

1 Bank vole immunoheterogeneity may limit Nephropatia Epidemica emergence in a
2 French non-endemic region

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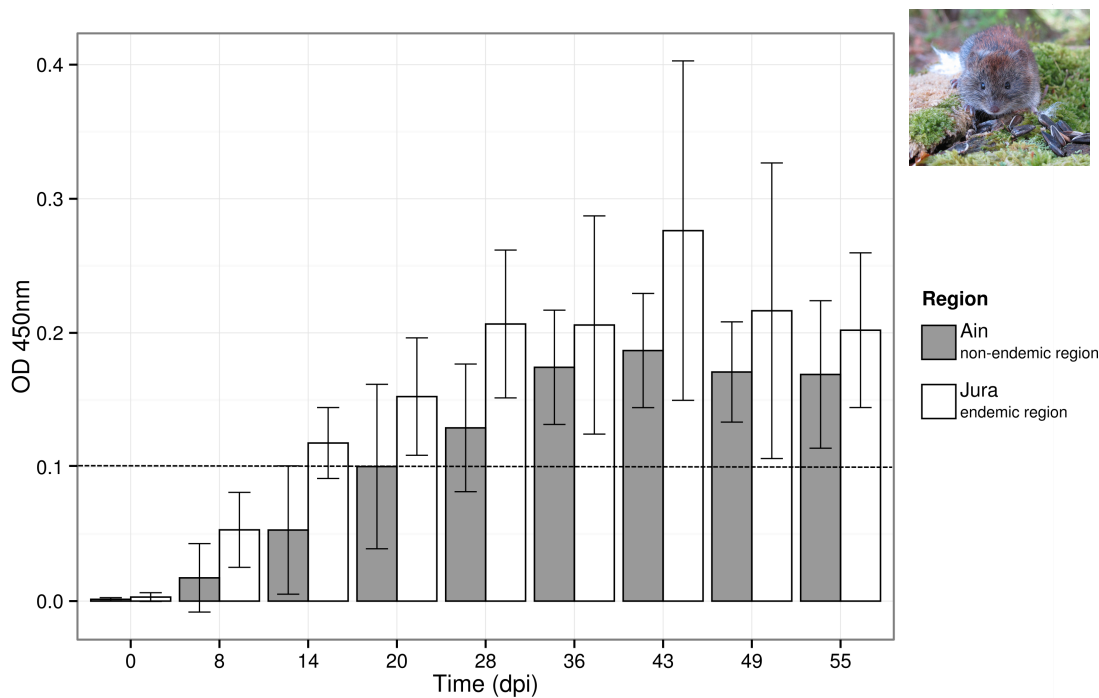
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23 **Abstract**

24 Ecoevolutionary processes affecting hosts, vectors and pathogens are important drivers of zoonotic
25 disease emergence. In this study, we focused on nephropathia epidemica (NE), which is caused by
26 Puumala hantavirus (PUUV) whose natural reservoir is the bank vole, *Myodes glareolus*. Despite
27 the continuous distribution of the reservoir in Europe, PUUV occurrence is highly fragmented. We
28 questioned the possibility of NE emergence in a French region that is considered to be NE-free but
29 that is adjacent to a NE-endemic region. We first confirmed the epidemiology of these two regions
30 using serological and virological surveys. We used bank vole population genetics to demonstrate
31 the absence of spatial barriers that could have limited dispersal, and consequently, the spread of
32 PUUV into the NE-free region. We next tested whether regional immunoheterogeneity could
33 impact PUUV chances to establish, circulate and persist in the NE-free region. Immune
34 responsiveness was phenotyped both in the wild and during experimental infections, using
35 serological, virological and immune related gene expression assays. We showed that bank voles
36 from the NE-free region were sensitive to experimental PUUV infection. We observed high levels
37 of immunoheterogeneity between individuals and also between regions. In natural populations,
38 antiviral gene expression (*Tnf* and *Mx2* genes) reached higher levels in bank voles from the NE-free
39 region. During experimental infections, anti-PUUV antibody production was higher in bank voles
40 from the NE endemic region. Altogether, our results indicated a lower susceptibility to PUUV for
41 bank voles from this NE-free region, what might limit PUUV circulation and persistence, and in
42 turn, the risk of NE.

43

44 Graphical abstract



45

46 Keywords

47 *Myodes glareolus*; Rodent; Puumala hantavirus; Population genetics; Experimental infections;
48 ecoimmunology

49

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50 1. Introduction

51 Emerging infectious diseases (infections that have newly appeared in a population, or have
52 existed but are rapidly increasing in incidence or geographic range, \ (Morse and Schluederberg,
53 1990) have become an important public health threat these last decades (Daszak et al., 2000; King
54 et al., 2006). The need for integrating evolutionary concepts to better understand and prevent them
55 has recently been advocated (Vander Wal et al., 2014). Hence, hosts, vectors and pathogens evolve
56 in response to environmental selective pressures but also as a result of inter-specific interactions,
57 what, in turn, strongly impacts infectious disease dynamics and emergence.

58 Among important zoonotic (re-)emerging agents are hemorrhagic fever-causing hantaviruses of
59 the Bunyaviridae family (Vapalahti et al., 2003). They are mainly rodent-borne (Henttonen et al.,
60 2008) and are responsible for two kinds of human syndromes, hemorrhagic fever with renal
61 syndrome (HFRS), mainly in Eurasia, and hantavirus cardiopulmonary syndrome (HCPS) in the
62 Americas. For twenty years, since reliable tools for hantavirus infection diagnostics have become
63 available, the (re-)emergence of zoonoses associated with hantaviruses has been recognized as a
64 growing public health concern worldwide. In Europe, an increase in amplitude and frequency of
65 epidemics due to hantaviruses has been observed these last 20 years (Heyman et al., 2011).
66 Moreover, the geographic distribution of hantaviruses is expanding (e.g. for Seoul virus Jameson et
67 al., 2013; Mace et al., 2013). Although this may be potentially and partly explained by increased
68 clinician awareness and easier access to diagnostic tests, a significant rise in the real number of
69 human cases is very likely (Heyman et al., 2012; Reynes et al., 2015). In France, Puumala virus
70 (PUUV) is the main agent of hantavirus infections. It induces an attenuated form of HFRS called
71 nephropathia epidemica (NE). PUUV is transmitted to humans via aerosols contaminated by excreta
72 of its unique and specific natural reservoir host, the bank vole *Myodes glareolus*, which is a forest
73 dwelling rodent species (Brummer-Korvenkotio et al., 1982). Although *M. glareolus* exhibits a
74 spatially continuous distribution all over France, except on the Mediterranean coast, human cases
75 are mostly being reported in the northeast part of the country (National Reference Center for
76 Hantavirus <http://www.pasteur.fr/fr/sante/centres-nationaux-reference/les-cnr/hantavirus>, but see
77 Reynes et al., 2015). Elsewhere, PUUV can be absent or the prevalence of PUUV in bank vole
78 populations can reach high levels without any human case being reported yet (Castel et al., 2015).
79 Niche modeling approaches based on climatic and environmental factors have been conducted to

80 better predict the spatial distribution of NE disease (Zeimes et al., 2015). They highlighted
81 geographic areas of high risk of NE emergence, although neither seropositive bank voles nor human
82 cases had been detected yet. They also failed to detect geographic areas with high levels of PUUV
83 prevalence in reservoir populations and no human case reported. These results suggest that the
84 drivers of NE emergence are just beginning to be elucidated, and that other important factors than
85 abiotic ones, including evolutionary processes and PUUV and/or host genetic characteristics, could
86 be relevant. In particular, selective pressures affecting host-pathogen interactions may promote the
87 evolution of different defense strategies in hosts, ranging from resistance, *i.e.* the ability to reduce
88 pathogen burden to tolerance, *i.e.* the ability to limit the damage caused by a given parasite burden
89 (Schneider and Ayres, 2008; Raberg et al., 2009). Previous field and laboratory studies have
90 revealed some variability in the susceptibility of bank voles to PUUV infection, as reflected by the
91 probability to be infected with PUUV (Olsson et al., 2002; Kallio et al., 2006; Deter et al., 2008b;
92 Dubois et al., 2017a) and the pattern of PUUV excretion once bank voles are infected (Hardestam et
93 al., 2008; Voutilainen et al., 2015). Moreover, Guivier et al. (2010; 2014) have shown significant
94 differences in the level of immune related gene expression in bank voles from NE endemic and
95 non-endemic regions at the European and regional scales. It was suggested that this
96 immunoheterogeneity could reflect some balance of resistance / tolerance to PUUV. Indeed PUUV
97 infection in bank vole is chronic and mainly asymptomatic (but see Tersago et al., 2012) and
98 mounting immune responses is energetically costly and can induce immunopathologies (e.g. in
99 human infections, Vaheri et al., 2013). Guivier et al. (2010; 2014) therefore proposed that bank
100 voles from NE endemic areas would be more tolerant to the virus as a result of co-adaptation
101 whereas those from NE non-endemic ones would be more resistant.

102 In this context, we proposed to investigate the hypothesis that immunoheterogeneity between
103 bank vole populations could influence the geographic distribution range of PUUV, using two
104 complementary approaches, natural population surveys and experimental infections. We focused on
105 bank vole populations settled on both parts of the southern limit of PUUV distribution in France
106 (Fig. 1), *i.e.* in the NE endemic area (human cases regularly reported, Jura, see National Reference
107 Center for Hantavirus data) and in the NE non-endemic area (no human case ever reported yet, Ain).
108 Our first objective was to discard the possibility of a spatial barrier that could prevent or limit the
109 transmission of PUUV from the Jura to the Ain bank vole populations. Our second objective was to

110 test the assumption of an important regional immunoheterogeneity between bank vole populations
111 that could reflect different levels of sensitivity to PUUV infection. We predicted bank voles from
112 Ain to be less sensitive to PUUV infection than those from Jura. Under this hypothesis, we
113 expected bank voles from Ain to exhibit lower probability of infection and higher levels of immune
114 responses to PUUV infection, hence reflecting their higher levels of resistance to this hantavirus.

115

116 **2. Material and methods**

117 *2.1. Ethical statement*

118 All animal works have been conducted according to the French and European regulations on care
119 and protection of laboratory animals (French Law 2001-486 issued on June 6, 2001 and Directive
120 2010/63/EU issued on September 22, 2010). Experimental protocols have been evaluated and
121 approved by the Animal Ethics Committee C2EA-16 (ANSES/ENVA/UPEC, CNREEA n°16).

122

123 *2.2. Natural population studies*

124 *2.2.1. Rodent sampling*

125 Bank voles were trapped in June and September 2014 in two adjacent regions of eastern France
126 that have contrasted status in regard of human cases of NE. Jura is considered as an endemic zone
127 as NE cases are regularly reported (National Reference Center for Hantavirus) whereas Ain is
128 considered to be non-endemic as human case has never been reported yet (Fig. 1). These two areas
129 are part of the same geological massif (Sommaruga, 1997) and they experience highly similar
130 climatic conditions (WorldClim data, Supplementary Fig. 1).

131 Six sites were sampled in mixed forest of coniferous and deciduous tree species (Fig. 1, Table 1).
132 The minimum distance between sites was 8.7 km. Six to ten lines of 20 live-traps (INRA) with
133 about five meters interval were set up so that each sampling site consisted of a few km² area. Traps
134 were baited with sunflower seeds. Each trap was geolocated. The traps were checked daily and
135 early in the morning. Trapping session per site lasted at least three nights so that a minimum of 35
136 voles were caught.

137 Once trapped, animals were bled through retro-orbital sinus, killed by cervical dislocation,
138 weighed, measured, sexed and dissected. Whole blood was stored at 4°C until centrifugation to
139 collect sera 24 hours later. Sera, lungs and urine were immediately placed in dry ice and then stored

140 at -80°C for virological and immunological analyses. Spleens were placed in RNAlater solution
141 (Sigma) at 4°C during one night then stored at -20°C for population genetics and gene expression
142 analyses.

143

144 2.2.2. *Microsatellite genotyping*

145 Genomic DNA was extracted from a piece of spleen using the the EZ-10 96-Well Plate Genomic
146 DNA Isolation Kit for Animal (BioBasic) according to manufacturer's instructions, with a final
147 elution of 400µL in elution buffer. Genotyping was performed at 19 unlinked microsatellites loci
148 previously published by Rikalainen et al. (2008) using primers and cycling conditions described in
149 Guivier et al. (2011) using an ABI3130 automated DNA sequencer (Applied Biosystems). Alleles
150 were scored using GENEMAPPER software (Applied Biosystems).

151

152 2.2.3. *Serological and virological analyses*

153 Serum samples were screened by IgG ELISA as described in Klingstrom et al. (2002). Due to the
154 high cross-reactivity between the different hantavirus serotypes (Kruger et al., 2001), the plates
155 were coated with Tula virus infected and non-infected cell lysates. Previous experiments have
156 shown that anti-PUUV positive and negative sera reacted similarly with lysates of PUUV and
157 TULV infected cells and with recombinant PUUV nucleocapsid protein (Dubois et al., 2017a).

158 Viral RNA was extracted from serum, lung and liver, which are target organs for PUUV
159 (Gavrilovskaya et al., 1983; Bernshtein et al., 1999), and urine, as it is a main route of excretion for
160 PUUV (Hardestam et al., 2008), using the QIAamp Viral Mini Kit (Qiagen). Quantitative RT-PCR
161 (qRT-PCR) were performed using 2.5µL of viral RNA amplified using the SuperScript III One-Step
162 RT-PCR system with Platinum Taq High Fidelity (Invitrogen) on LightCycler 480 (Roche
163 Diagnostics) as described in Suppl. Table S1. Relative amounts of viral RNA (expressed in RNA
164 copy per mg of tissue or per µl of liquid) were calculated using a standard curve obtained with *in*
165 *vitro* transcribed RNA. All samples were tested in duplicate to avoid false positives and negatives.

166

167 2.2.4. *Immunological analyses*

168 We quantified gene expression for three candidate immune related genes, namely *Tlr7*, *Tnf-α* and
169 *Mx2*, that are relevant with regard to PUUV infections (Rohfritsch et al., 2013; Charbonnel et al.,

170 2014; Dubois et al., 2017b). TLR7 is a receptor to virus and is probably involved in the detection of
171 ssRNA viruses like hantaviruses (Bowie and Haga, 2005). The proinflammatory cytokine tumor
172 necrosis factor alpha (TNF) and the antiviral protein Mx2 are known to limit PUUV replication in
173 humans and in cell cultures (Kanerva et al., 1996; Temonen et al., 1996; Jin et al., 2001). We used
174 *β-actin* as the endogenous reference gene, as previously validated by Friberg et al. (2011) in wood
175 mice.

176 We analysed a subset of 170 bank voles including 10 adult PUUV-seropositive individuals and a
177 number of PUUV-seronegative individuals that enabled to reach about 30 voles per site with an
178 equal sex ratio. Total RNA was extracted from a piece of spleen using the NucleoSpin© 96 RNA
179 kit (Macherey-Nagel) following manufacturer's instructions. As secondary lymphoid organ, the
180 spleen has important immune functions and is the site of low levels of PUUV replication (Korva et
181 al., 2009). RNA extractions were electrophoresed on 1.5% agarose gels and visualized using
182 ethidium bromide staining to check for quality, then total RNA concentrations were measured using
183 a NanoDrop 8000 spectrophotometers (Thermo Scientific) and normalized to 200 ng/μL using
184 RNase free water. For *β-actin*, *Tnf-α* and *Mx2*, we used specific primers described in previous
185 studies (Guivier et al., 2010; Guivier et al., 2014). For *Tlr7*, we designed the following specific
186 primers based on the cDNA consensus sequences obtained for 12 *M. glareolus* samples (GenBank
187 Accession Numbers: KX463605 - KX463616): Tlr7-Mg2-F
188 (5'-TACCAGGACAGCCAGTTCTA-3'), Tlr7-Mg2-R (5'-GCCTCTGATGGGACAGATA-3').
189 We generated cDNA from 4 μL of extracted RNA (800 ng per reaction), in a 20 μL reaction, using
190 the Improm-II Reverse Transcription System (Promega), according to the conditions specified by
191 the manufacturer for oligo (dT)15 primers. We performed a quantitative PCR on a LightCycler 480
192 (Roche Diagnostics), using the 384-multiwell plate format, as previously described in Guivier et al.
193 (2010; 2014). PCR efficiencies were estimated using the LinRegPCR software (Ruijter et al., 2009).
194 Candidate gene mRNA relative expression levels were estimated for each sample using the method
195 developed by Pfaffl (2001).

197 2.3. Experimental studies

198 2.3.1. Rodent sampling

199 Bank voles were trapped in May 2015 in two sites already sampled in 2014 (Cormaranche forest

200 in Ain and Arbois forest in Jura) and using the same sampling protocol as described above. They
201 were brought back to the laboratory, tested for the presence of anti-PUUV IgG, held during three
202 weeks in quarantine and then retested for the presence of anti-PUUV IgG. Ten voles from Ain and
203 nine voles from Jura, all of them being seronegative, were transferred in a ABSL-3 capacity and
204 held individually in ISOcages N (Tecniplast). Food and water were provided *ad libitum*, with fresh
205 vegetables once a week.

206

207 2.3.2. *Experimental infections*

208 1.7×10^3 f.f.u of PUUV Sotkamo strain 1:10 diluted in DMEM (ThermoFisher Scientific) were
209 injected by subcutaneous route in each of the 19 bank voles. From 7 to 55 days post-infection (*dpi*),
210 blood, saliva and feces were collected once a week for each bank vole. We did not succeed
211 collecting urine samples. Approximately 200 μ L of whole blood was sampled through the
212 retro-orbital sinus and stored at 4°C until centrifugation to collect sera 24 hours later. Saliva was
213 collected using sterile swabs subsequently placed in 300 μ L of Hank's Balanced Salt Solution (Life
214 Technologies) and vortexed for 10 seconds. Feces were sampled directly from the anus. All samples
215 were stored at -80°C until analyses. At the end of the experiment (55 *dpi*), all the bank voles were
216 euthanized by cervical dislocation. Blood, lungs, liver, kidneys, urine and feces were collected and
217 stored at -80°C until analyzes. Three bank voles, two from Ain and one from Jura, died during the
218 course of experimental infection (at 28 *dpi*) and were not considered for further analyzes.

219

220 2.3.3. *Serological and virological analyses*

221 PUUV IgG detection was performed on sera as described in 2.2.2. Viral RNA was extracted
222 from all samples (sera, lung, liver, kidney, feces, saliva and urine) and PUUV RNA detection was
223 realized using qRT-PCR for all the samples as described earlier. To improve the sensitivity of viral
224 detection, nested RT-PCR were applied to sera, feces and saliva, using the Titan One Tube RT-PCR
225 System (Roche) and Taq DNA Polymerase (Qiagen).

226 Gene expression could not be performed on bank voles from this experiment as spleens could
227 only be collected at 55 *dpi*. It was too far from the infection to expect any difference in gene
228 expression between bank voles (Hardestam et al., 2008; Schountz et al., 2012), and it did not enable
229 to study gene expression kinetics.

230

231 2.4. Statistical analyses

232 2.4.1. Natural population analyses

233 We analysed the genetic structure of bank vole populations using microsatellite data. We tested
234 the conformity to Hardy-Weinberg equilibrium (HWE) for each locus and each population. We
235 analyzed linkage disequilibrium (LD) for each pair of loci using GENEPOP v4.2 (Raymond and
236 Rousset, 1995). Corrections for multiple tests were performed using the false discovery rate (FDR)
237 approach as described in Benjamini and Hochberg (1995). We described the spatial genetic
238 structure by estimating the genetic differentiation between each pair of sites using Weir and
239 Cockerham's pairwise F_{ST} estimates (1984). Significance was assessed using exact tests and FDR
240 corrections. Several complementary analyses were performed to test for the existence of spatial
241 barriers that could limit vole dispersal in this area, especially between Jura and Ain. We first
242 computed a discriminant analysis of principal components (DAPC), which is a multivariate,
243 model-free approach to clustering (Jombart et al., 2010). Because DAPC is sensitive to the number
244 of principal components used in analysis, we used the function *optim.a.score* to select the correct
245 number of principal components. We next performed an analysis of molecular variance (AMOVA)
246 in Arlequin v3.5.1.2 (Excoffier and Lischer, 2010). Compared to the DAPC, it enabled to test
247 specifically for genetic differences between regions, considering the potential variability between
248 sites within each region. Finally models of isolation by distance (IBD) were applied to test for
249 geographic-genetic correlations. IBD models were analyzed independently for each region and for
250 the whole dataset, so that disruption of gene flow between regions could also be detected. Genetic
251 differentiation was estimated for each pair of individuals and Mantel tests were applied to test for a
252 correlation between matrices of genetic differentiation and of Euclidean distances between
253 individuals, using 10000 permutations, in GENEPOP. We also calculated confidence intervals for the
254 slope of the regression line by bootstrapping over loci (ABC intervals, Di Ciccio and Efron, 1996).

255 We tested for variations of serological and immunological characteristics in natural populations
256 between regions using generalized linear mixed models with the GLMER function implemented in
257 the LME4 package for R 3.1.0 (R Core Team, 2013). The fixed variables included in the models
258 were *region*, *sex* and *weight* and all pairwise interactions. When they were non-significant, they
259 were removed from the model. The factor *site* was included as a random factor. Chi-square tests

260 with Bonferroni correction were applied to analyze the effect of significant variables using the
261 package PHIA for R. We analysed independently PUUV serological status (using a binomial
262 response variable) and each gene expression level Q_n (using a log-transformed response variable
263 for the three genes). To prevent confounding effects due to the potential induction of the three
264 candidate genes expression following PUUV infection, we limited our statistical analyses to
265 PUUV-seronegative bank voles (Guivier et al., 2014).

266

267 2.4.2. Experimentation analyses

268 We tested for regional differences in bank vole responses to PUUV experimental infection by
269 examining the levels of anti-PUUV IgG (OD_{450nm} , using a normal response variable) between Jura
270 and Ain through time. Generalized mixed linear models were applied as described above. The fixed
271 variables included in the models were *region*, *dpi* and their interaction. Bank vole identity was
272 included as a random effect. Possible variation in the level of viral RNA between regions was tested
273 using a linear model with the fixed effect *region*.

274

275 3. Results

276 3.1. Natural population studies

277 3.1.1. Population genetic structure

278 Three loci, Cg16H5, Cg16E4 and Cg6G11, were excluded from the genetic analyses due to the
279 poor quality profiles obtained. Six bank voles were also excluded because they could not be
280 amplified for most loci. Two other microsatellite loci, Cg2F2 and Cg3F12, showed deviations of
281 HWE in four and six sites respectively, suggesting the presence of null alleles. They were removed
282 from further analyses. Five out of 84 tests for deviation from HWE were significant with a different
283 loci involved each time. 25 of 546 pairs of loci (4.58%) exhibited significant linkage disequilibrium
284 but the loci involved were not consistent among sites. Thus, our final dataset included 271 bank
285 voles genotyped at 14 microsatellites loci.

286 Spatial pairwise F_{ST} ranged between 0.009 and 0.03. All of these estimates were significant after
287 FDR correction (Table 2). DAPC clustering showed that the genetic variation of the six sites
288 overlapped (Fig. 2), although the first discriminant component tended to slightly separate the two
289 regions. The second discriminant component revealed that one site in Jura (Mignovillard) tended to

290 be different from the three others and that the two sites in Ain tended to be separated from each
291 other (Fig. 2). AMOVA analyses revealed an absence of significant differentiation between the two
292 regions, and significant variation between sites within regions (Table 3). IBD patterns were highly
293 significant whatever the dataset considered. Genetic differentiation between bank voles decreased
294 with spatial distance, and the slope of these regressions were similar, respectively 0.003, 0.004 and
295 0.005 for Ain, Jura and the whole dataset, confirming an absence of spatial barrier between the two
296 regions (Table 4).

297

298 *3.1.2. Variations of serological, virological and immunological features in natural populations*

299 277 bank voles were trapped in the six sites surveyed (Table 1). Among them, 12 captured in
300 Jura were PUUV seropositive. Two of these individuals had very low OD. Moreover, they had a
301 low body mass, so that we thought they were juveniles carrying maternal antibodies and not PUUV
302 infected individuals (weight lower than 17.5g, see Voutilainen et al., 2016). They were therefore
303 excluded from further analyses. None of the bank vole captured in Ain was PUUV seropositive.
304 Seroprevalence levels in Jura sites ranged between 7.1 % and 10.0 %, except in Mignovillard where
305 no PUUV seropositive bank vole was detected. The best model that explained bank vole serological
306 status only included *weight* ($X^2_1 = 5.541$, $p = 0.019$). Heavier bank voles were more likely to be
307 PUUV seropositive.

308 The serum, lung, liver and urine of the 10 adult seropositive individuals were tested using
309 qRT-PCR. Three sera were PUUV negative and the seven other samples showed viral RNA ranging
310 between $1.01e4$ and $1.58e5$ copies.mg⁻¹. The viral RNA load in the lung and liver ranged
311 respectively between $7.50e6$ and $4.88e9$ copies.mg⁻¹, and $1.79e6$ and $8.63e8$ copies.mg⁻¹ (Suppl.
312 Table S2). Urine samples could be collected for only five of these 10 adult bank voles. Two of these
313 excreta were PUUV positive as their quantitative RT-PCR cycle thresholds, C_T , were lower than 35
314 cycles. The viral load ranged between $2.08e4$ and $2.26e4$ copies.mg⁻¹.

315 Extreme values of gene expression levels were detected for the three candidate genes (60%
316 higher than the other values). The models were run with or without these extreme values. *Mx2* gene
317 model revealed a significant difference between regions (without outliers: $X^2_1 = 4.56$, $p = 0.033$;
318 with outliers: $X^2_1 = 6.00$, $p = 0.01$ Fig. 3a). Bank voles from Ain over-expressed *Mx2* compared to
319 those from Jura. *Tlr7* gene model included the variable *sex* (without outliers: $X^2_1 = 13.67$, $p = 2.10^{-4}$;

320 with outliers $X^2_1 = 7.80$, $p = 0.005$, Fig. 3b), with higher values of *Tlr7* expression detected in
321 females than in males. When including outliers, *Tnf- α* gene model revealed a significant effect of
322 *region*, *weight* ($X^2_1 = 9.173$, $p = 0.002$; $X^2_1 = 4.474$, $p = 0.034$; respectively, Figs. 3 c, d) and of the
323 interaction *sex * region* ($X^2_1 = 5.783$, $p = 0.016$). *Tnf- α* expression was higher in bank voles from
324 Ain than those from Jura. This effect was stronger when considering males only. These results have
325 to be taken cautiously as they turned to be non-significant when the outliers were not included in
326 the model. Then we only found a significant effect of *weight* ($X^2_1 = 11.50$, $p = 6.10^{-4}$), with heavier
327 bank voles exhibiting higher levels of *Tnf- α* expression than lighter ones.

328

329 3.2 Variations of serological and virological features during experimental infections

330 Three bank voles died before the end of the experiment and were not considered in the analyses.
331 Among the 16 remaining bank voles experimentally infected with PUUV (eight from Ain and 8
332 from Jura), 14 seroconverted by the end of the study. The two bank voles that did not seroconvert at
333 the end of the experiment (55 *dpi*) originated from Ain (Suppl. Table S3). Considering bank voles
334 that seroconverted, our model showed a lower OD_{450nm} in voles from Ain than those from Jura (X^2_1
335 = 4.126, $p = 0.042$, see Fig. 4), and a significant variation of OD_{450nm} through time ($X^2_1 = 195.129$, p
336 = 2.10^{-16}).

337 Viral RNA was detected by qRT-PCR in the sera of a single bank vole from Ain at 14 and 20 *dpi*.
338 Nested RT-PCR allowed detecting PUUV RNA at 8 *dpi* in the sera of two bank voles from Ain and
339 of four bank voles from Jura (Suppl. Table S4). In addition, PUUV RNA could not be detected in
340 any feces or saliva samples tested using qRT-PCR or nested RT-PCR.

341 At the end of the experiment (55 *dpi*), viral RNA was detected by qRT-PCR in the lungs of 13
342 bank voles (including one that did not seroconvert) over the 16 infected during the experiment. The
343 relative amount of PUUV RNA ranged between $4.34e5$ and $4.07e7$ RNA copies. mg^{-1} (Suppl. Table
344 S5). The statistical model revealed that PUUV viral load in lungs did not vary between the two
345 regions. The amounts of viral RNA detected in the other organs were lower and concerned less
346 individuals, in particular in liver : only four individuals showed positive results, and less than
347 $3.07e6$ RNA copies. mg^{-1} were detected (Suppl. Table S5). Finally, no viral RNA could be detected
348 in saliva or urine samples collected at 55 *dpi*, and only two bank voles (one from each region) had
349 PUUV RNA in their feces (Suppl. Table S5).

350 Finally, our results revealed a strong inter-individual variability in the serological / virological
351 relationships. All bank voles but one that seroconverted had at least one organ/excreta with viral
352 RNA at 55 dpi. Moreover, one bank vole that had not seroconverted at 55 dpi exhibited viral RNA
353 in its lungs and kidneys (Suppl. Table S5).

354

355 **4. Discussion**

356 *4.1 – Contrasted PUUV epidemiological situations between the French regions Ain and Jura*

357 To our knowledge, this is the first bank vole serological study ever conducted in the French Ain
358 region with regard to PUUV. It is probably because no NE case had been reported there yet
359 (National Reference Center for Hantaviruses) and PUUV studies remained focused on endemic
360 areas. Deepening our understanding of infectious disease emergence requires to broaden our
361 research out of the areas where diseases are well described, to better identify the factors limiting or
362 facilitating emergence success (e.g. for plague in Madagascar Gascuel et al., 2013; Tollenaere et al.,
363 2013). None of the 90 bank voles captured in Ain during this field survey was seropositive for
364 PUUV antibodies. This result tends to confirm the absence of PUUV circulation in these bank vole
365 populations, or at such a low level that it can not be detected using ecological surveys. An extensive
366 survey in more sites or in different years could still be performed to assert more firmly the absence
367 of PUUV circulation in bank vole populations settled south from Jura. In the opposite, and as
368 expected from the existence of known NE cases, we found PUUV seropositive bank voles in all but
369 one site from Jura. Seroprevalence levels were very similar to those observed in the Ardennes
370 epidemic region or in previous studies conducted in Jura (ranging from 0 to 18%, Guivier et al.,
371 2011; Deter et al., 2008a). Although no bank vole from Mignovillard forest had anti-PUUV
372 antibodies, we know from previous studies that PUUV may circulate in this site (Deter et al.,
373 2008a). The existence of these two adjacent regions with contrasted epidemiological situations with
374 regard to PUUV reinforced the need to better assess the risk of PUUV emergence and persistence in
375 Ain. It emphasized the necessity to question whether PUUV infected bank voles from Jura could
376 migrate to Ain, thus enhancing the chance for PUUV to be introduced there, and whether PUUV
377 could persist in bank vole populations from Ain once introduced.

378

379 *4.2 – High bank vole gene flow between the two French regions Ain and Jura*

380 We developed a population genetic approach to evaluate the possibility that a spatial barrier
381 could prevent bank vole gene flow from Jura to Ain, what would limit the chance of PUUV
382 introduction in this latter region through the dispersal of infected animals. Indeed PUUV is
383 transmitted directly between bank voles, from contaminated excreta or through bites, without any
384 vector (Korpela and Lähdevirta, 1978; Yanagihara et al., 1985). PUUV introduction from one site to
385 another may therefore strongly result from the introduction of an infected bank vole, either through
386 wood transport or natural dispersal. For example, Guivier et al. (2011) showed that despite frequent
387 local extinction of PUUV in hedge networks, the virus was frequently re-introduced in these
388 habitats through the emigration of seropositive bank voles coming from close forests.

389 Our results revealed weak spatial genetic structure between sites and regions, with levels of
390 genetic differentiation ranging between 1 and 3%. These estimates are very similar to the levels of
391 genetic differentiation observed in previous studies conducted at the same geographical scale on
392 bank voles using the same genetic markers (Guivier et al., 2011) or on other rodent species
393 (*Arvicola scherman*, Berthier et al., 2005). These results revealed high population size and / or high
394 gene flow between populations (Wright, 1951). We did not find any signature of disrupted gene
395 flow between bank voles from the two regions. The spatial structure observed was mainly due to
396 isolation by distance, *i.e.* migration rates being inversely proportional to the geographic distance
397 between populations. This pattern is also frequently observed in micromammals, including rodents,
398 when studied at this geographical scale (Aars et al., 2006; Berthier et al., 2006; Bryja et al., 2007).
399 The fact that the rates of isolation by distance did not differ within region and over the whole
400 dataset corroborated the hypothesis of an absence of spatial barrier disrupting gene flow between
401 Jura and Ain. As such, the possibility for PUUV infected bank voles to disperse from Jura to Ain,
402 and consequently, the possibility for PUUV introduction in Ain seemed likely.

403 Considering this possibility, the reason why PUUV, which is known to circulate in Jura since the
404 early 2000's, has not reached the Ain NE-free region needs to be questioned. It could be explained
405 by the low progression of PUUV from northern to southern populations, and the emergence of NE
406 in Ain would only be a question of time. It could also be due to differences in metapopulation
407 functioning between bank vole populations in Ain and Jura. Higher genetic drift, lower migration
408 rates between populations could account for lower probability of PUUV persistence in Ain (Guivier
409 et al., 2011). However, we did not find any evidence supporting this hypothesis based on the

410 population genetic analyses conducted using microsatellites. We therefore explored the possibility
411 that bank voles from Ain and Jura could present different levels of sensitivity to PUUV infections,
412 that would in turn lead to contrasted PUUV epidemiology in these two regions.

413

414 4.3 – High levels of immunoheterogeneity between bank voles

415 The assessment of immunological variations in wild populations has recently been at the core of
416 eco-epidemiological studies, enabling the analysis of their environmental and evolutionary causes
417 as well as the prediction of their epidemiological consequences (Jackson et al., 2011). Here we
418 described strong individual variability of bank vole immune responses from both natural population
419 survey and experimental infections. In the wild, susceptibility, defined here as the probability of
420 being infected with PUUV, strongly depended on the weight of bank voles, which is a proxy for age
421 and/or body condition. Heavier/older bank voles are likely to disperse more than younger ones and
422 have accumulated more opportunities to be exposed to PUUV (see for details Voutilainen et al.,
423 2016). In addition, these heavier/older bank voles could also suffer from immunosuppression due to
424 high levels of corticosterone associated with breeding or to decreased body condition (Beldomenico
425 et al., 2008). Moreover, we revealed variable amounts of viral RNA between bank voles, for all
426 organs and excretas considered, what may result from differences in the time since infection or
427 individual characteristics. The low number of animals that could be handled in highly secured
428 animal facilities (ABSL3) did not enable to test the impact of individual factors on the probability
429 of getting infected and on their specific sensitivity. Nevertheless, the experiments conducted
430 allowed controlling for PUUV strain or quantity injected, and some variability was still detected
431 both in anti-PUUV antibody production (e.g. 2 out of 16 bank voles did not seroconvert 55 days
432 post-infection) and in the amount of viral RNA detected in organs and excretas. It was therefore
433 relevant to analyse whether immune responsiveness also differed between bank voles. Considering
434 three immune related candidate genes, namely *Tlr7*, *Mx2* and *Tnf- α* , we found that sex was an
435 important factor mediating inter-individual immunoheterogeneity. In the wild, females
436 over-expressed *Tlr7* gene compared to males. These observations corroborated common patterns
437 observed in vertebrates showing that females are more immunocompetent than males (Klein and
438 Flanagan, 2016). Indeed, TLR7 is involved in the recognition of diverse viruses (Heil et al., 2004;
439 Lund et al., 2004) and impaired expression and signaling by TLR7 may contribute to reduced innate

440 immune responses during chronic viral infections (Hirsch et al., 2010). The sexual dimorphism in
441 *Tlr7* gene expression is observed in bank voles for the first time. This gene is linked to the X
442 chromosome, and it belongs to the 15% of X-linked genes escaping inactivation and being
443 expressed from both the active and inactive X chromosome (Plath et al., 2002). This could explain
444 the over-expression of *Tlr7* gene observed in females compared to males. Surprisingly, we did not
445 find any evidence of higher levels of *Tnf- α* gene expression in female compared to male bank voles,
446 as was previously detected by Guivier et al. (2014). Higher expression levels were even detected in
447 males compared to females in the region Ain. This pattern was driven by three bank voles with
448 extreme values, that might correspond to recently infected males and blur the general picture
449 observed in the Ardennes (Guivier et al., 2014). Altogether, this high inter-individual
450 immunoheterogeneity, which was observed in the wild and confirmed when performing
451 experimental infections, is important with regard to PUUV epidemiology as it highlighted bank
452 voles with increased likelihood of being infected, excreting and further transmitting the virus (e.g.
453 super-spreaders, Lloyd-Smith et al., 2005).

454
455 *4.4. Regional bank vole immunoheterogeneity - an explanation for the contrasted PUUV*
456 *epidemiology observed in adjacent French regions ?*

457 Evidence of immunoheterogeneity was found between Jura and Ain, two regions with contrasted
458 NE epidemiological statuses. It concerned several immune pathways, including the antiviral and
459 adaptative immune responses of bank voles to PUUV infections. Bank voles from Ain mounted
460 lower levels of anti-PUUV antibodies than those from Jura during PUUV experimental infections.
461 Unfortunately, this regional pattern could hardly be interpreted in terms of rodent sensitivity to
462 PUUV as the role of IgG during hantavirus infections remains unclear (review in Schonrich et al.,
463 2008). Indeed, the presence of IgG can protect rodents from subsequent challenges but does not
464 participate in eliminating the virus (review in Easterbrook and Klein, 2008; Schountz and Prescott,
465 2014). The similar amounts of viral RNA observed in bank voles from Ain and Jura at the end of
466 the experiment could support the hypothesis that sensitivity to PUUV did not differ between these
467 two regions. However, this result has to be taken cautiously as it is likely that variability in virus
468 replication and excretion might only be visible sooner after the infection (Hardestam et al., 2008;
469 Dubois et al., 2017a). Because we observed more bank voles from Jura than Ain with PUUV RNA

470 in their sera eight days after the infection, we can not refute the possibility of a regional
471 heterogeneity in sensitivity to PUUV.

472 The immune-related gene expression analyses conducted using wild bank voles also provide
473 arguments in favor of this hypothesis. Rodents from Ain over-expressed *Mx2* and *Tnf- α* genes
474 compared to bank voles from Jura. These results are congruent with previous studies conducted in
475 the French Ardennes and over Europe (Guivier et al., 2010; Guivier et al., 2014). These immune
476 related genes were chosen to represent some of the main pathways underlying bank vole immune
477 responses to PUUV infections. They encode respectively for TNF, a proinflammatory cytokine and
478 *Mx2*, an antiviral protein, that both limit PUUV replication (Kanerva et al., 1996; Temonen et al.,
479 1996; Jin et al., 2001). Besides, the overproduction of these molecules could induce
480 immunopathologies (Li and Youssoufian, 1997; Wenzel et al., 2005; Bradley, 2008), what could
481 drive a balance of resistance / tolerance to PUUV (Guivier et al., 2010; Guivier et al., 2014).
482 Altogether these regional differences in bank vole responses to PUUV could reflect potential
483 divergence with respect to resistance and tolerance strategies. These differences may be mediated
484 by a subset of individuals within populations, as suggested by bank voles exhibiting extreme values
485 of immune related gene expression and mostly explaining the significant differences observed.
486 Within population evolution from resistance to tolerance has previously been demonstrated in an
487 unmanaged population of Soay sheep (Hayward et al., 2014) and in wild populations of field voles
488 (Jackson et al., 2014). Further assessment of bank vole health and fitness would be required to
489 confirm this hypothesis of tolerant and resistant phenotypes with regard to PUUV infections
490 (Raberg et al., 2009). It would also be interesting to address the issue of potential physiological
491 trade-offs between inflammatory and antibody-mediated responses (Lee and Klasing, 2004). The
492 patterns observed in this study could suggest that bank voles from the NE endemic region would
493 promote an adaptative immunity in response to PUUV infection instead of a more costly and
494 potentially damaging inflammatory response, potentially as a result of co-adaptation between bank
495 vole and PUUV (Easterbrook and Klein, 2008).

496

497 **Conclusions**

498 This study highlighted the importance of combining natural population surveys and experimental
499 approaches in addressing questions related to immunoheterogeneity and its potential consequences

500 for epidemiological questions. Here the natural population survey enabled to study a large number
501 of animals and to describe inter-individual and inter-population variability in immune
502 responsiveness, that may further blur the results obtained from experimental infections. Besides, the
503 experiments enabled to control for the time since exposure to PUUV and to follow immune
504 responses kinetics. The diverse array of eco-evolutionary approaches developed here brought
505 important answers to evaluate the global risk of NE emergence in a non endemic region. Our results
506 supported the possibility for PUUV introduction in the region Ain via the dispersal of PUUV
507 seropositive bank voles from Jura. They also indicated the possibility of PUUV circulation in this
508 non endemic region as experimental infections revealed that bank voles from Ain are sensitive to
509 PUUV. Therefore, NE emergence in this region might only be a question of time. But several
510 arguments also indicated that PUUV persistence might be unlikely. Lower susceptibility to PUUV
511 could account for the absence or low number of PUUV infected bank voles in the Ain region, and
512 also potentially for the weak level of PUUV excretion in the environment. Further investigations are
513 now required to identify the mechanisms underlying bank vole immunoheterogeneity between
514 adjacent NE endemic and non endemic regions, in particular with regard to abiotic (e.g. climate,
515 resource availability) or biotic conditions (e.g. spatially varying pathogen communities, see for the
516 bank vole Behnke et al., 2001; Ribas Salvador et al., 2011; Razzauti-Feliu et al., 2015; Loxton et al.,
517 2016). Sociological and human behavioral factors would then be the ultimate step to analyse what
518 limits PUUV transmission between *M. glareolus* and humans.

519

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526

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758 **Legends to Figures.**

759

760 **Fig. 1.** Map of the study area. Each color represents a site of trapping and each circle or diamond
761 represents a trapping line. The location of the two regions (Jura and Ain) within France is shown on
762 top left.

763

764 **Fig. 2.** Scatterplot of the first two principal components discriminating *M. glareolus* populations by
765 sites using a discriminant analysis of principal components (DAPC) based on microsatellite data.
766 Points represent individual observations. Envelops represent population membership. Bank vole
767 populations from Ain (NE non-endemic region) are represented in green and those from Jura (NE
768 endemic region) in blue.

769

770 **Fig. 3.** Variations of immunological characteristics in natural bank vole populations. a) Variations
771 of *Mx2* gene expression between individuals of the two regions, Ain (NE non-endemic region) and
772 Jura (NE endemic region). b) Variations of *Tlr7* gene expression between males and females. c) and
773 d) Variations of *Tnf- α* gene expression between the two regions and between sex within the two
774 regions.

775

776 **Fig. 4.** Variation of the optical density in ELISA test (OD_{450nm}) over time for the 14 bank voles that
777 seroconverted during the experimental infections. Barplots and error bars represent the mean
778 OD_{450nm} value \pm SD for all the individuals in each region. The hatched line represents the threshold
779 above which the bank voles were considered to be PUUV seropositive.

780

781

Table 1.

Sampling site characteristics

Region	Sites	Dates of sampling (dd/mm/yyyy)	Number of captured voles	Number of infected voles (Number of infected juveniles)	Prevalence (Prevalence without juveniles)
Ain	Chatillon-en-Michailles	28/08/2014 - 31/08/2014	44	0	0 %
(non endemic)	Cormaranche-en-Bugey	01/09/2014 - 03/09/2014	46	0	0 %
		12/06/2014 - 15/06/2014			
	Chaux-des-Crotenay	23/08/2014	56	4 (0)	7.1 %
Jura	Mont-sous-Vaudrey	07/06/2014 - 10/06/2014	40	4 (2)	10.0 % (5 %)
(endemic)	Mignovillard	16/06/2014 - 18/06/2014	45	0	0 %
	Poligny	24/08/2014 - 26/08/2014	46	4 (0)	8.7 %

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Table 2.

F_{st} estimates between sites per year (lower side) and associated p -values of Fisher exact tests (upper side).

	Vaudrey	Crotenay	Mignovillard	Poligny	Chatillon	Cormaranche
Vaudrey		< 10 ⁻⁵	< 10 ⁻⁵	< 10 ⁻⁵	< 10 ⁻⁵	< 10 ⁻⁵
Crotenay	0.01676***		< 10 ⁻⁵	< 10 ⁻⁵	< 10 ⁻⁵	< 10 ⁻⁵
Mignovillard	0.02296***	0.01887***		< 10 ⁻⁵	< 10 ⁻⁵	< 10 ⁻⁵
Poligny	0.01501***	0.00926***	0.01453***		< 10 ⁻⁵	< 10 ⁻⁵
Chatillon	0.02142***	0.01844***	0.02279***	0.02071***		< 10 ⁻⁵
Cormaranche	0.03116***	0.02501***	0.02161***	0.02388***	0.01566***	

Significant p -values are in bold; *** $p \leq 0.001$

Table 3.

Results of the analysis of molecular variance (AMOVA). The AMOVA hierarchy was based on geographical features, comparing the two regions (Ain and Jura) and the sites within the regions.

Source of variation	<i>d.f.</i>	Variance of components	Percentage of variation	<i>p</i>
Among regions	1	0.04481	0.73	0.068
Among sites within regions	4	0.09903	1.61	< 10⁻⁵ ***
Within sites	536	6.00519	97.66	< 10⁻⁵ ***

Significant *p*-values are in bold; *** $p \leq 0.001$

1 **Table 4.**

2 Isolation by distance characteristics — probability (IBD p), intercept and slope calculated for each region and for the whole dataset. 95% confidence
3 intervals (CI) for the slope of the IBD were obtained by bootstrapping over the 14 microsatellite loci.

Dataset	IBD p	Intercept	Slope [95% CI]
Ain	< 10⁻⁵ ***	0.043	0.003 [2.10 ⁻⁵ , 0.006]
Jura	< 10⁻⁵ ***	0.059	0.004 [6.10 ⁻⁴ , 0.009]
Ain and Jura	< 10⁻⁵ ***	0.057	0.005 [0.003, 0.009]

4 Significant p -values are in bold; *** $p \leq 0.001$

Fig. 1.

Map of the study area. Each color represents a site of trapping and each circle or diamond represents a trapping line. The location of the two regions (Jura and Ain) within France is shown on top left.

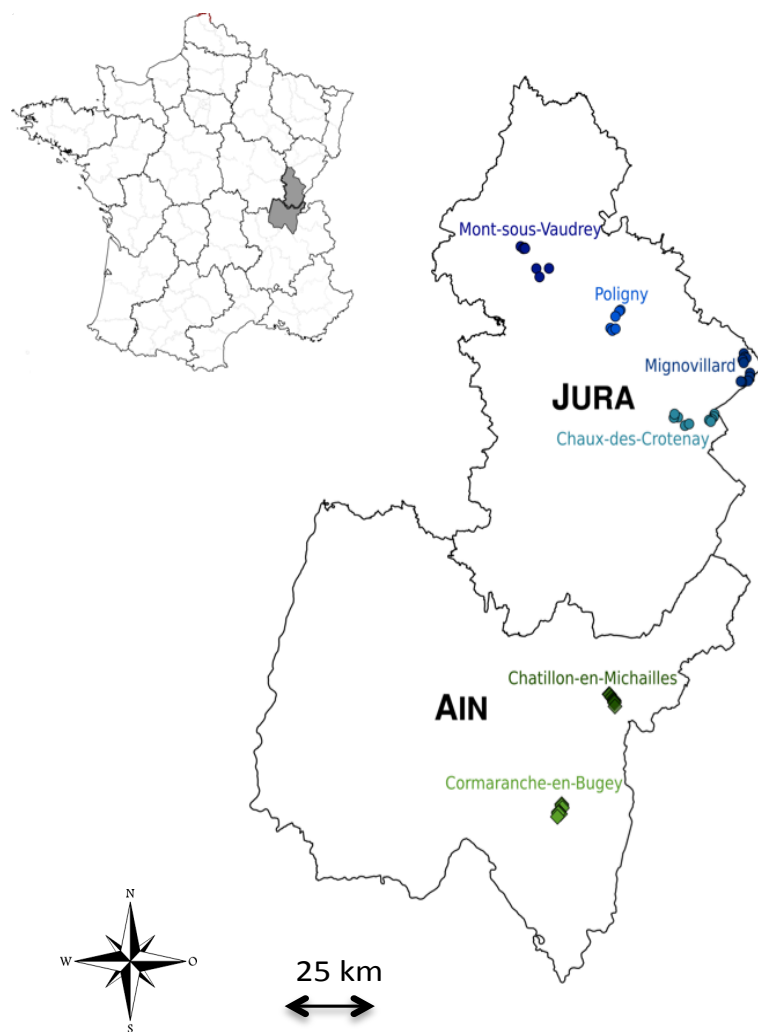
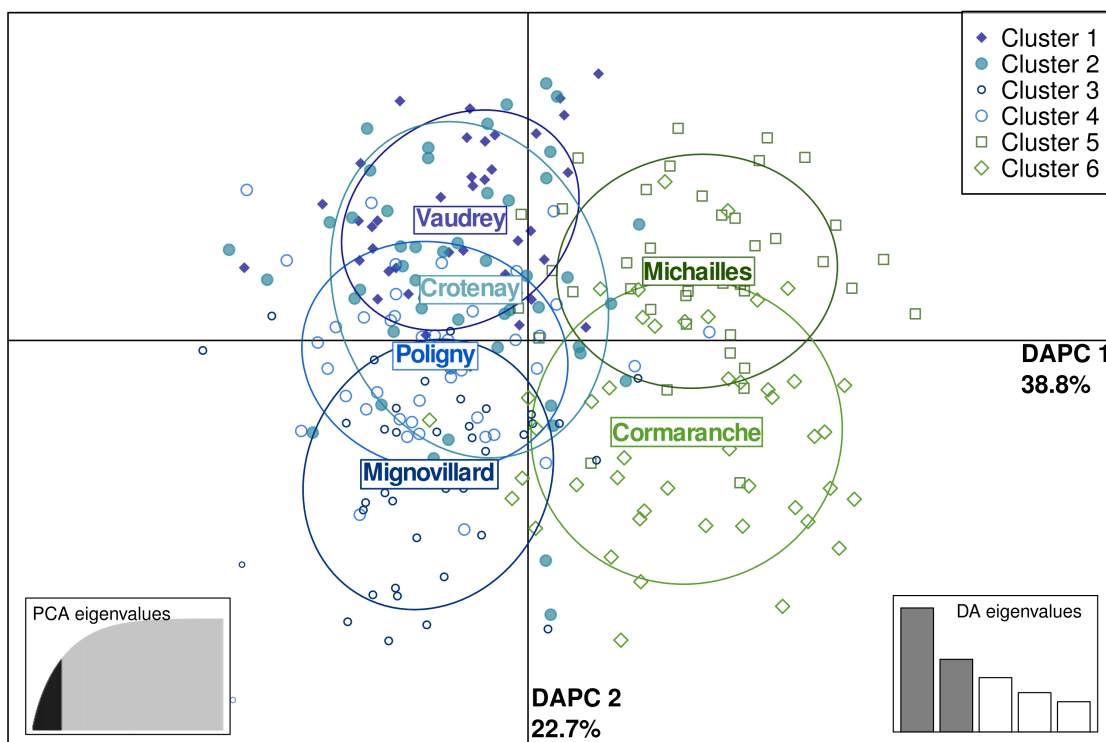


Fig. 2.

Scatterplot of the first two principal components discriminating *M. glareolus* populations by sites using a discriminant analysis of principal components (DAPC) based on microsatellite data. Points represent individual observations. Envelops represent population membership. Bank vole populations from Ain (NE non-endemic region) are represented in green and those from Jura (NE endemic region) in blue.



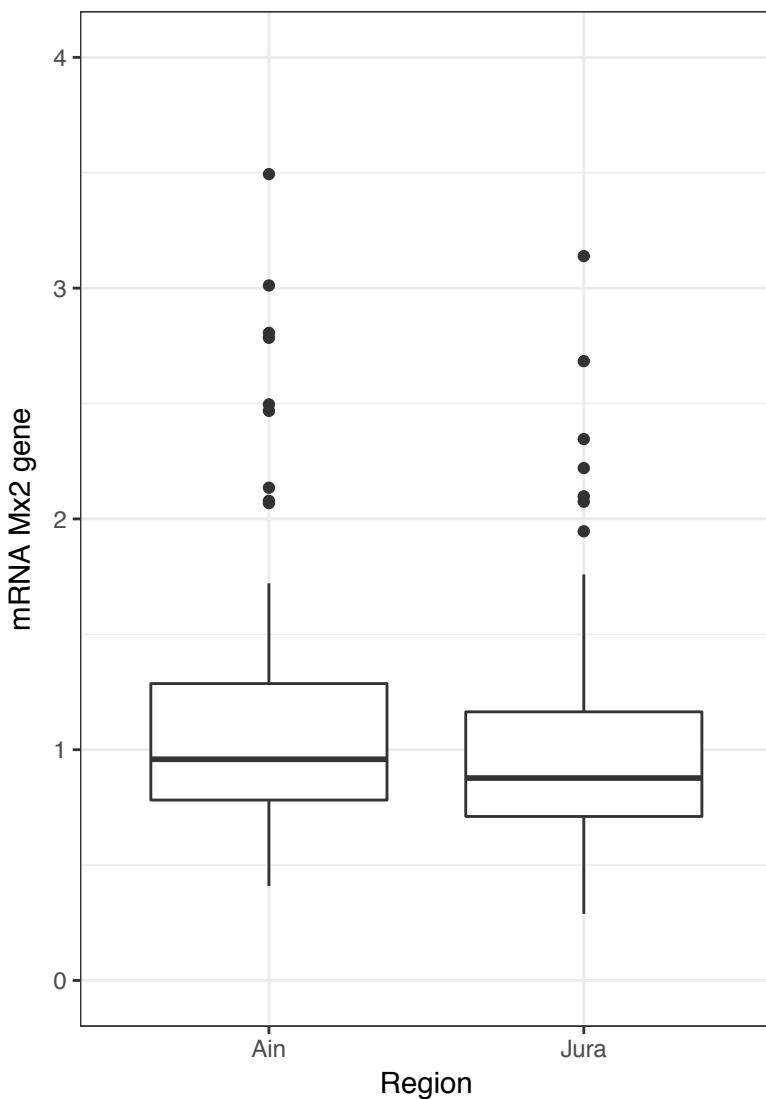
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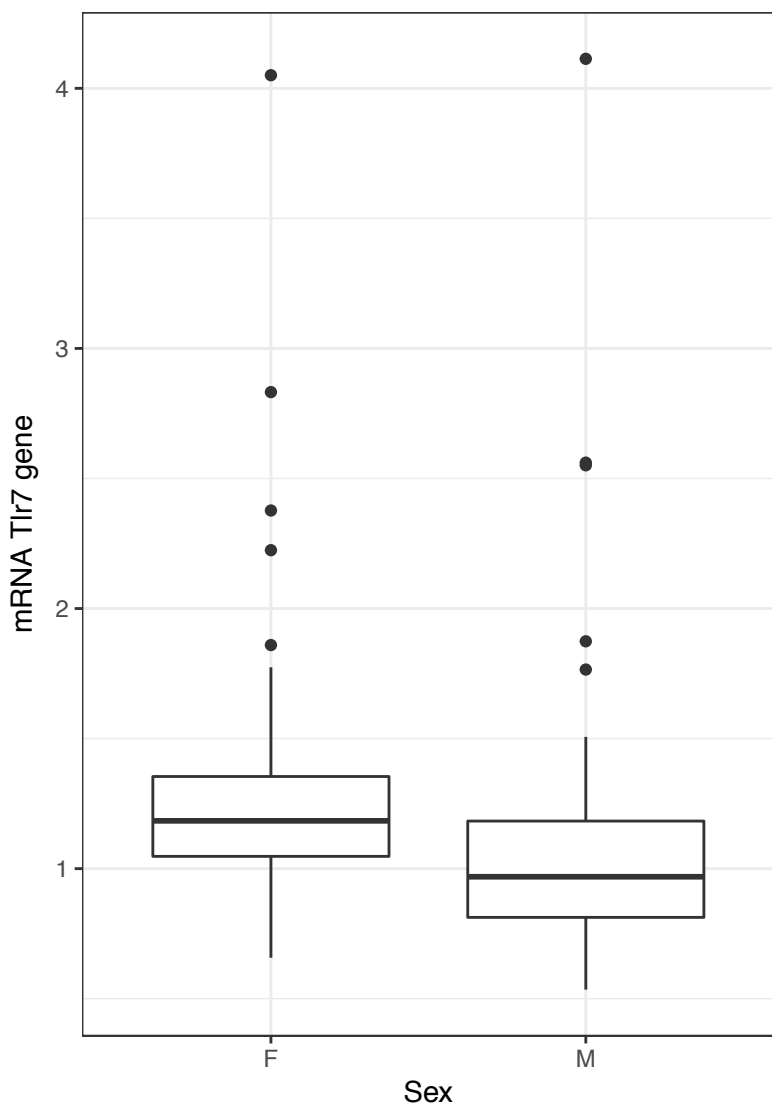
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Variations of immunological characteristics in natural bank vole populations. a) Variations of *Mx2* gene expression between individuals of the two regions, Ain (NE non-endemic region) and Jura (NE endemic region). b) Variations of *Tlr7* gene expression between males and females. c) and d) Variations of *Tnf- α* gene expression between the two regions and between sex within the two regions.

a)



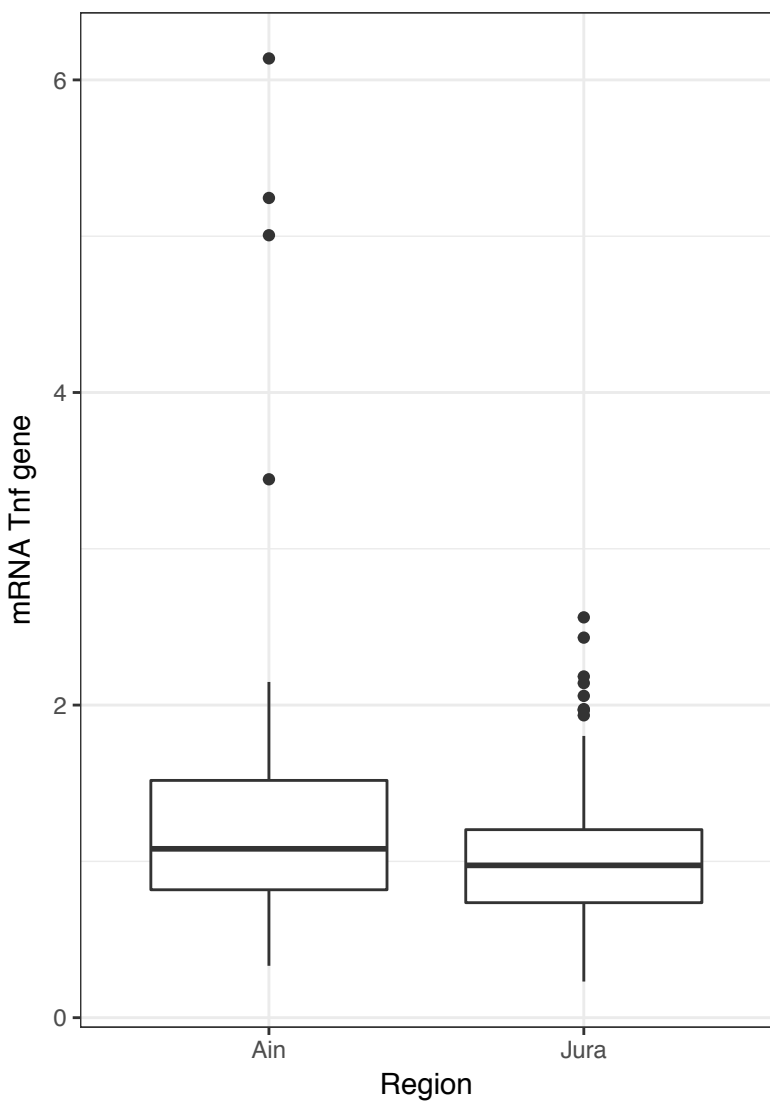
b)



c)

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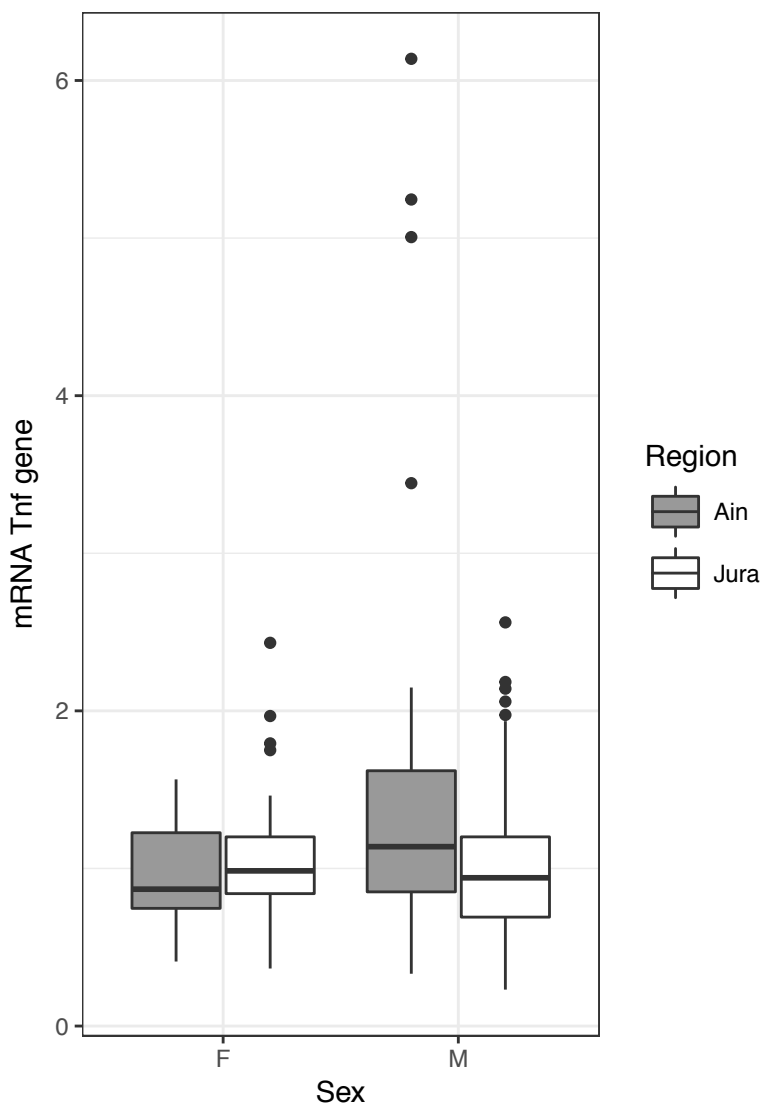
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d)

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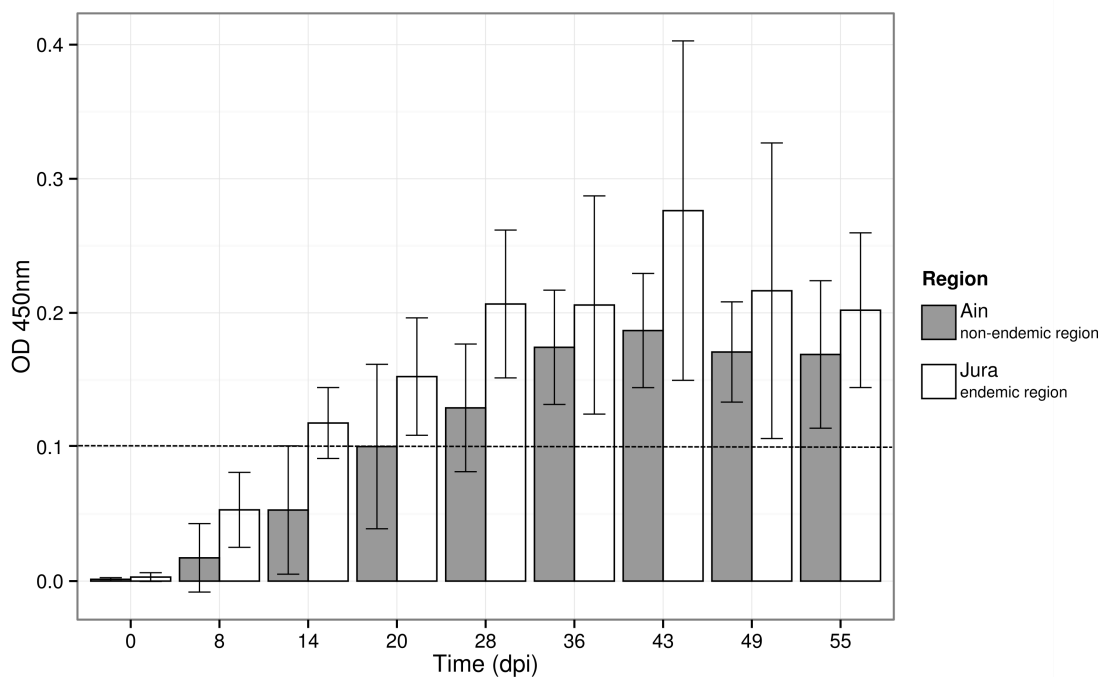


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Fig. 4.

Variation of the optical density (OD_{450nm}) over time for the 14 bank voles that seroconverted during the experimental infections. Barplots and error bars represent the mean OD_{450nm} value \pm SD for all the individuals in each region. The hatched line represents the threshold above which the bank voles were considered to be PUUV seropositive.



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Supplementary Table 1. Sequences of primers and probes and cycling conditions of quantitative and nested RT-PCR.

Method	Primer and probe name and sequence (5' to 3')	Cycling steps	Temperature (°C)	Time	Number of cycles
Quantitative RT-PCR	Primer PUU R (CCKGGACACAYCATCTGCCAT)	Reverse transcription	50	30 minutes	1
	Primer PUU F (GARRTGGACCCRGATGACGTTAA)	Hot Start activation	95	10 minutes	1
	Probe PUU 1 (CAACAGACAGTGTCAGCA)	Denaturation	95	15 seconds	45
	Probe PUU 2 (CAACARACAGTGTCAGCA)	Annealing /Extension	60	60 seconds	45
		Cooling	40	30 seconds	1
RT-PCR	Primer PPT334C (TATGGIAATGTCCTTGATGT)	Reverse transcription	50	60 minutes	1
		Denaturation	94	2 minutes	40
		Annealing	44	60 seconds	40
	Primer PPT986R (GCACAIGCAAAIACCCA)	Extension	68	2 minutes	40
		Final elongation	68	7 minutes	1

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Supplementary Table 2. Detection of PUUV RNA in sera, lung, liver and urine of the 10 adult seropositive bank voles detected in natural populations.

Region	Individual number	Sera (RNAcopies.μl ⁻¹)	Lung (RNA copies. mg ⁻¹)	Liver (RNA copies. mg ⁻¹)	Urine (RNA copies.μl ⁻¹)
Jura	NCHA00014	N	1.21e8	3.42e7	N
	NCHA00041	N	7.27e8	1.95e7	N
	NCHA00071	8.90e4	7.13e8	5.79e8	N
	NCHA00073	1.91e4	6.61e8	8.09e7	x
	NCHA00086	2.61e4	9.72e7	3.93e8	x
	NCHA000143	N	7.50e6	1.79e6	x
	NCHA000150	1.01e4	1.35e8	1.48e8	x
	NCHA000178	1.33e6	4.88e9	8.63e8	2.26e4
	NCHA000180	1.58e5	3.29e8	6.94e8	x
	NCHA000181	6.77e4	8.33e7	7.00e7	2.08e4

x : samples not available. N indicates that the qRT-PCR has been done but the cycle threshold was too high (above 45).

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Supplementary Table 3. Anti-PUUV antibody responses of bank voles to PUUV infection through time.

Region	Individual number	OD _{450nm}								
		0 dpi	8 dpi	14 dpi	20 dpi	28 dpi	36 dpi	43 dpi	49 dpi	55 dpi
Ain non-endemic	15.129_63	0.0000	0.0050	0.0700	0.0890	0.1270	0.2440	0.258	0.24	0.264
	15.129_67	0.0010	0.0025	0.0000	0.1090	0.1640	0.1510	0.155	0.144	0.118
	15.129_89	0.0000	0.0040	0.0380	0.0320	0.0750	0.1270	0.146	0.137	0.135
	15.129_92	0.0020	0.0210	0.0810	0.1430	0.1550	0.1650	0.217	0.172	0.196
	15.129_96	0.0030	0.0040	0.0050	0.0380	0.0700	0.1540	0.172	0.178	0.129
	15.129_98	0.0020	0.0675	0.1240	0.1910	0.1840	0.2050	0.173	0.154	0.172
	15.129_100	0.0030	0.0025	0.0090	0.0380	0.0850	0.0520	0.034	0.039	0.04
	15.129_66	0.0010	0.0310	0.0970	0.0420	0.0100	0.0150	0.018	0.028	0.042
Jura endemic	15.131_52	0.0000	0.0410	0.0850	0.1180	0.1120	0.0870	0.097	0.061	0.099
	15.131_53	0.0060	0.0615	0.1710	0.2450	0.2460	0.2950	0.441	0.405	0.27
	15.131_68	0.0050	0.0600	0.1020	0.1490	0.2550	0.3260	0.467	0.308	0.241
	15.131_70	0.0000	0.0310	0.1150	0.1330	0.2580	0.1890	0.285	0.25	0.206
	15.131_71	0.0070	0.0015	0.1310	0.1430	0.2550	0.2630	0.296	0.222	0.257
	15.131_72	0.0060	0.0925	0.1220	0.1830	0.1950	0.1820	0.235	0.213	0.198
	15.131_73	0.0000	0.0710	0.0950	0.1060	0.1680	0.1500	0.216	0.098	0.143
	15.131_74	0.0000	0.0665	0.1220	0.1430	0.1640	0.1550	0.173	0.175	0.202

The amount of anti-PUUV IgG is expressed using the optical density measured at 450 nm (OD_{450nm}). A sample is considered as seropositive when the OD_{450nm} exceeds 0.1 (values are then indicated in bold).

The three animals that died at 28 dpi are not included in the Table.

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Supplementary Table 4. Detection of PUUV RNA in excretas collected at 8 and 20 days post-infection using nested RT-PCR.

Region	Individual number	Serological status	Sera		Feces		Saliva	
			8dpi	20dpi	8dpi	20dpi	8dpi	20dpi
Ain non- endemic	15.129_63	+	POS	N	N	N	N	N
	15.129_67	+	N	N	N	N	N	N
	15.129_89	+	N	N	N	N	N	N
	15.129_92	+	N	N	N	N	N	N
	15.129_96	+	N	POS	N	N	N	N
	15.129_98	+	N	N	N	N	N	N
	15.129_100	-	N	N	N	N	N	N
	15.129_66	-	POS	N	N	N	N	N
Jura endemic	15.131_52	+	POS	N	N	N	N	N
	15.131_53	+	N	N	N	N	N	N
	15.131_68	+	POS	N	N	N	N	N
	15.131_70	+	N	N	N	N	N	N
	15.131_71	+	N	N	N	N	N	N
	15.131_72	+	POS	N	N	N	N	N
	15.131_73	+	POS	N	N	N	N	N
	15.131_74	+	N	N	N	N	N	N

- indicates bank voles that did not seroconvert during the experiment; + indicates bank voles that seroconverted during the experiment. POS indicates that the nested RT-PCR enabled to amplify PUUV RNA.

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Supplementary Table 5. Detection of PUUV RNA in organs and excretas collected 55 days after experimental infections using quantitative RT-PCR.

Region	Individual number	Serological status	Sera	Lung	Liver	Kidney	Saliva	Urine	Feces
Ain non- endemic	15.129_63	+	N	4.07e7	3.07e6	1.76e6	N	N	N
	15.129_67	+	N	4.34e5	N	N	N	N	N
	15.129_89	+	N	5.03e6	1.11e6	1.48e6	N	N	N
	15.129_92	+	N	N	N	N	N	N	N
	15.129_96	+	N	3.32e6	1.69e6	1.71e7	N	N	1.64e7
	15.129_98	+	N	4.89e6	N	8.59e5	N	N	N
	15.129_100	-	N	N	N	N	N	N	N
	15.129_66	-	N	7.65e6	N	9.58e6	N	N	N
Jura endemic	15.131_52	+	N	1.03e7	N	N	N	N	N
	15.131_53	+	N	6.35e6	N	2.00e6	N	N	3.55e6
	15.131_68	+	N	3.54e6	N	5.30e5	N	N	N
	15.131_70	+	N	4.30e6	N	N	N	N	N
	15.131_71	+	N	3.17e7	1.32e6	6.50e5	N	N	N
	15.131_72	+	N	8.90e5	N	5.19e5	N	N	N
	15.131_73	+	N	N	N	4.35e5	N	N	N
	15.131_74	+	N	5.74e5	N	N	N	N	N

- indicates bank voles that did not seroconvert during the experiment; + indicates bank voles that seroconverted during the experiment. Viral RNA is expressed in RNA copies. mg⁻¹. N indicates that the qRT-PCR has been done but the cycle threshold was too high (above 45).

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