direct associations between OTUs of the same *Mycoplasma* species, thus
671 facilitation interactions are unlikely to explain the high diversity of *Mycoplasma* taxa both within
672 and between host species.

673 Infections by *Bartonella* species are also known to often result in subclinical and persistent
674 bacteremia in mammals, including rodents (Birtles *et al.* 2001; Kosoy *et al.* 2004). The positive
675 association detected in *Ar. scherman* between *Bartonella* spp. and anti-CPXV antibodies might
676 therefore be explained by, for example, joint accumulation of both chronic bacterial infections and
677 long-lived antiviral antibodies used to test for prior exposure to relatively short-lived CPXV
678 infections. However, if the same processes governing association of the chronic infections described

679 above were at play here, we would have expected to find both pathogens implicated in positive
680 associations (i) with other chronic infections, and (ii) across host species given their ubiquitous
681 prevalence. While the failure to recover the association in *Mi. agrestis* (previously described (Telfer
682 *et al.* 2010)) was likely due to low statistical power, the lack of a general pattern across other host
683 species despite adequate sampling suggests a more specific, and potentially immune-mediated,
684 ecological process between these two pathogens. Indeed, pox virus infections, including CPXV,
685 have been shown to induce immunomodulation that increases host susceptibility to other parasites
686 (Johnston & McFadden 2003). These interactions could be of variable intensities according to the
687 rodent species considered, due to potential differences in impacts of CPXV infection on immunity
688 across host species, or to the influence of other infections not examined here on host immune
689 responses during pox infections (e.g., helminths (Cattadori, Albert & Boag 2007), protozoa (Telfer
690 *et al.* 2010)). Furthermore, *Bartonella* spp. infection was negatively associated with *Mycoplasma*
691 spp. infections in *My. glareolus*, corroborating negative interactions reported in co-infection
692 experiments in gerbils (Eidelman *et al.* 2019). This association may therefore originate from an
693 interaction mediated by specific (immune) genetic features of *My. glareolus*, and not ecological
694 conditions as proposed by Eidelman et al. (2019). The antagonistic and host-specific nature of this
695 association lends further support to the interpretation that *Bartonella* spp. infections do not behave
696 in similar ways to other chronic infections in the community. However, few studies have
697 investigated the robustness of within-host interactions across different host species (e.g., (Lello *et*
698 *al.* 2018)), and this question deserves further investigation.

699 Our results suggest that intrinsic ecological interactions could help shape the composition of the
700 pathogen community within hosts. However, this suggestion provides only a hypothesis that
701 requires further investigation. Interpretation of associations can be misleading, as they may arise
702 from unmeasured co-factors such as exposure to shared transmission routes, and may even run
703 counter to the underlying ecological process (Fenton *et al.* 2014). The associations we found here
704 were not visible (or even misleading, in the case of a 3-way interaction between hantavirus, Myco1

705 and Myco3), for instance, when ignoring extrinsic factors using the SCN analysis, despite the
706 increased statistical power it offered. Evidence for interactions between pathogens within hosts
707 initially came from laboratory studies (e.g., in the development of vaccines, reviewed in
708 (Casadevall & Pirofski 2000)), and until recently, many studies conducted in the wild could not
709 detect such interactions (e.g., (Behnke 2008)). Developments of statistical approaches have
710 contributed to improve sampling designs and analyses, in particular by better controlling for
711 confounding factors, enabling the detection of associations resulting from these within-host
712 interactions (e.g., (Lello *et al.* 2004; Telfer *et al.* 2010)). However, it is unlikely any statistical
713 approach can ever solve the problem of an unmeasured explanatory variable. Experiments
714 conducted in semi-controlled environments have been used to confirm the importance of
715 interactions suggested by the associations (e.g., (Knowles *et al.* 2013)). Both facilitation mediated
716 by immune responses (e.g., (Ezenwa *et al.* 2010)) and competition mediated by shared resources
717 (e.g., (Brown 1986; Budischak *et al.* 2018)) have been emphasized.

718 There remain additional important limits to the interpretation of snapshot observational studies
719 from wild populations such as ours. For instance, they can not provide information about the
720 sequence or duration of infection, although these features strongly affect the outcome of within-host
721 interactions (Eidelman *et al.* 2019). Moreover, both the 16S metabarcoding approach and
722 serological antibody tests can only be interpreted in terms of presence/absence of exposure to
723 pathogens, although co-infection may rather impact parasite abundance (e.g., (Thumbi *et al.* 2013;
724 Gorsich, Ezenwa & Jolles 2014)). Lastly, we also acknowledge several caveats to consider with our
725 methods. We removed animals from which fewer than 500 reads were amplified in one or both
726 bacterial metabarcoding PCR replicates. While 16 of these samples removed were due to random
727 failure of PCR amplification from just one of the two replicates, 12 of the animals had poor
728 amplification in both PCR replicates. In the absence of an internal positive control, e.g., a spike-in
729 standard (Zemb *et al.* 2020), we were unable to verify whether a lack of reads was due to poor DNA
730 extraction or a true lack of infections. Although this has a risk of artificially inflating prevalence

731 rates by selectively removing uninfected individuals, it is unlikely to have had a qualitative effect
732 on our results. Similarly, limiting our analyses to OTUs with 500 reads or more in the entire dataset
733 may select against detection of very rare or low-burden infections. We also removed many OTUs
734 corresponding to bacteria normally occurring in external or internal microbiomes of healthy
735 animals, some of which were represented by a high abundance of reads in positive animals. This
736 was due mainly to the fact that 16S data cannot often distinguish between pathogenic and
737 commensal taxa of many such genera. We know that, for instance, *Helicobacter* species are
738 naturally found in the digestive tract, but can also cause pathogenic infections. Parasitism can affect
739 host microbiome composition (Gaulke *et al.* 2019), and this in turn can have impacts on host health
740 and disease susceptibility (reviewed in (Murall *et al.* 2017; Rosshart *et al.* 2017)). Thus, our choice
741 to ignore OTUs corresponding to microbes typical of healthy flora contributes to the problem of
742 missing data, such as information on intestinal helminth infections or other viruses, which may
743 explain or alter the associations we were able to detect. Furthermore, the evaluation of diversity
744 measures (e.g., Shannon diversity index) based only on a selection of taxa violates the assumption
745 that all species are represented in the sample; thus, patterns of diversity could also be influenced by
746 missing data. These caveats are common problems for disease surveillance and community ecology
747 studies, irrespective of the diagnostic methods, and it is difficult to speculate about their overall
748 impacts on the present study. Finally, it is well-understood that this bias towards detection of
749 common pathogens and difficulty in interpreting evidence for the absence of a pathogen in a given
750 individual or population can make testing for negative associations driven by antagonistic
751 ecological interactions incredibly difficult, if not impossible (Weiss *et al.* 2016; Cougoul *et al.*
752 2019).
753
754
755 **Conclusions**
756 Our results add to a growing number of studies finding that (i) rodents host many important
757 zoonotic human pathogens and (ii) pathogen communities are shaped primarily by host species

identity. We also detected a number of previously undescribed associations among pathogens within these rodent communities, and we also confirmed previously identified associations, sometimes in other rodent species than those in which they were previously described. These associations can be considered in the future as hypotheses for pathogen-pathogen interactions within rodent hosts, and that participate in shaping the community of pathogens in rodent communities. Long-term survey and experimental studies are now required to confirm these interactions and understand the mechanisms underlying the patterns of co-infection detected. In addition to these biological results, we have identified several methodological caveats, with regard to both pathogen and association detection, that deserves further investigation to improve our ability to make robust inference of pathogen interactions.

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None of the rodent species investigated here has protected status (see list of the International Union for Conservation of Nature). All procedures and methods were carried out in accordance with relevant regulations and official guidelines from the American Society of Mammalogists. All protocols presented here were realized with prior explicit agreement from relevant institutional committee (Centre de Biologie pour la Gestion des Populations (CBGP): 34 169 003).

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Data Accessibility

Supplementary data deposited in Dryad (https://doi.org/XXXXXXXXXXX) include the following 16S metabarcoding data: (i) raw sequence reads (fastq format), (ii) raw output files generated by the mothur program (iii) raw abundance table (iv) filtered occurrence table and (v) scripts and data files for statistical analyses. Items iii-v are also provided in Supplemental Materials Appendix 2 to directly accompany this publication.

792 Table 1. BLAST search results for OTUs suspected of belonging to pathogenic genera.
793

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

794

795 Table 1 (continued). BLAST search results for OTUs suspected of belonging to pathogenic genera.
796

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

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