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 incidence 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

47 **1. Introduction**

48 Infectious diseases are among the most important global threats to biodiversity, wildlife and
49 human health, and are associated with potential severe socioeconomic consequences (Daszak,
50 Cunningham & Hyatt 2000; Smith, Sax & Lafferty 2006; Jones *et al.* 2008). Although combatting
51 these risks is a main worldwide priority, our understanding of the processes underlying disease
52 emergence still remains too limited for developing efficient prediction, prevention and management
53 strategies. In humans, the majority of emerging pathogens originate as zoonoses from animal host
54 populations in which they naturally circulate (Taylor, Latham & Woolhouse 2001; Jones *et al.*
55 2008). Thus, identifying the epidemiological features (e.g., prevalence, diversity, host specificity,
56 geographic distribution) of zoonotic pathogen communities in their wild hosts, and the factors that
57 influence pathogen occurrence in those communities, is as important to human health as it is to
58 understanding the fundamentals of disease ecology (Garchitorena *et al.* 2017).

59 Both extrinsic and intrinsic factors can contribute to the composition of natural pathogen
60 communities within and between wild animal species, populations and individuals. Extrinsic factors
61 include geographic location, climate, periodicity of epidemic cycles and abiotic features influencing
62 inter-specific transmission opportunities (e.g., (Harvell et al. 2002; Burthe et al. 2006; Poulin et al.
63 2012)). Intrinsic factors such as host species identity, sex, age, and body condition as well as
64 genetic and immunogenetic features have also been intensively studied (e.g., (Beldomenico *et al.*
65 2008; Streicker *et al.* 2010; Streicker, Fenton & Pedersen 2013; Salvador *et al.* 2011; Charbonnel *et*
66 *al.* 2014; Bordes *et al.* 2017)). Although less investigated, inter-specific ecological interactions
67 (e.g., competition, facilitation) among pathogens within animal hosts are also likely to be an
68 important intrinsic force in determining the composition of pathogen communities. Ecological
69 interactions between free-living species are well-known to play a part in the distribution,
70 abundance, and many other qualitative and quantitative features of populations; the application of
71 this basic tenant of community ecology to pathogen incidence and expression of disease has become
72 recognized as imperative for assessing both risks and potential benefits posed to human health,

73 agriculture, wildlife, and conservation (Pedersen & Fenton 2007). Simultaneous infection by
74 multiple parasite species is ubiquitous in nature (Petney & Andrews 1998; Cox 2001; Moutailler *et*
75 *al.* 2016). Interactions among co-infecting parasites may have important consequences for disease
76 severity, transmission and community-level responses to perturbations (Jolles *et al.* 2008; Telfer *et*
77 *al.* 2010). Henceforth, and through the advent of sequencing technologies in particular, it is now
78 possible and essential to investigate disease emergence from a multi-host / multi-pathogen
79 perspective (Galan *et al.* 2016), considering the potential influence of pathogen interactions on
80 current and future disease distributions (Cattadori, Boag & Hudson 2008; Jolles *et al.* 2008;
81 Budischak *et al.* 2015; Abbate *et al.* 2018).

82 Rodent communities are relevant models for developing this community ecology approach to
83 disease distribution and emergence. They harbor a wide variety of pathogenic taxa (e.g., Bordes *et*
84 *al.* 2013; Pilosof *et al.* 2015; Koskela *et al.* 2016; Diagne *et al.* 2017) and are important reservoir
85 hosts of agents of zoonoses that have severe implications for human health. Han et al. (2015) have
86 revealed that about 10% of the 2277 extant rodent species are reservoirs of 66 agents of zoonoses,
87 including viruses, bacteria, fungi, helminths, and protozoa. They also described 79 hyper-reservoir
88 rodent species that could be infected by multiple zoonotic agents. Among the most important
89 zoonotic pathogens, we find *Echinococcus* spp., *Schistosoma* spp., *Toxoplasma* spp., *Trypanosoma*
90 spp., *Yersinia pestis*, *Leptospirosa* spp., *Bartonella* spp., hantaviruses (e.g. Puumala virus) and
91 arenaviruses (e.g. *Lassa virus*) in particular (https://www.gideononline.com/). Strong ecological
92 interactions have been shown among some of these pathogenic taxa (Telfer *et al.* 2010; Kreisinger
93 *et al.* 2015), as well as between micro- and macroparasites (eg., (Salvador *et al.* 2011; Ezenwa &
94 Jolles 2015)). These interactions have been shown to be dependent on the landscape (or habitat
95 features) from which hosts were sampled (Ribas Salvador *et al.* 2011; Guivier *et al.* 2014). In
96 addition, rodents share a number of habitats with humans, including urban settings, agricultural
97 lands, and forests, providing opportunities for human-rodent contact and pathogen transmission
98 (Davis, Calvet & Leirs, 2005). Describing the distribution and composition of natural pathogen

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

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

124 likely to be accompanied by differences in transmission dynamics and host responses to infection
125 (Davies & Pedersen 2008; Singer 2010; Dallas, Laine & Ovaskainen 2019).
126

127 ## 2. Materials and methods

128 *2.1. Study area and host sampling*

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

150 *2.2. Detecting virus exposure and bacterial infection*

151 Among the 713 rodents sampled for this study, indirect fluorescent antibody tests (IFATs; see for
152 details (Kallio-Kokko *et al.* 2006)) were successfully performed on 677 animals to detect
153 immunoglobulin G (IgG) specific to or cross-reacting with cowpox virus (CPXV, *Orthopoxvirus*),
154 Puumala or Dobrava-Belgrade virus (respectively PUUV and DOBV, *Orthohantavirus*, collectively
155 referred to henceforth as "hantavirus"), lymphocytic choriomeningitis virus (LCMV,
156 *Mammarenavirus*), and Tick-borne encephalitis virus (TBEV, *Flavivirus*). We refer to these
157 antiviral antibody tests as indicating a history of past exposure, but hantavirus and LCMV
158 antibodies also likely indicate continued chronic infection. In contrast, current or very recent
159 exposure to bacterial infection was tested via 16S rRNA gene amplicon sequencing of splenic
160 tissue, giving no indication of past exposure history. Funding was available to test for bacteria in
161 just half of the animals, chosen haphazardly to equally represent all host species, study sites and
162 years, resulting in successful analysis for 332 rodents (see Figure 1 for a breakdown of number of
163 individuals sampled per focal host species). For each individual animal, the DNA from splenic
164 tissue was extracted using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer
165 recommendations. Each DNA extraction was analyzed twice independently. We followed the
166 method described in Galan *et al*. (2016) to perform PCR amplification, indexing, pooling,
167 multiplexing, de-multiplexing, taxonomic identification using the SILVA SSU Ref NR 119
168 database as a reference (http://www.arb-silva.de/projects/ssu-ref-nr/). Briefly, DNA samples were
169 amplified by PCR using universal primers targeting the hyper variable region V4 of the 16S rRNA
170 gene (251 bp) and sequencing via Illumina MiSeq. The V4 region has been proven to have
171 reasonable taxonomic resolution at the genus level (Claesson *et al.* 2010). A multiplexing strategy
172 enabled the identification of bacterial genera in each individual sample (Kozich *et al.* 2013). Data
173 filtering was performed as described in Galan et al. (2016) to determine presence/absence of
174 bacterial infections (summarized in Figure S3). Briefly, we discarded all bacterial OTUs containing
175 fewer than 50 reads in the entire dataset and animals for which one or both individual PCR samples

176 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
178 either 0.012% of the total number of reads in the run for that OTU (i.e. filtering using the rate of
179 indexing leak) or the maximum number of reads for that OTU in any negative control sample (i.e.
180 filtering using the presence of reads in the negative controls due to contaminations) (Galan *et al.*
181 2016). For each OTU suspected as pathogenic, Basic Local Alignment Search Tool (BLAST)
182 searches of the most common sequences were conducted to infer species identity where possible.
183 OTUs with at least 500 reads across all animals in the dataset were considered reliably detectable,
184 allowing us to assign absent status to these OTUs in animals failing to meet the criteria for OTU
185 presence. Only OTUs for which there were at least 500 reads across all animals in the dataset (for
186 which present and absent statuses could be assigned), and where reasonable certainty of
187 pathogenicity could be established from the literature, were considered in analyses of the pathogen
188 community.
189
190 *2.3. Statistical Methods*
191 All statistical analyses were implemented in R version 3.2.2 (R Core Team 2015).
192
193 *2.3.1. Testing for extrinsic drivers of pathogen community composition across the rodent*
194 *community*
195 We analyzed pathogen community composition across the whole rodent community. We first
196 estimated pathogen community richness using the Shannon diversity index (alpha diversity)
197 considering bacterial OTUs and anti-viral antibodies found in each study year, study site, habitat,
198 host species, host sex and host age group. We evaluated a linear regression model using analysis of
199 deviance ('lm' and 'drop1' functions from the basic *stats* package) to test for significance of
200 differences in pathogen diversity due to the fixed factors listed above after first correcting for all
201 other factors in the model (marginal error tested against the *F*-distribution). Post-hoc comparisons

202 and correction for multiple tests were performed using function 'TukeyHSD' from package *stats*
203 and 'HSD.test' from package *agricolae* to regroup significantly distinct factor levels.
204 We next tested for differences in pathogen community composition (beta diversity) between host
205 species, habitats, study sites, years, age groups, and sexes. We used multiple correspondence
206 analysis (MCA) to reduce variance in presence/absence of each bacterial pathogen species and anti-
207 viral antibody, implemented with the function 'MCA' in the *FactoMinR* package and visualization
208 tools found in the *factoextra* package. This produced a down to a set of quantitative and orthogonal
209 descriptors (dimensions) describing the pathogen community composition. With each MCA
210 dimension as a continuous dependent response variable, we then evaluated linear regression models
211 using analysis of deviance with post-hoc comparisons as detailed above.
212
213 *2.3.2. Detecting and testing for associations between co-circulating pathogens*
214 Because we identified a large number of pathogens, the number of potential association
215 combinations to consider was excessively high, especially with regard to the relatively small
216 number of rodents sampled. We therefore decided to test the significance only of those
217 associations (i) clearly suggested by the community-wide MCA or (ii) previously described in
218 the literature: positive association between *Bartonella* spp. and CPXV (Telfer *et al.* 2010), positive
219 associations between *Mycoplasma* species (Sykes *et al.* 2008; Tagawa *et al.* 2012; Fettweis *et al.*
220 2014; Volokhov *et al.* 2017), and both positive (Kedem *et al.* 2014; Eidelman *et al.* 2019) and
221 negative (Cohen, Einav & Hawlena 2015) associations between *Bartonella* spp. and hemotropic
222 *Mycoplasma* species. Given the *a priori* assumption that associations would differ between host
223 species, we analyzed each host species separately; where evidence suggested no significant
224 differences between host species, we pooled individuals into a single analysis to gain statistical
225 power.
226 We tested the significance of each association using both association screening (SCN) analysis
227 (Vaumourin *et al.* 2014) and multiple logistic regression analysis (GLMs, modeling the binomial

228 ‘presence/absence’ status of each pathogen as a function of the occurrence of other pathogens). We
229 first performed SCN analysis, as this approach is among the most suitable for detecting pathogen
230 associations in cross-sectional studies (Vaumourin et al. 2014). Briefly, given the prevalence of
231 each pathogen species in the study population, SCN analysis generates a simulation-based 95%
232 confidence envelope around the expected frequency of each possible combination of concurrent
233 infection status (a total of 2^{NP} combinations, where NP = the number of pathogen species) under the
234 null hypothesis of random pathogen associations. Observed frequencies of co-infection
235 combinations falling above or below this envelope are considered to occur more or less frequently,
236 respectively, than in 95% of the random simulations. Significance of the association is given as a *p*-
237 value, calculated as the number of instances in which the simulated co-infection frequency differed
238 (above or below the upper or lower threshold, respectively) from the observed frequency divided by
239 the total number of simulations (Vaumourin *et al.* 2014).

240 The benefit of the SCN approach is a relatively high level of statistical power and the ability to
241 identify precisely which combinations of pathogens occur outside the random expectations
242 (Vaumourin et al. (2014)). However, the SCN is sensitive to heterogeneity in the data due to
243 extrinsic factors (e.g., host specificity, or structuring in space, time, age or sex), which can both
244 create and mask true associations. A multiple logistic regression (GLM) approach was thus also
245 systematically employed, as it has the benefit of explicitly taking into account potentially
246 confounding extrinsic factors. Binomial exposure (presence/absence of either bacterial infection or
247 anti-viral antibodies) to a single pathogen was set as the dependent variable with exposure to the
248 hypothetically associated pathogen(s) treated as independent variable(s) and extrinsic factors (host
249 sex, host age, study year, study site, and where appropriate, habitat) were specified as covariates
250 using function ‘glm’ in the *stats* package with a binomial logit link. When there was no *a priori*
251 assumption concerning timing of exposure (e.g. anti-viral anti-body presence is more likely to affect
252 current acute bacterial infection than the reverse), the occurrence of each pathogen implicated in a
253 given association was set as the dependent variable in reciprocal GLMs. As for the MCA

254 dimensions above, statistical significance of the association was assessed after first correcting for all
255 covariates in the model using the 'drop1' function (-2 log likelihood ratio tests via single-term
256 deletions compared to the full model). Despite a large number of *a priori* hypotheses, we regarded a
257 p-value of < 0.05 as significant due to the very low number of individuals of each host species
258 sampled. Though conceivably important, we also did not have sufficient power to test for
259 interaction terms.
260

261 **3. Results**

262 *3.1. Taxonomic identification and prevalence of pathogens*

263 *3.1.1. Viral exposure*

264 The most abundant virus detected was CPXV, infecting 222 (32.8%) of the 677 animals tested. It
265 was detected in all focal host species. However, significant variation in prevalence was observed
266 between focal host species (highly prevalent (43-70%) in *Ar. scherman*, *Mi. agrestis*, and *My.*
267 *glareolus*; Figure 1; χ^2=111.1, *df*=6, $p<10^{-4}$). Anti-hantavirus antibodies were detected in 16 animals
268 (2.4%), and were significantly structured among host species (with exposure highest in *Mi. arvalis*
269 (9.7%), *R. norvegicus* (3.3%) and *My. glareolus* (3.1%); Figure 1; χ^2=12.7, *df*=6, *p*=0.048). Anti-
270 LCMV antibodies were detected in two *Mi. arvalis* individuals (Figure 1). No animals tested
271 positive for anti-TBE antibodies.
272

273 *3.1.2. Bacterial pathogens*

274 Out of 952 bacterial OTUs represented by at least 50 reads in the dataset, 498 were considered
275 positive in at least one animal after data filtering (presented in Supporting Information Table S1).
276 We checked manually for potential chimera that would not have been removed from the dataset by
277 the *Uchime* program implemented in *mothur.* Two OTUs (00024 & 00037) identified as *Bartonella*
278 with low bootstrap values (74 and 92 respectively) appeared to represent chimeric sequences
279 between the two highly amplified genera (*Bartonella* and *Mycoplasma*) in co-infected samples.

280 Two OTUs (00009 & 00117) which were unclassified but which had a large number of reads in
281 positive animals were also found to represent chimeric sequences between the two genera, despite
282 high bootstrap values (100). Three additional chimeric *Mycoplasma* OTUs with under 500 reads
283 were also excluded (OTUs 00076, 00159, and 00316). Two OTUs (00002 & 00059) were found to
284 be redundant with OTUs Myco1 and Myco3, respectively, and two more (00134 & 00220) were
285 chimera between Myco OTUs. These 11 OTUs were manually removed from the database, and are
286 not included in Table S1.

287 We identified 43 OTUs belonging to bacterial genera with members known or thought to be
288 pathogenic in mammals (Table 1). After BLAST queries, we found 16 of these OTUs (representing
289 7 distinct genera) which could be considered as reliably detectable pathogens in the focal host
290 species (Figure 1). An additional 24 OTUs were considered potentially pathogenic but excluded
291 from analyses because they were only observed in rare host species, presence-absence could not be
292 reliably established due to a low total number of reads (<500 in the dataset, e.g., *Borrelia* spp. and
293 *Leptospira* spp.), inability to rule out contamination by natural sources of non-pathogenic flora
294 during dissection (e.g., *Helicobacter* spp., *Streptococcus* spp.) or by known contaminants of
295 sequencing reagents (e.g., *Williamsia* spp.; (Salter *et al.* 2014)), or because their identity to a
296 pathogenic species was uncertain due to insufficient genetic variation at the 16S rRNA locus (e.g.,
297 *Yersinia* spp.) (Table 1). We also identified three OTUs belonging to the eukaryotic family
298 Sarcocystidae (98% sequence similarity to the coccidian parasite *Sarcocystis muris*); though each
299 OTU was represented by >500 reads, there are currently no data on the reliability of this method for
300 detection (Table 1). Individual infection status for each of these OTUs is given in Table S2.

301 The 16 reliably detectable pathogenic OTUs included *Bartonella* spp., 10 *Mycoplasma* spp.
302 OTUs, *Rickettsia canadensis*, "*Candidatus* Neoehrlichia mikurensis", *Orientia* spp., *Brevinema*
303 *andersonii*, and *Spiroplasma* spp. Phylogenetic analysis including published sequences from
304 BLAST queries revealed that the 10 *Mycoplasma* spp. OTUs belonged to three distinct species: *M.*
305 *haemomuris* (Myco1-3,5,7-9), *M. coccoides* (Myco4 and Mco6), and "*Candidatus* M.

306 ravipulmonis" (Myco10) (Figure S4). In general, these bacterial infections were present in all but 30

307 of the 332 animals tested (90.4% prevalent), and were not concentrated in any particular focal host

308 species (χ^2=9.7, *df*=6, *p*=0.139). Prevalence of each pathogen in each focal host species is presented

309 in Figure 1.

310

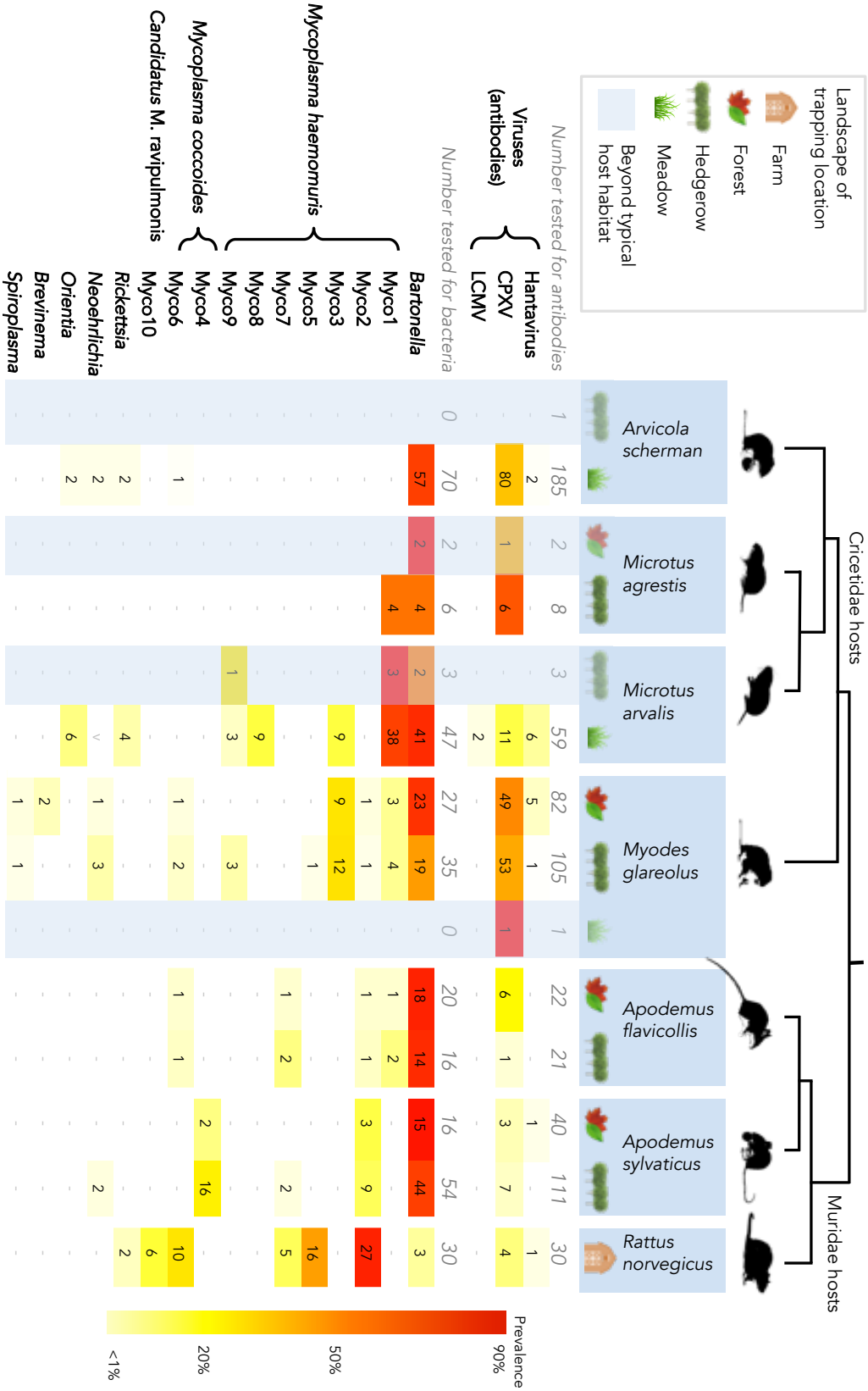


Figure 1. Pathogen occurrence across the rodent species community.

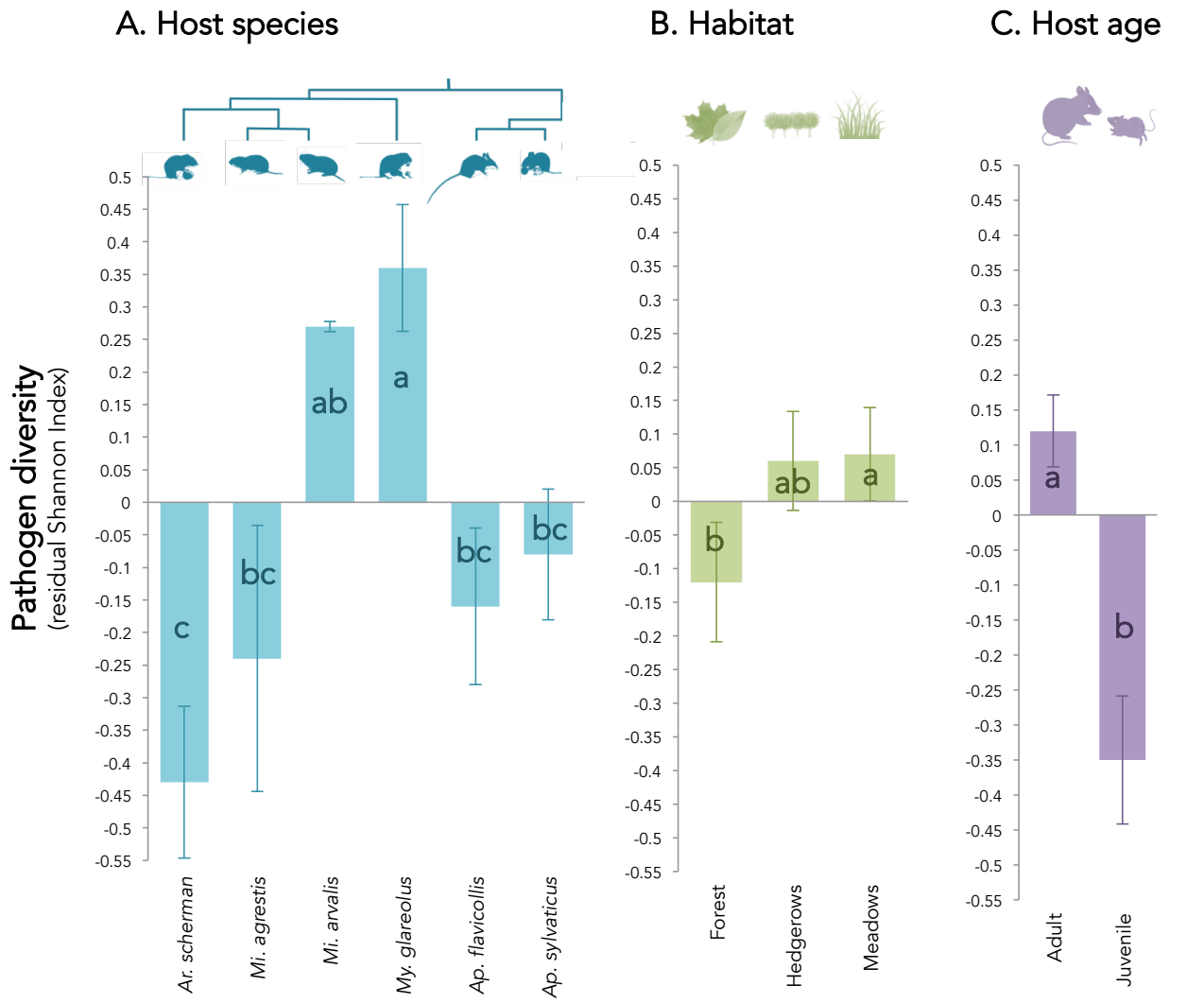
311

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

3.2.1. Analyses of pathogen diversity

We found evidence for the co-circulation of between 3 (in *Mi. agrestis*) and 12 (in *My. glareolus*) pathogen taxa per host species across the rodent community (Figure 1). Using multiple regression analysis on the Shannon diversity index, we found that pathogen diversity differed significantly between habitats ($F_{2,78} = 5.63$, $p = 0.005$) and host species ($F_{5,78} = 7.12$, $p < 10^{-4}$). It was significantly higher in adults than in juveniles ($F_{1,78} = 32.29$, $p < 10^{-7}$). It did not differ between study sites, years, or host sexes (Figure 2, Table S3). Post-hoc Tukey tests showed that after

Figure 2: Extrinsic drivers of pathogen diversity in a rodent species community. Significant differences in Shannon diversity index were tested on residual variance of the multiple regression model controlling for all other extrinsic factors. Different letters signify statistically significant differences at $p < 0.05$, with post-hoc Tukey adjustments for multi-level factors.



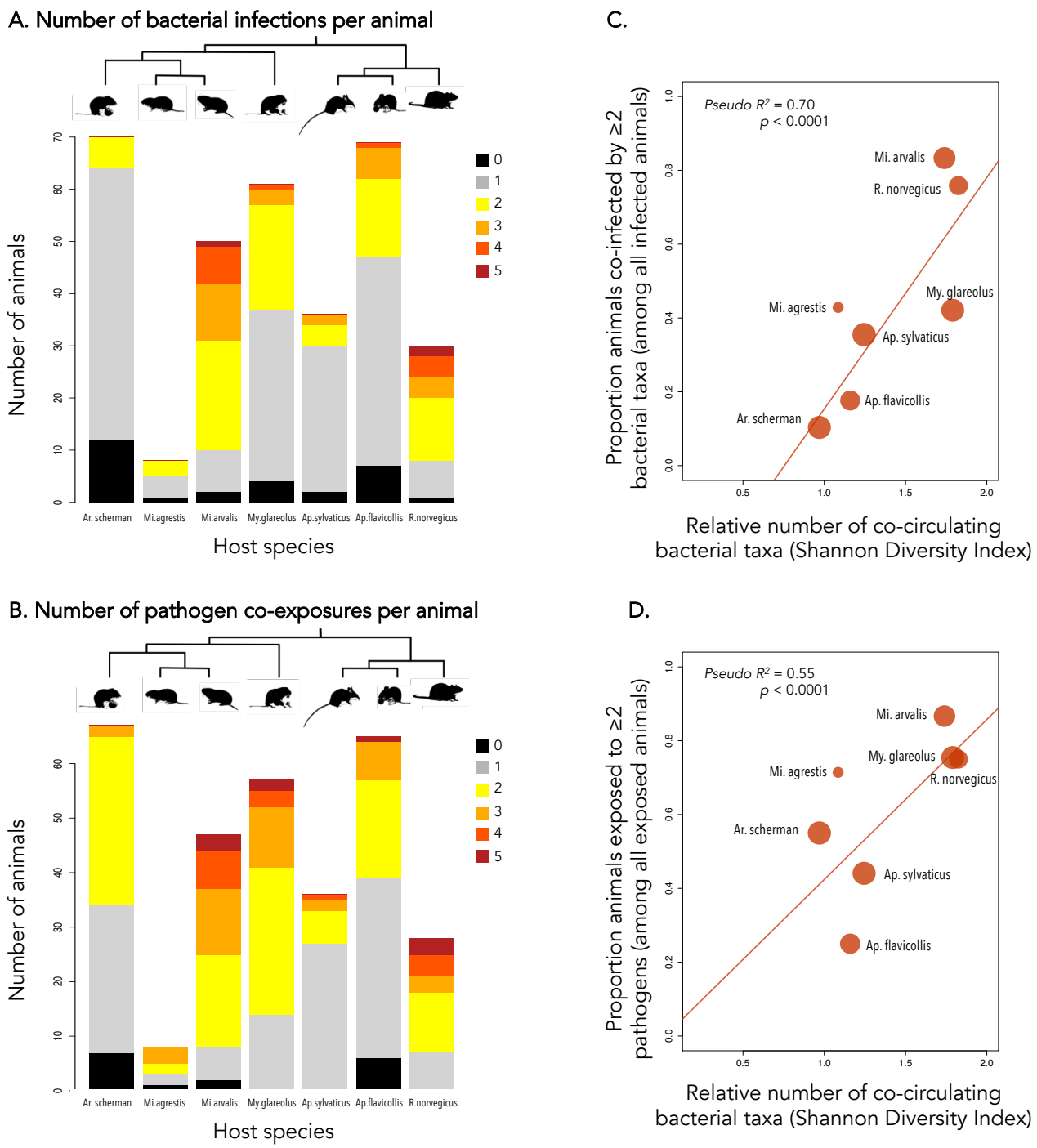
321 correcting for all other factors in the model, meadow habitats had higher diversity of pathogen
322 exposure than forest habitats, and the diversity of pathogen communities in host species fell along a
323 continuum between *My. glareolus* (high) and *Ar. scherman* (low) extremes (Figure 2, Table S3). To
324 understand the relative pathogen diversity of *R. norvegicus* hosts, excluded from the model because
325 they were entirely confounded with farm habitats, we analyzed two additional modified models :
326 one excluding host habitat and the other excluding host species. Post-hoc Tukey tests from these
327 models respectively showed that *R. norvegicus* hosts had the second most diverse pathogen
328 community, and that farm habitats were thus in the same high-diversity category as meadow
329 habitats (Table S4).

330 We also found an enormous amount of both bacterial co-infections and concurrent history of
331 viral exposures (Figures 3A, 3B). The percentage of animals co-infected with two or more reliably
332 detectable pathogenic bacterial OTUs among all those infected in each host species ranged between
333 84.4% (in *Mi. arvalis*) and 10.5% (in *Ar. scherman*). This co-infection frequency was significantly
334 correlated with the diversity (Shannon Index) of bacteria circulating in each rodent species (Figure
335 3C; *Pseudo-*R^2 = 0.70, $p < 0.0001$, calculated using logistic regression weighted by the number of
336 infected animals per species). Bacterial co-infections were more frequent than expected in *Mi.*
337 *agrestis*, and less frequent than expected in *My. glareolus* (according to Cook's Distance, Figure
338 S5A). Results were similar when co-occurrence of anti-viral antibodies were considered along with
339 bacterial OTU exposure (Figure 3D; *Pseudo-*R^2 = 0.55, $p < 0.0001$). While *Mi. arvalis* had both
340 more bacterial co-infections and slightly more pathogen co-exposures than expected based on
341 pathogen diversity, other outliers differed between the two measures (Figure S5): *My. glareolus* co-
342 exposure frequencies were not lower than expected, and both *Apodemus* species had lower than
343 expected co-exposures.

344 *3.2.2. Analyses of pathogen community composition*

345 Many pathogen taxa were found only in a single host species (*Mycoplasma haemomuris* OTU
346 Myco8, "*Candidatus* Mycoplasma ravipulmonis*"* (Myco10), *Brevinema* spp., *Spiroplasma* spp.,

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



and LCMV), and each host species had a unique combination of co-circulating pathogens (Figure 1). In order to identify extrinsic factors (host species, host sex and age class, habitat, study site, year) potentially driving the composition of pathogen communities within the rodent community,

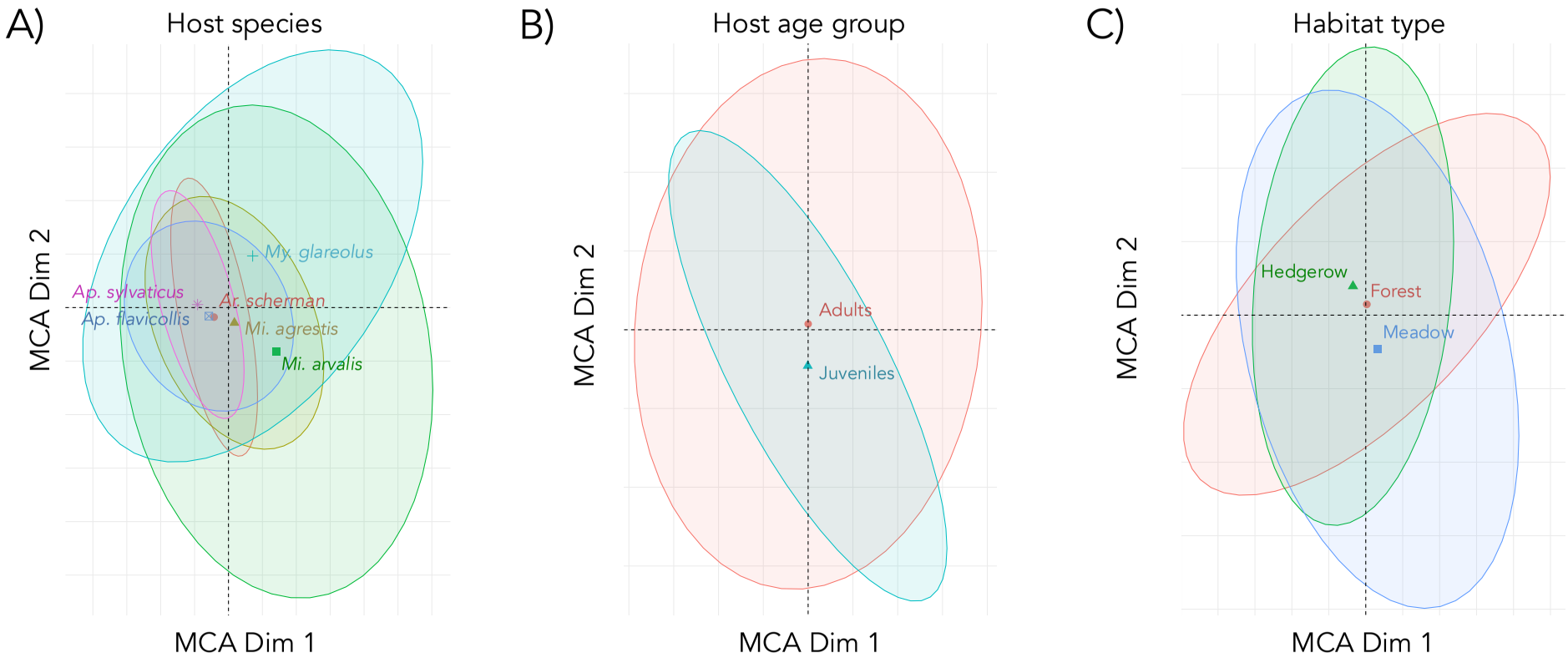
353 we performed a single MCA on occurrence data for exposure to both bacteria and viruses followed
354 by logistic regression analyses. Due to competing *a priori* hypotheses that host species and habitat
355 would be important factors, we excluded *R. norvegicus* individuals (as this host species was the
356 only one found in farm habitats, confounding these two variables; but see MCA results when *R.*
357 *norvegicus* was included in Figures S6, S7). Likewise, we excluded pathogens that occurred only in
358 one habitat type of one host species (not including *R. norvegicus*). Two additional individuals were
359 excluded due to missing sex and age information. Analyses were performed on the remaining 280
360 individuals from six host species and their 14 pathogens (*Bartonella*, Myco1, Myco2, Myco3,
361 Myco4, Myco6, Myco7, Myco9, *Rickettsia*, *Neoehrlichia*, *Orientia*, *Spiroplasma*, and antibodies
362 against CPXV and hantaviruses) (Figure S8).

363 Out of 14 orthogonal MCA dimensions returned, the first two captured 23.0% of the variation in
364 pathogen and antibody occurrence, and the first seven explained a cumulative 63.4% of the total
365 variance (Figure S9). Further dimensions captured less variance than would be expected if all
366 dimensions contributed equally to overall inertia in the data. Dimension 1 (MCA Dim1; explaining
367 13.1% of the variation in pathogen community and loading heavily with the presence of Myco1,
368 Myco3 and anti-hantavirus antibodies) differed significantly between host species ($F_{5,268} = 23.83$, p
369 < 0.0001) and age classes ($F_{1,268} = 6.27$, $p = 0.013$; Table S5). Dimension 2 (MCA Dim2;
370 explaining 9.9% of the variation in pathogen community and primarily describing the occurrence of
371 *Bartonella*) was also structured significantly by host species ($F_{5,268} = 3.89$, $p = 0.002$; Table S5).
372 While these first two dimensions varied by host species (Figure 4A) and host age class (Figure 4B),
373 variance in host habitats (Figure 4C) was not significant after accounting for the other factors. Host
374 species was the most consistently important extrinsic driver of pathogen community composition,
375 significantly explaining variation captured in six of the first seven dimensions, MCA Dim1 – MCA
376 Dim7 (except for MCA Dim 5; Table S5).

377

378

Figure 4. Pathogen community structure among extrinsic factors (A) host species (B) host age groups and (C) habitat types, represented by plotting the mean values for the first and second dimensions described by multiple correspondence analysis (MCA). MCA Dim1 and MCA Dim 2 collectively accounted for 23% of total variance in the data. Ellipses include 95% of individual values for each factor group.



379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404

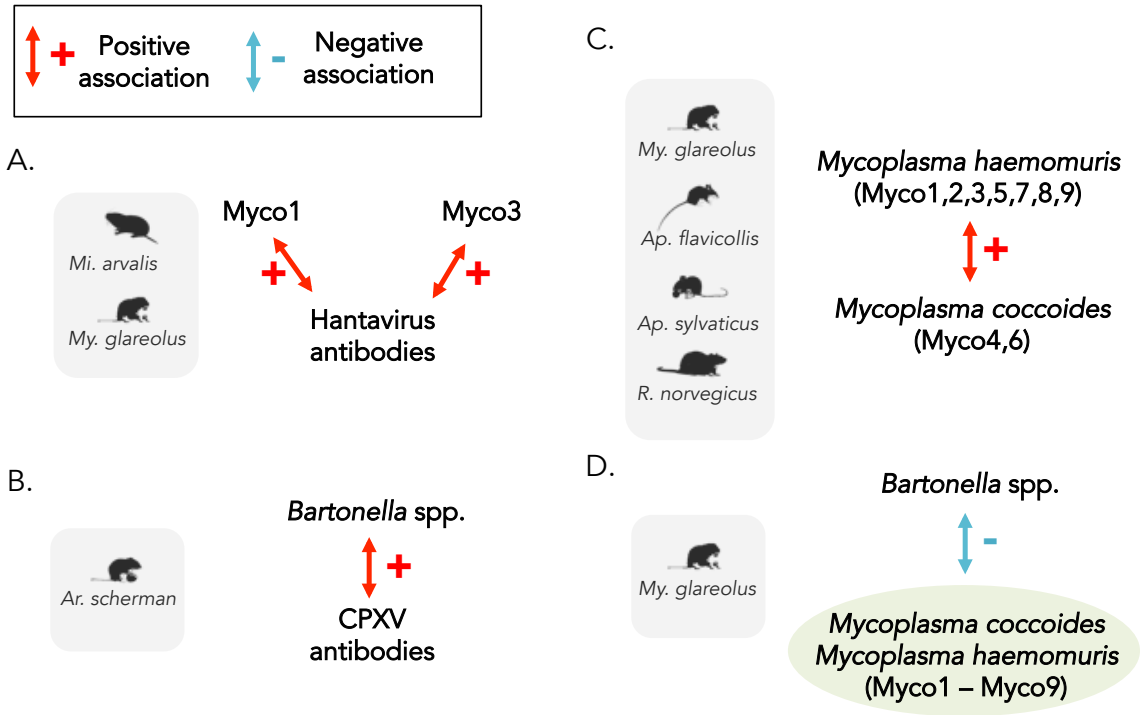
405 *3.3. Associations between pathogens*

406 *3.3.1. Validation of the associations detected by MCA*

407 We applied SCN and GLM analyses to further characterize patterns detected using MCA. Strong
408 and relatively equal loading of MCA Dim1 with Myco1, Myco3, and anti-hantavirus antibody
409 presence indicated that these three pathogens were positively associated with one-another. Indeed,
410 the six animals with anti-hantavirus antibodies were found exclusively in animals infected with
411 Myco3, and Myco1 was found in 2/3 of hantavirus-exposed animals but in just 1/3 of those without
412 hantavirus exposure. The MCA also revealed significant differences among host species and host
413 age classes for Dim 1; hantavirus and Myco 3 only circulated in two host species (*Mi. arvalis* and
414 *My. glareolus*) and 34 of those 35 occurrences were in adults. To exclude positive associations
415 arising from mutual host specificity and age-related accumulation of exposure probability, we
416 focused our analyses on the dataset restricted to adults of the two host species in which all three
417 pathogens co-circulated (*Mi. arvalis* and *My. glareolus*). Individual SCN analyses performed on
418 adults of each host species revealed no associations (Table S6A). However, since values of MCA
419 Dim 1 did not differ between the two host species (according to post-hoc tests given in Table S5),
420 we also ran a single SCN analysis on the pooled data from adults of both species to improve
421 statistical power (Table S6A). According to this pooled SCN analysis, the three-way co-occurrence
422 of Myco1, Myco 3 and anti-hantavirus antibodies was significantly more frequent than would be
423 expected by random chance (p = 0.008), with a trend for anti-hantavirus antibodies occurring by
424 themselves more rarely than expected (p = 0.13; Table 6A, Figure S10). We also investigated this
425 association using GLM. However given the small number of hantavirus exposures and perfect
426 association with Myco3 infection, there was insufficient statistical power to explicitly test for an
427 association between all three pathogens and extrinsic factors. We therefore ran three reciprocal
428 GLM models on the restricted dataset, one for each pathogen as a function of extrinsic factors
429 controlling for heterogeneous host groups (host species, host sex, study site, habitat, and year
430 sampled) and exposure to the two other pathogens (Table S6B). These models showed that there

remained significant unexplained positive associations between hantavirus exposure and Myco1 infection (Hantavirus antibodies ~ Myco1: $\chi^2 = 5.67$, p = 0.017; Myco1 ~ Hantavirus antibodies: $\chi^2 = 2.89$, p = 0.09) and between hantavirus exposure and Myco3 infection (Myco3 ~ Hantavirus antibodies: $\chi^2 = 12.11$, p < 0.001), but that there was no evidence of direct association between Myco1 and Myco3 infections (Myco1 ~ Myco3: $\chi^2 = 0.07$, p = 0.8; Myco3 ~ Myco1: $\chi^2 < 0.01$, p = 0.96; 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.



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 (*M. haemomuris*) and Myco4 (*M. 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 7A), and after correcting for all extrinsic factors, there

446 remained only a non-significant trend (Myco2 ~ Myco4: χ^2 = 2.47, p = 0.12; Myco4 ~ Myco2: χ^2 =
447 2.47, p = 0.12; Table S7B) for a positive association between the two OTUs. No additional
448 associations with sufficient variance for statistical tests were clearly suggested by the MCA
449 analysis.
450
451 *3.3.2. Validation of associations described in the literature*
452 We tested the *a priori* hypothesis that seropositivity to CPXV would be positively associated
453 with *Bartonella* infection, previously detected in *Mi. agrestis* (Telfer *et al.* 2010). The whole dataset
454 was considered as these two pathogens co-circulated in all host species (Figure 1). SCN analyses
455 performed independently for each host species revealed no associations (Table S8A), and the MCA
456 results suggested that pooling data across host species would be inappropriate. After correcting for
457 extrinsic factors using GLM, we found reciprocal evidence for a positive association in *Ar.*
458 *scherman* hosts (*Bartonella* ~ CPXV antibodies: χ^2 = 5.37, p = 0.020; CPXV antibodies: χ^2 = 5.21,
459 p = 0.022; Figure 5B), but not in any other host species (Table S8B). It is of note that there were
460 only eight *Mi. agrestis* individuals, rendering statistical power to test for the association while
461 controlling for extrinsic factors insufficient in this host species where the association was
462 previously described. While prevalence of both pathogens in *Mi. agrestis* was relatively high
463 compared to other host species, one of the two animals without *Bartonella* infection was positive
464 for CPXV antibodies, also precluding evidence for a within-species trend.
465 We next focused on the potential associations between OTUs identified as belonging to two
466 different species of hemotropic *Mycoplasma, M. haemomuris* (HM) and *M. coccoides* (HC), within
467 the four host species in which they both circulated (*My. glareolus*, *Ap. flavicollis*, *Ap. sylvaticus*, *R.*
468 *norvegicus*; Figure 1; Figure S4). We found no significant associations using independent SCN
469 analyses for each host species (Table S9A). However, after controlling for extrinsic factors using
470 GLM, a significant positive association was detected (HM ~ HC: χ^2 = 10.27, p = 0.0013; HC ~ HM:
471 χ^2 = 9.92, p = 0.0016), and did not differ between host species (non-significant interaction term

472 between host species by explanatory pathogen occurrence in each reciprocal model, Table S9B;
473 Figure 5C). We note that only one *R. norvegicus* animal was uninfected with *M. haemomuris*, and
474 that animal also had no *M. coccoides* infection; thus the trend for the association in this host species
475 was also positive but lacked sufficient variance for independent statistical analysis.

476 Finally, we tested for associations between *Bartonella* spp. and hemotropic *Mycoplasma* species,
477 grouping the occurrence of different OTUs of the latter (Myco1 – Myco9) into a single presence-
478 absence variable. There was no association detected by SCN analyses (Table S10A), and marginal
479 evidence that any association may differ by host species after correcting for extrinsic factors using
480 GLM (Table S10B). After controlling for extrinsic factors using independent GLMs for each host
481 (where possible), we found a negative association between the two pathogen groups only in *My.*
482 *glareolus* hosts (*Bartonella* ~ *Mycoplasma*: $\chi^2 = 5.73$, $p = 0.017$; *Mycoplasma* ~ *Bartonella*: $\chi^2 =$
483 5.65, $p = 0.017$, Table S10B, Figure 5D).

484

485 **4. Discussion**

486 Rodents have long been recognized as important reservoirs of infectious agents, with a high
487 transmission potential to humans and domestic animals. Europe is identified as a hotspot of rodent
488 reservoir diversity and one third of rodent species are considered hyper-reservoirs, carrying up to 11
489 zoonotic agents (Han *et al.* 2015). Nevertheless, associations between these pathogens have still
490 only rarely been investigated (but see, for example, studies from field voles in the UK (Telfer *et al.*
491 2010) and in Poland (Pawelczyk *et al.* 2004), gerbils in Israel (Cohen *et al.* 2015), across a rodent
492 community in North America (Dallas *et al.* 2019), and co-infection frequencies of zoonotic
493 pathogens from rodents in Croatia (Tadin *et al.* 2012)).

494 In this study, we confirmed that rodent communities in northern France may harbor a large
495 diversity of potential zoonotic pathogens, with at least 10 bacterial genera (23 OTUs) and
496 antibodies against four genera of viruses circulating in the areas examined. Some of these pathogens
497 have already been reported in this region or in geographic proximity, including viruses

498 (*Orthohantavirus*, *Orthopoxvirus*, *Mammarenavirus* (Charbonnel *et al.* 2008; Salvador *et al.*
499 2011)); and bacteria (e.g. *Bartonella, Mycoplasma, Rickettsia, "Candidatus* Neoehrlichia*",*
500 *Orientia, Spiroplasma, Treponema, Leptospira, Borrelia, Neisseria, Pasteurella*; see (Vayssier-
501 Taussat *et al.* 2012; Razzauti *et al.* 2015)). Several previously unseen agents were also detected,
502 such as Ljungan virus, a picornavirus found in other European rodent populations (e.g., Salisbury *et*
503 *al.* 2014), and a relative of the putatively pathogenic spirochaete *Brevinema andersonii* that infects
504 short-tailed shrews and white-footed mice in North America (Defosse *et al.* 1995). In addition, we
505 detected a relatively high prevalence of anti-hantavirus antibodies in *Mi. arvalis*, explained by
506 cross-reactivity between the anti-PUUV antibodies used in our assay and those elicited against the
507 related *Tula orthohantavirus* (TULA) virus common to European voles (Deter *et al.* 2007;
508 Tegshduuren *et al.* 2010).

509 Three pathogens were highly prevalent. Anti-*Orthopoxvirus* antibodies were detected in all
510 seven focal rodent species, with prevalence levels reaching up to 70%. This corroborates prior
511 evidence that cowpox virus could be widespread in European rodents, particularly voles (Bennett *et*
512 *al.* 1997; Essbauer, Pfeffer & Meyer 2010; Forbes *et al.* 2014). An astounding 77% of all
513 individuals in the study were infected by *Bartonella sp.*, with *R. norvegicus* being the notable
514 exception (with only 10% infected, leaving an average of 84% prevalence in all other species). The
515 bacterial genus *Bartonella* is a diverse group of hemotrophs known to commonly infect rodents and
516 other mammals (Breitschwerdt & Kordick 2000; Bai *et al.* 2009) and which have also been
517 implicated in both zoonotic and human-specific disease (Iralu *et al.* 2006; Breitschwerdt 2014;
518 Vayssier-Taussat *et al.* 2016). We could not assess the specific diversity of *Bartonella* sp.
519 circulating in these rodent communities because accurate resolution in this genus requires additional
520 genetic markers (Matar *et al.* 1999; Guy *et al.* 2013). Hemotropic and pneumotropic *Mycoplasma*
521 spp. were also highly prevalent, collectively infecting 43% of all hosts, with nearly all *R.*
522 *norvegicus* and *Mi. arvalis* samples infected. On the contrary, we found just one *Mycoplasma*
523 infection in *Ar. scherman,* despite ample sampling (70 animals tested) and its previous detection in

524 *A. scherman* from Eastern France (Villette *et al.* 2017). These *Mycoplasma* species are also known
525 pathogens of humans and rodents (Harwick, Kalmanson & Guze 1972; Baker 1998). Here, we
526 found 15 different OTUs corresponding to two distinct hemotropic *Mycoplasma* species (*M.*
527 *haemomuris* and *M. coccoides*) and the pneumotropic *Mycoplasma* species *M. pulmonis* and
528 "*Candidatus* M. ravipulmonis". The former two are both hemotropic mycoplasmas responsible for
529 vector-transmitted infectious anaemia of wild mice, rats, and other rodent species (Neimark *et al.*
530 2001, 2005; Messick 2004). However, *M. pulmonis* and "*Candidatus* M. ravipulmonis" cause
531 respiratory infections, are more closely related to other pneumotropic mycoplasmas, and
532 "*Candidatus* M. ravipulmonis" has only ever before been described as spontaneously occurring in
533 laboratory mice and rats exposed to unknown microbes of other animals and humans via passaging
534 experiments (formerly termed Grey Lung virus; (Andrews & Glover 1945; Neimark, Mitchelmore
535 & Leach 1998; Graham & Schoeb 2011; Piasecki, Chrzastek & Kasprzykowska 2017)).
536 Lastly, our data corroborated the status of hyper-reservoir (more than two zoonotic pathogens
537 carried by a reservoir species) for all seven of the focal rodent species studied here (Han *et al.*
538 2015), with the addition of *Mi. subterraneous* that we also found to carry two potentially zoonotic
539 pathogens (*Bartonella* spp. and *Brevinema* spp.; Appendix 1). Overall, we found a high variability
540 in the number of pathogens circulating in each species despite correction for sampling effort, with
541 low levels observed in *Apodemus* species and *Arvicola scherman*, and high levels detected in *Mi.*
542 *arvalis, My. glareolus*, and *R. norvegicus*.
543 The search for factors that drive parasite species richness, diversity and community composition
544 has been at the core of numerous studies (Poulin 1995; Poulin & Morand 2000; Nunn *et al.* 2003;
545 Mouillot *et al.* 2005; Krasnov *et al.* 2010). Here, we emphasized that both pathogen diversity and
546 community composition was mainly structured by host species identity, despite both shared habitats
547 and shared pathogen taxa. Using multiple correspondence analysis (MCA) together with logistic
548 regression and the novel computational method of association screening (SCN) analysis, we found
549 no evidence that any specific pathogen-pathogen associations were likely to be as important as host

550 species identity in determining pathogen distributions across the community of rodents. The strong
551 influence of host characteristics (Cohen *et al.* 2015) and host species identity (Dallas *et al.* 2019) on
552 pathogen community composition has recently been described in comparison to intrinsic pathogen-
553 pathogen associations in other rodents. Moreover, the pathogen community composition provided a
554 unique signature of each rodent species, even among those most closely related (e.g. *Ap. flavicolis*
555 and *Ap. sylvaticus*). This result is in line with the conclusions of recent meta-analyses showing that
556 phylogeny, over other host traits, had a minimal impact on pathogen diversity in rodent species
557 (Luis *et al.* 2013; Guy *et al.* 2019).

558 The importance of host species identity in shaping pathogen community composition may not
559 stem from strict host-pathogen specificity, as most pathogens were found to infect multiple host
560 species – a broad result echoed across animal communities (Taylor *et al.* 2001; Woolhouse, Taylor
561 & Haydon 2001; Cleaveland, Laurenson & Taylor 2001; Pedersen *et al.* 2005; Streicker *et al.*
562 2013). However, we might be cautious as more precise molecular analyses are necessary to test
563 whether different species of a bacteria genus or divergent populations of the same bacteria species
564 may circulate independently in different rodent host species, with little or no transmission. For
565 example, two genera seemed to be largely shared among the rodent species studied here, *Bartonella*
566 and *Mycoplasma*. But previous studies have shown strong host-specificity when considering the
567 genetic variants of *Bartonella* (Buffet *et al.* 2013; Withenshaw *et al.* 2016; Brook *et al.* 2017)
568 Evidence in the literature for host specificity of *Mycoplasma* species has led to a mix of conclusions
569 (Pitcher & Nicholas 2005), as cases of cross-species transmission are commonly reported –
570 particularly in humans – despite a general consensus that most species are highly host-specific. We
571 found that some *Mycoplasma* taxa were dominant contributors to prevalence in a single host
572 species, and that when shared, they were shared with just a few other specific host species. Rare
573 infections in unexpected host species (e.g., Myco6 in *Ar. scherman* and Myco1 in *Ap. flavicollis*)
574 were represented by fewer sequence reads compared to positive samples in host species where they
575 were more prevalent, suggesting a low potential for amplification and sustained transmission from

576 these occasional hosts (Figure S4). On the other hand, while the Cricetidae appeared to be
577 susceptible only – with rare exception – to taxa within the *M. haemomuris* group, host species in the
578 Muridae family were susceptible to all three distinct *Mycoplasma* species detected, The biggest
579 exception to this pattern was that three of 62 *Myodes glareolus* (the most basal member of the
580 Cricetidae included in the study) animals were found to be infected by both hemotropic
581 *Mycoplasma* species. These results both support the observation that cross-species transmission
582 naturally occurs among wild rodents and suggest that the degree of host specificity may be driven
583 by both host and pathogen factors.

584 Several studies have emphasized the influence of host habitat specialization on parasite species
585 richness, low habitat specialization being associated with both high species richness of macro- and
586 micro-parasites (e.g. (Morand & Bordes 2015)). Our results did not fully corroborate this
587 association; while the grassland-specific *Ar. scherman* had the lowest pathogen diversity and the
588 multi-habitat spanning *My. glareolus* had the highest pathogen diversity, entirely farm-dwelling *R.*
589 *norvegicus* had high pathogen diversity nearly equal to that of *My. glareolus*, and the two
590 *Apodemus* hosts (neither with significantly higher pathogen diversity than *Ar. scherman*) were
591 found across both meadows and hedgerows. We also found no influence of sampling sites or years
592 on pathogen diversity at the small spatio-temporal scale considered here. Hence, further research is
593 required to decipher the eco-epidemiological features that explain this strong influence of rodent
594 species on pathogen community composition, such as host densities, home ranges, behaviours, or
595 genetics (e.g. (Morand 2015).

596 We found that a large proportion of rodents from Northern France were co-infected with two or
597 more bacterial pathogens (max = 5 bacterial pathogens in *Mi. arvalis* and *R. norvegicus* hosts), and
598 also had concurrent histories of exposure to multiple viruses (max = 5 bacterial and/or viral co-
599 exposures in *Mi. arvalis*, *My. glareolus*, *Ap. sylvaticus*, and *R. norvegicus* hosts). The percentage of
600 pathogen co-exposed hosts was as high as 89 % (in *Mi. arvalis* hosts), dropping only to 83% when
601 considering only presently co-infecting bacterial taxa. These results are in line with recent studies

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

623 Here, we tested for pathogen-pathogen associations suggested by multiple correspondence
624 analysis (MCA) of the structure of pathogen exposures in this specific rodent community, as well as
625 those previously described in the literature. We used multiple logistic regression analyses (via
626 analysis of deviance on generalized linear models (GLMs)) to determine whether pathogen-
627 pathogen associations were present after extrinsic factors creating heterogeneity among hosts were

628 taken into account. We found evidence in support of three previously identified associations
629 (positive association between *M. haemomuris* and *M. coccoides* infections; positive association
630 between *Bartonella* infection and the presence of CPXV antibodies; negative association between
631 *Bartonella* and hemotropic *Mycoplasma* infections), and characterized one set of associations not
632 previously described (positive associations between the presence of hantavirus antibodies and
633 infections by two specific *M. haemomuris* OTUs) – each in a unique subset of host species.

634 The positive association between *Mycoplasma haemomuris* and *M. coccoides* was found in the
635 four rodent species where these bacteria co-circulate (i.e., *My. glareolus, Ap. flavicollis*, *Ap.*
636 *sylvaticus* and *R. norvegicus*). Transmission of *Mycoplasma* blood infections is not yet well
637 understood, but given a lack of support for density-dependent transmission and high incidence of
638 spill-over events, it is likely that these bacteria are transmitted through bites of blood-sucking
639 arthropod vectors (Volokhov *et al.* 2017). The positive associations detected between *M.*
640 *haemomuris* and *M. coccoides* could also result from similarities in rodent susceptibility. Indeed
641 *Mycoplasma* infection can lead to acute or chronic infection, and the establishment of chronic
642 bacteremia seems to occur in immunosuppressed or immunocompromised individuals (Cohen *et al.*
643 2018). Co-infections with multiple *Mycoplasma* spp. might therefore be more likely to be detected
644 in these immunocompromised rodents with chronic infections. The existence of chronic infections
645 might also lead to additional co-infections and positive associations as a result of disease-induced
646 changes in population dynamics, immune system function, or through direct pathogen-pathogen
647 interactions (Fenton 2008; Aivelo & Norberg 2018; Fountain-Jones *et al.* 2019).

648 Whether through the accumulation of exposure probabilities or increased susceptibility, the
649 previously-undocumented positive association we found here between *M. haemomuris* OTUs
650 (Myco1 and Myco3) and hantavirus antibodies may similarly be explained by the chronic nature of
651 both *Mycoplasma* and hantavirus infections in rodents (e.g. for Puumala hantavirus in bank voles
652 (Yanagihara, Amyx & Gajdusek 1985; Meyer & Schmaljohn 2000; Vaheri *et al.* 2013)). This
653 positive association was found in both host species where the majority of hantavirus exposures

654 occurred (*Microtus arvalis* and *Myodes glareolus*), consistent with the generality of association
655 between *Mycoplasma* species across host taxa detailed above, suggesting the intrinsic ecology of
656 these pathogens contributes to shaping variation in the pathogen community independent of host
657 species identity. Curiously, we found no evidence for direct associations between OTUs of the same
658 *Mycoplasma* species, thus facilitation interactions are unlikely to explain the high diversity of
659 *Mycoplasma* taxa both within and between host species.

660 Infections by *Bartonella* species are also known to often result in subclinical and persistent
661 bacteremia in mammals, including rodents (Birtles *et al.* 2001; Kosoy *et al.* 2004). The positive
662 association detected in *Ar. scherman* between *Bartonella* spp. and cowpox virus antibodies might
663 therefore be explained by, for example, joint accumulation of both chronic bacterial infections and
664 long-lived antibodies used to test for prior exposure to relatively short-lived CPXV virus infections.
665 However, if the same processes governing association of the chronic infections described above
666 were at play here, we would have expected to find both pathogens implicated in positive
667 associations (i) with other chronic infections, and (ii) across host species given their ubiquitous
668 prevalence. While the failure to recover the association in *Mi. agrestis* (where it was previously
669 described (Telfer *et al.* 2010) was likely due to low statistical power, the lack of a general pattern
670 across other host species despite adequate sampling suggests a more specific, and potentially
671 immune-mediated, ecological process between these two pathogens. Indeed, pox virus infections,
672 including CPXV, have been shown to induce immunomodulation that increases host susceptibility
673 to other parasites (Johnston & McFadden 2003). These interactions could be of variable intensities
674 according to the rodent species considered, due to potential differences in impacts of CPXV
675 infection on immunity across host species, or to the influence of other infections not examined here
676 on host immune responses during pox infections (e.g. helminths (Cattadori, Albert & Boag 2007),
677 protozoa (Telfer *et al.* 2010)). Furthermore, *Bartonella* infection was negatively associated with
678 *Mycoplasma* infections in *My. glareolus*, corroborating negative interactions revealed in co-
679 infection experiments in gerbils (Eidelman *et al.* 2019). This association may therefore originate

680 from an interaction mediated by specific (immune-) genetic features of *My. glareolus*, and not
681 ecological conditions as proposed by Eidelman et al. (2019). The antagonistic and host-specific
682 nature of this association lends further support to the interpretation that *Bartonella* infections do not
683 behave in similar ways to other chronic infections in the community. However, few studies have
684 investigated the robustness of within-host interactions across different host species (e.g., (Lello *et*
685 *al.* 2018)), and this question deserves further investigation.

686 Our results suggest that intrinsic ecological interactions could help shape the composition of the
687 pathogen community within hosts. However, this suggestion provides only a hypothesis that
688 requires further investigation to establish its provenance. Interpretation of associations can be
689 misleading, as they may arise from unmeasured co-factors such as exposure to shared transmission
690 routes, and may even run counter to the underlying ecological process (Fenton *et al.* 2014). We
691 noted that the associations we found here were considered properly characterized only once
692 extrinsic factors had been taken into account using multiple logistic regression analyses, and were
693 not visible (or even misleading, in the case of a 3-way interaction between hantavirus, Myco1 and
694 Myco3) when ignoring these factors using the SCN analysis, despite the increased statistical power
695 it offered. Evidence for interactions between pathogens within hosts initially came from laboratory
696 studies (e.g. in the development of vaccines, reviewed in (Casadevall & Pirofski 2000)), and until
697 recently, many studies conducted in the wild could not detect such interactions (e.g. (Behnke
698 2008)). Developments of statistical approaches have contributed to improve sampling designs and
699 analyses, in particular by better controlling for confounding factors, enabling the detection of
700 associations resulting from these within-host interactions (e.g., (Lello *et al.* 2004; Telfer *et al.*
701 2010)). Experiments conducted in semi-controlled environments have been used to confirm the
702 importance of interactions suggested by the associations (e.g. (Knowles *et al.* 2013)). Both
703 facilitation mediated by immune responses (e.g., (Ezenwa *et al.* 2010)) and competition mediated
704 by shared resources (e.g., (Brown 1986; Budischak *et al.* 2018)) have been emphasized.

705 There remain additional important limits to the interpretation of snapshot observational studies
706 from wild populations such as we have presented here. For instance, they can not provide
707 information about the sequence and timing of infection, although these features strongly affect the
708 outcome of within-host interactions (Eidelman *et al.* 2019). Moreover, both the 16S metabarcoding
709 approach and serological antibody tests can only be interpreted in terms of presence/absence of
710 exposure to pathogens, although co-infection may rather impact parasite abundance (e.g., (Thumbi
711 *et al.* 2013; Gorsich, Ezenwa & Jolles 2014)). Lastly, we also acknowledge several caveats to
712 consider with our methods. We removed animals from which fewer than 500 reads were amplified
713 in one or both bacterial metabarcoding PCR replicates. While 16 of these samples removed were
714 due to random failure of PCR amplification from just one of the two replicates, 12 of the animals
715 had poor amplification in both PCR replicates. In the absence of an internal positive control, e.g. a
716 spike-in standard (Zemb *et al.* 2020), we were unable to verify whether a lack of reads was due to
717 poor DNA extraction or a true lack of infections. Although this has a risk of artificially inflating
718 prevalence rates by selectively removing uninfected individuals, it is unlikely to have had a
719 qualitative effect on our results. Similarly, limiting our analyses to OTUs with 500 reads or more in
720 the entire dataset may select against detection of very rare or low-burden infections. We also
721 removed many OTUs corresponding to bacteria normally occurring in external or internal
722 microbiomes of healthy animals, some of which were represented by a high abundance of reads in
723 positive animals. We know that, for instance, *Helicobacter* species are naturally found in the
724 digestive tract, but can also cause pathogenic infections. We also know that parasitism can affect
725 host microbiome composition (Gaulke *et al.* 2019), and this in turn can have impacts on host health
726 and disease susceptibility (reviewed in (Murall *et al.* 2017)). Thus, our choice to ignore OTUs
727 corresponding to microbes typical of healthy flora contributes to the problem of missing data, such
728 as information on intestinal helminth infections, which may explain or alter the associations we
729 were able to detect. These caveats are common problems for disease surveillance and community
730 ecology studies, irrespective of the diagnostic methods, and it is difficult to speculate about their

731 overall impacts on the present study. Finally, it is well-understood that this bias towards detection
732 of common pathogens and difficulty in interpreting evidence for the absence of a pathogen in a
733 given individual or population can make testing for negative associations driven by antagonistic
734 ecological interactions incredibly difficult, if not impossible (Weiss *et al.* 2016; Cougoul *et al.*
735 2019).

736

737 **Conclusions**

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

750

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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 and (iv) filtered occurrence table.

771 Table 1. BLAST search results for OTUs suspected of belonging to pathogenic genera.
772

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 *Mycoplasm*a 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

773

774 Table 1 (continued). BLAST search results for OTUs suspected of belonging to pathogenic genera.
775

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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