Role of individual and population heterogeneity in shaping dynamics of multi-pathogen excretion in an island endemic bat

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S.A. and M.D. designed research. S.A., M.T., G.L.M., C.L., A.O.H., C.T., A.D., L.J., R.V.R., P.T., and M.D. performed data samplings. S.A., M.T. and A.D. performed laboratory works. S.A. and M.D. analyzed data. S.A. and M.D. led the writing, with final approval from all co-authors. P.M. and M.D. supervised work and led the project administration.

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We have no competing interests to declare.

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Short title

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Abstract

Understanding processes driving pathogen transmission in bats is critical to prevent spillovers and emergence events. However, it requires fine-scale ecological investigations, which are rare because of the complexity and heterogeneity of bat populations. Here, based on the monitoring of 5,949 Reunion free-tailed bats (*Mormopterus francoismoutoui*) in 17 roosts over 24 months, we studied the simultaneous excretion of paramyxovirus and *Leptospira* bacteria in urine, and herpesvirus in saliva. We analysed the influence of individual and roost-level factors on co-excretion dynamics. We evidenced all year long excretion with high prevalence (37% - 87%), with low levels of temporal synchrony among roosts and no signal of density-dependent transmission. Recapture data support persistent infections for all infectious agents, alongside with clearance followed by reinfection for paramyxovirus and *Leptospira*. Our results suggest that sex-specific social behaviour or physiological adaptations drive herpesvirus excretion, while paramyxovirus and *Leptospira* excretion reactivate during reproduction. Our data also suggest the protection of juveniles by maternal antibodies, but different waning dynamics, much shorter for HSV infection, that could lead to a pathogen-dependent role of juveniles in the spread of infection. Interestingly, the excretion of one infectious agent was positively correlated to the excretion of a second one. However, the probability of excreting a third one was reduced for individuals already bi-excreting, suggesting negative interactions for cumulative infections. Connecting such spatio-temporal data with ecosystem alterations in Reunion Island could further contribute to our understanding of the links between human-mediated changes in the ecology of bats and infectious diseases emergence processes.

66 ## Significance Statement

67 Understanding risks of bat-borne pathogen spillover is challenging because of the
68 difficulty in studying excretion dynamics in wild bat populations. Here, we used an original
69 island-endemic model to build up a fine-scale spatio-temporal analysis of the excretion of
70 two viruses (paramyxovirus and herpesvirus) and a bacterium (*Leptospira*) at both
71 population- (roost) and individual- (through recaptured bats) levels. Excretion patterns
72 are specific to each infectious agent and driven by the age and the reproduction of both
73 females and males. Results also suggest that a combination of viral persistence and
74 clearance, as well as interactions between infectious agents, are important within-host
75 processes that contribute to the transmission of infections in bat populations. This
76 information is crucial to better predict shedding pulses and prevent potential future
77 spillovers.

Main Text

Introduction

Recent human outbreaks, such as those caused by the SARS-CoV-2 virus, have highlighted the importance of understanding processes driving the transmission of infectious agents within bat populations (1). Indeed, a direct link between infection dynamics in reservoir bats and zoonotic spillovers has been pointed out for several viral human diseases (2–4). Thus, identifying the ecological drivers of pathogen transmission within bat populations, at both within- and between-host levels, is a key element for predicting spillover events (5, 6). Unfortunately, ecological studies investigating infection dynamics in bats often lack of robust spatio-temporal datasets (7). This gap mainly results from logistical constraints due to the elusive and roaming nature of bats that make these animals difficult to study in long-term periods, and limits the breadth of data acquisition. Moreover, as gregarious mammals, bats often display metapopulation dynamics (8). Such spatio-temporal heterogeneity is characterized by periodically interacting spatial discrete patches, with various sex and age-structure, roost size and function (9, 10), which adds complexity to disentangle individual and populational drivers of infection dynamics in bats (11–13).

With molecular tools becoming increasingly accessible for the study of infectious agents, it is now recognized that co-infections are common in bats (14–18). However, most of the previous longitudinal studies on bats have focused on a single infectious agent, or different variants within the same viral family (e.g. 19–22). Thus, our knowledge on spatio-temporal co-infection dynamics with different groups of infectious agents (DNA/RNA viruses, bacteria, fungi) remains limited (23–25). Theory predicts that co-infections can have major consequences on within-host infection dynamics because of potential interactions that might be antagonistic or mutualistic (17, 26). For example, the infection of field voles with the bacterium *Anaplasma phagocytophilum* increased by five times the susceptibility to cowpox virus (27). Examining the spatio-temporal patterns of simultaneous excretion of infectious agents within bat roosts (co-circulation) and

individual hosts (co-infection) is likely to provide much-needed further insights into underlying processes driving infection dynamics (21).

Island are widely considered as relevant model systems in ecology and evolutionary biology studies (28, 29), including those focusing on emerging diseases (e.g. (30, 31). Islands are well-known ecosystems hosting limited species number and high levels of endemism (32). Due to their ability to flight over water, bats are often the only native mammals in remote oceanic islands and among the 1,456 recognized bat species, 60% live in island and 25% are even endemic (33). The clear boundaries and often reduced surface of islands can help sample island endemic bat populations at a fine scale, which is crucial to make reliable inferences about spatial and temporal infection dynamics (34). Moreover, the reduced number of bat species on islands may also simplify ecological interactions, by minimizing interspecies contacts, and by sampling monospecific bat roosts, which can contribute to a more in-depth understanding of infection dynamics at the population level. Due to the reduced species richness typical of small territories, insular ecosystems may also lack predators or competitors and offer empty ecological niches, which can favor the spread of island endemic bats and the expansion of their population size. This is especially true for bat families with highly gregarious behavior such as free-tailed bats (Molossidae), for which roosts can host hundreds of thousands of individuals (35, 36). High bat densities favor the sampling of large numbers of individuals, a prerequisite for epidemiological studies. Island endemic bats, with such an abundant, simplified, and well-defined system, represent a rare opportunity to collect extensive spatio-temporal data to capture a comprehensive picture of subtle mechanisms underlying infection dynamics in bats (37).

The Reunion free-tailed bat (*Mormopterus francoismoutoui*; 38) is a small tropical insectivorous bat endemic to Reunion Island. This volcanic territory is located in the southwestern Indian Ocean and has emerged about 3 million years ago (39). Although small in size (2,512 km^2), Reunion Island is shaped by a mountainous landscape and has undergone significant landscape changes following 350 years of human colonization associated with intensive urbanization and agriculture (40). Reunion free-tailed bats are

134 broadly distributed on the island and monospecific roosts occur in a diversity of natural
135 habitats, such as caves and cliffs. These mollosid bats have also adapted to urbanization
136 and use numerous anthropogenic roosts such as buildings and bridges, hosting from a
137 few hundred to more than 100,000 individuals (9, 38). A recent longitudinal monitoring
138 of several roosts revealed highly dynamic roosting behaviors, with roosts of various sizes,
139 sex-ratio, and functions (female-biased maternities, mixed colonies or male-biased
140 roosts) (9). Specifically, large female aggregations (up to 50,000 pregnant females) are
141 observed synchronously every year in a few roosts, before parturition in austral summer
142 (December), which coincides with a female-biased sex-ratio and a spatial sexual
143 segregation during this period of the year (9, 41). Moreover, the end of austral summer
144 (May) is characterized by a decrease in roost size and a change in sex-ratio in the studied
145 roosts (from female- to male-biased), suggesting important seasonal sex-specific
146 movements within the island, which is corroborated by the absence of spatial genetic
147 structure in this metapopulation (42).

148 Reunion free-tailed bats are natural hosts for several infectious agents (19, 23,
149 43), including paramyxovirus and *Leptospira* bacteria (41). Previous non-invasive
150 sampling of bat faeces and urine underneath roosts highlights that shedding pulses
151 occurred during the austral summer, followed by a sustained transmission during winter
152 (23, 41, 43). However, these studies were based on a reduced number of samples, which
153 were not associated to information on bat individuals (because no bat capture was
154 performed), precluding direct insights into the role of individual factors (e.g. sex, age,
155 reproductive status, body condition) shaping these infection dynamics. Moreover,
156 investigations were respectively performed on a single (41) and two maternity roosts (23),
157 and the role of population-level factors (e.g. roost size, sex-ratio) thus remains poorly
158 understood. Here, we used data from a fine-scale spatio-temporal monitoring and
159 sampling of *M. francoismoutoui*, based on the study of 17 roosts all over Reunion Island
160 during two consecutive years. We measured the simultaneous excretion (nucleic acid
161 detection) of three infectious agents in each individual bat, focusing on paramyxovirus
162 (viral RNA) and *Leptospira* (bacteria DNA) both shed in urine, and herpesvirus (viral DNA)

163 in the saliva. Specifically, we first aimed at analysing spatio-temporal trends in excretion
164 prevalence and at testing for roost synchronicity. Then, we evaluated whether individual
165 (sex, age, reproductive status) and roost-level (roost size, sex-ratio and level of sexual
166 spatial segregation) variables explain prevalence of single infectious agents, co-excretion
167 patterns, and the probability of changing excretion status (within-host dynamics).
168

169 ## Results

170 **Spatio-temporal dynamics of excretion prevalence.**

171 Between January 2019 and December 2020 (24 months), we captured 5,949 Reunion
172 free-tailed bats in 17 roosts all over Reunion Island (Fig. 1), including 526 recaptures (up
173 to four recaptures were recorded for the same individual). Excretion data were obtained
174 from 5,518 urine samples for paramyxoviruses (PMV) and *Leptospira* bacteria (LEPTO) and
175 from 3,981 saliva samples for herpesviruses (HSV), including 3,784 individuals for which
176 all three infectious agents were tested. Over the entire study period, we measured a high
177 global prevalence with 36.63% ± 1.27% of bats shedding PMV, 46.48% ± 1.32% shedding
178 LEPTO and 87.14% ± 1.04% shedding HSV (Table 1). We used generalized additive mixed
179 models (GAMM, *SI Appendix,* Tables S1 and S2) to characterize variation in prevalence
180 over space and time. Results revealed no difference in prevalence between the two
181 consecutive years, except for HSV (M3: $\chi^2_{0.8} = 4.64$, $P < 0.01$). Globally, we found statistical
182 evidence of intra-annual variations for PMV and HSV prevalence (M1: $\chi^2_{6.7} = 16.30$, $P =$
183 0.04; M3: $\chi^2_{8.2} = 40.06$, $P < 0.001$), but when investigating data in adults only, similar
184 temporal patterns were observed for all three infections, with a higher prevalence in
185 October-December and a decrease from March to June (Fig. 2A).

186 Excretion of the three infectious agents was detected in all tested roosts, except
187 PMV in the TM5 roost (temporary roost exclusively composed of juveniles; see 9).
188 Globally, no variation of PMV prevalence was observed among roosts, although we
189 detected a significant difference for HSV and LEPTO (M2: $\chi^2_{8.2} = 96.73$, $P < 0.001$; M3: $\chi^2_{4.4}$
190 $= 9.61$, $P = 0.02$). Finally, a low level of temporal synchrony in prevalence was detected
191 among roosts, as only 11.1 % of pairwise comparisons were significant (*SI Appendix,* Fig.

192 S1), and synchrony did not increase among nearby roosts (PMV: r^2 = -0.11; LEPTO: r^2 =
193 0.42; HSV: r^2 = -0.06; all $P > 0.05$).
194
195 **Roost-level drivers of excretion.**
196 Roost size varied from 15 to 100,000 individuals but was not correlated with the
197 prevalence of PMV and LEPTO (M1: z = 0.62; M2: z = 1.41; all $P > 0.05$), while HSV
198 prevalence slightly decreased in larger roosts (M3: z = -2.92, $P < 0.01$). However, this
199 negative correlation was driven by the low HSV prevalence in juveniles (the period when
200 roost sizes were the highest), and disappeared when juveniles were removed from the
201 model (M3 without juveniles: z = -0.99, P = 0.32). Moreover, prevalence was not
202 correlated with the sex-ratio within roosts (M1: z = 0.01; M2: z = -1.05; M3: z = -1.81; all
203 $P > 0.05$) nor with sexual segregation levels of adults among roosts (PMV: r^2 = 0.39; LEPTO:
204 r^2 = 0.43; HSV: r^2 = 0.51, all $P > 0.05$).
205
206 **Individual variation of excretion.**
207 Models revealed that adults were systematically more frequently shedding viruses and
208 bacteria than juveniles (M1: z = -5.82; M2: z = -6.13; M3: z = -11.56, all $P < 0.001$). Mean
209 prevalence of excretion in adults was 43.00 ± 1.42% for PMV, 52.85 ± 1.43% for LEPTO
210 and 96.20 ± 0.65% for HSV. At the time when juveniles started to fly (January-February;
211 9), excretion prevalence in juveniles was either null (PMV) or low (LEPTO: 12.03 ± 4.11%
212 and HSV: 15.03 ± 5.66%). Then, over the following six months, HSV prevalence increased
213 drastically in juveniles to reach 60.67 ± 7.82%, while it remained low, or only slightly
214 increased, for PMV and LEPTO (Fig. 2A). Excretion prevalence also varied according to sex,
215 but only in adult bats, and differently according to the targeted infectious agent. Indeed,
216 adult males were more frequently shedding HSV than females (Tukey's post hoc test: t =
217 -4.10, $P < 0.001$), although the opposite pattern was observed for PMV (Tukey's post hoc
218 test, t = 4.57, $P < 0.001$).
219 To test the influence of reproduction on the excretion status in adults, we focused
220 our analysis on adults during two reproductive periods: pregnancy (November-

221 December) and mating (March-April). During pregnancy, the detection of the two urine-
222 excreted agents, PMV and LEPTO, was higher in pregnant than in non-pregnant females
223 (M4: χ_1 = 37.49; M5: χ_1 = 24.99, all $P < 0.001$, Fig. 2B). Similarly, during the mating period,
224 reproductively active males were more frequently found shedding than non-reproductive
225 males, but only for LEPTO (M8: χ_1 = 6.76, $P < 0.01$, Fig. 2B). Higher HSV prevalence was
226 also observed in both reproductively active females and males, but the difference was
227 not statistically significant, probably because of a high variability within the non-
228 reproductive groups.
229
230 **Co-excretion dynamics.**
231 Among the 3,784 bats with paired samples (both urine and saliva collected), 36.89% (n =
232 1,396) were shedding two infectious agents and 22.46% (n = 850) were shedding three (*SI*
233 *Appendix,* Fig. S2). Only 399 bats (10.54%) did not excrete any of the three screened
234 infectious agents, with 79.2% of them being noteworthily juveniles. The excretion of one
235 infectious agent was strongly positively correlated with the excretion of any other
236 infection (for all combinations in M1, M2, M3, all $P < 0.001$). Indeed, when considering
237 bats shedding one infectious agent, the probability of shedding a second infectious agent
238 was multiplied from 1.29 to 31.9 folds, for HSV when shedding LEPTO and for PMV when
239 shedding HSV, respectively (Fig. 3). By contrast, individuals already shedding two
240 infectious agents were less frequently shedding the third one (M1: z = -3.12, $P < 0.01$; M2:
241 z = -2.42, $P = 0.02$; M3: z = -2.42, $P = 0.02$). For example, *Leptospira* prevalence increased
242 by 3.89 times when bats were already shedding HSV, and by 6.50 times when already
243 shedding PMV, but only by 2.60 times when shedding both HSV and PMV (Fig. 3).
244 Models showed no significant intra-annual variations for bi- and tri-excretions,
245 except for PMV-HSV excretion (M12: $\chi_{6.7}$ = 19.03, $P = 0.02$), and no effect either of sex-
246 ratio, sexual segregation or roost size. By contrast, we detected a strong influence of age
247 and sex, with bi- and tri-excretions being more frequent in adults (M10: z = -4.74; M11: z
248 = -10.70; M12: z = -7.37; M13: z = -4.71; all $P < 0.001$), especially in adult females (Tuckey's
249 post hoc test, M10: t = 4.68, $P < 0.001$; M12: t = 3.68, $P < 0.01$, M13: t = 4.30, $P < 0.001$).

250 Finally, pregnancy increased the probability of bi- and tri-excretions in females (M14: χ_1 =
251 19.85; M15: χ_1 = 23.04; M16: χ_1 = 28.81; M17: χ_1 = 17.39; all $P < 0.001$) and reproductively
252 active males had a higher probability of shedding simultaneously LEPTO and HSV
253 compared to non-reproductive males (M19: χ_1 = 8.36; $P < 0.01$).
254
255 **Within-host dynamics of excretion.**
256 All captured bats were marked (by tattooing on the wing) allowing to collect excretion
257 data at the individual level. We tested 471 and 457 samples for PMV and LEPTO
258 respectively, including initial capture and samples from recaptured bats. We also tested
259 86 saliva samples from 31 recaptured bats (from 2 to 5 capture events) for the presence
260 of HSV. All bats initially testing positive for HSV (n = 29) remained positive over time
261 (between 25 and 405 days between recapture events), while the only two initially-
262 negative bats were testing positive at their first recapture. Thus, within-host analyses
263 using multinomial logistic regressions of excretion status changes were only performed
264 on PMV and LEPTO data. Recapture intervals ranged from 25 to 719 days (*SI Appendix,*
265 Fig. S3A), during which we defined four conversion classes: negative to negative (no
266 conversion), negative to positive, positive to negative, and positive to positive (no
267 conversion). For many recaptured animals, bats kept the same excretion status over time.
268 Indeed, between 50.74% (PMV) and 40.04% (LEPTO) of recaptured bats remained
269 negative or, to a lesser extent (PMV: 25.69%, LEPTO: 34.79%) positive. Interestingly, when
270 looking at short windows of time (from 1 to 6 month-intervals), a major proportion (about
271 75%) of PMV or LEPTO-positive bats remained positive (Fig. 4A). Some bat individuals
272 remained positive across all their recapture events (Fig. 4B).
273 The probability of the four excretion status changes was dependent on the time
274 interval for LEPTO (M23: χ^2_3 = 17.60, $P < 0.001$, Fig. 4C and *SI Appendix,* Fig. S3B), but not
275 for PMV (M22, χ^2_3 = 5.11, P = 0.16, *SI Appendix,* Fig. S3C). Indeed, Figure 4 showed that
276 LEPTO-negative bats had a higher probability of remaining negative over short periods of
277 time, although the probability of becoming positive increased with time. However, when
278 individuals were shedding LEPTO at the time of their first capture, the probability of

279 becoming negative was not dependent on the time. In contrast, the probability of being
280 still shedding LEPTO did increase with time. We also found a significant effect of changing
281 reproductive status on the probability of changing both PMV and LEPTO excretion status
282 (M22: $\chi^2_9 = 32.45$, $p < 0.001$; M23: $\chi^2_9 = 20.11$, $P = 0.02$, *SI Appendix,* Fig. S4). Indeed, bats
283 that remained non-reproductively active over recapture events (reproduction: 0/0), also
284 mainly remained LEPTO- and PMV-negative (conversion: 0/0). Similarly, most bats
285 successively captured when reproductively active (reproduction: 1/1) remained LEPTO-
286 and PMV-positive (conversion: 1/1). Most bats becoming positive across recaptures also
287 became reproductively active, especially for PMV (20.97% for PMV and 11.67% for
288 LEPTO).
289

290 **Discussion**

291 Using an original island-endemic bat model, we demonstrated the widespread and all-
292 year-long co-circulation of three viral and bacterial infections in Reunion free-tailed bat
293 populations, which confirms the circulation of PMV and LEPTO (23, 41) and describes for
294 the first time HSV infection in this bat species. We revealed high global prevalence of PMV
295 (37%) and LEPTO (46%), and extremely high prevalence of HSV (87%). This is coherent
296 with the high prevalence of HSV generally found in other bat species, as recently reported
297 in the Mexican free-tailed bat *Tadarida brasiliensis* (44) and in the common vampire bat
298 *Desmodus rotundus* (12, 45). High infection prevalence in Reunion free-tailed bats could
299 be explained by a strong gregarious behaviour of these bats, which can aggregate up to
300 900 individuals per m^2 (46). The proximity of individuals within high-density roosts can
301 facilitate transmission through bat fluids (saliva, urine), in which case, roost size is often
302 assumed to positively correlate with prevalence (4, 47, 48). However, we did not find such
303 a correlation, suggesting that density-dependent transmission may not play a major role
304 in the circulation of paramyxovirus, *Leptospira* and herpesvirus in Reunion free-tailed
305 bats. However, we cannot exclude that if prevalence is biased towards certain groups of
306 individuals that aggregate in high density within area, then transmission is likely to be
307 related to the density of these high-risk bat groups, rather than the overall roost size

308 measured in our study (49). Interestingly, we did not find evidence of spatial synchrony
309 between roosts, despite probably frequent intra- and inter-seasonal movements within
310 this bat metapopulation (9, 42). Thus, transient epidemics, passing as waves of bat-to-bat
311 transmission between roosts, are probably not the main mechanism involved in
312 maintenance (4, 11, 50) and the probability of excretion seems therefore more related to
313 individual factors than population-level characteristics.

314 Chronic or episodic shedding from persistently infected bats can explain high
315 prevalence across roosts and time. Indeed, our results shows that nearly all (96%) of
316 recaptured bats, initially HSV-positive, were still shedding HSV in saliva over recaptures,
317 supporting lifelong HSV infection in Reunion free-tailed bats. HSV are known to establish
318 latent infection in humans (51) and rodents (52), and the same mechanism is also
319 suspected in bats (53, 54). Interestingly, in *D. rotundus* infected with a betaherpesvirus,
320 the length of the active periods (when shedding occurs) appears to be longer than the
321 latency state (when shedding resumes) (54), which is coherent with our recapture data
322 showing almost always HSV-positive bats over recaptures. For PMV and LEPTO, we
323 showed that viral RNA and bacterial DNA were present in urine for up to 3 months after
324 their initial detection in a large proportion of recaptured bats (75%). Together with
325 examples of bats remaining positive over multiple recapture events, this supports that
326 persistent infection can also explain PMV and LEPTO temporal dynamics. However, we
327 also evidenced positive to negative excretion switches for PMV and LEPTO, suggesting
328 that cycles of clearance followed by reinfection also occur in the bat population.
329 Persistent LEPTO infection in bats is not surprising as this bacterial infection is often
330 considered chronic in other reservoir hosts, bacteria forming protective biofilms in renal
331 tubules (55). Experimental infections have shown that *Leptospira interrogans* can stably
332 colonize the kidneys of rats and mice over their lifespan, with intermittent excretion of
333 the bacteria that can last for at least 3 months (56, 57). Our data show that when
334 individuals are shedding LEPTO at the first capture, the probability of becoming negative
335 is not dependent on the time, which is in keeping with an intermittent excretion. On
336 contrary, mechanisms underlying within-host PMV dynamics are still debated (5, 58).

337 Several field and lab evidence support that henipavirus, for example, can persist within
338 bat individuals with long-term continuous or intermittent excretion (11, 37, 59–62). A
339 modelling study also suggests that latent henipavirus infections can occur alongside with
340 occasional viral clearance, followed by temporary immunity and reinfection (58). This
341 same study supports that bats are much more likely to develop a latent infection than
342 clearing it. Finally, it has been hypothesized that a small portion of super-long-shedder
343 individuals with a long infectious period could maintain Hendra virus in bat populations
344 (63). Distinguishing between these different infection processes is complex. Finally, since
345 because we only measured the presence of viral and bacterial nucleic acids in our study,
346 an important step will be to assess the infectiousness of excreted samples to finely
347 validate the role of long-term continuous and intermittent excretion in transmission (4).

348 In persistent infections, the reactivation process is often linked to several stressors
349 that may affect host immunity (64, 65). Reproduction, by imposing immunological
350 challenges, is recognized as a major stressor that could lead to the reactivation of viral
351 and bacterial infections (66). Such processes are expected to be particularly pronounced
352 in female mammals, for whom reproduction requires large and prolonged energetic
353 investments (67, 68). We showed that pregnancy increased the probability of PMV and
354 LEPTO (but not HSV) excretions, as well as bi- and tri-excretions in Reunion free-tailed
355 bats. Variable patterns between infectious agents are coherent with the increased
356 seroprevalence observed in reproductively active females of *P. scapulatus* infected with
357 Hendra virus (69), and the absence of such correlation for HSV-infected *Rousettus*
358 *aegyptiacus* and *Miniopterus natalensis* (70). Moreover, recapture data showed that most
359 bats becoming PMV and LEPTO positive across recaptures were also those becoming
360 reproductively active. We thus hypothesize that both PMV and LEPTO excretions are
361 reactivated during female's pregnancy in Reunion free-tailed bats. In humans, such
362 process has been described for the Varicella Zoster Virus, for which reactivation occurs
363 commonly during the third trimester of pregnancy (71). Reactivation during bat
364 pregnancy could explain the enhanced prevalence observed during the pregnancy period
365 (October to December), a pattern reported in other field studies (19, 20, 23, 41, 70, 72).

366 Such reactivation could also lead to horizontal transmission to non-immune bats.
367 However, we cannot exclude that immigration of infected bats also has a role in
368 transmission during late pregnancy, as massive aggregations of bats occur at this time of
369 the year (9). Our study therefore supports the role of a trade-off between pregnancy and
370 immunity. Some studies have hypothesized that such process, beside conferring
371 protection to newborns through the transfer of protective maternal antibodies, could
372 benefit to the mothers through a boost of immunity (71).

373 Although less documented, male reproduction trade-offs have been suggested in
374 bats, with higher Hendra seroprevalence observed in *Pteropus scapulatus* males during
375 the mating season (69). Our study revealed that, during the mating period, reproductively
376 active males had a higher probability of shedding LEPTO and LEPTO-HSV, as compared to
377 non-reproductive males. By contrast, no difference was observed for PMV excretion. This
378 suggests that trade-offs between reproduction and immunity in males could drive
379 excretion dynamics, but that differences exist depending on the considered infectious
380 agent. Such pattern could be mediated by reduced immune function during
381 spermatogenesis, a period of high energy demand and elevated testosterone (73, 74). As
382 well, reproductively active males of *Myotis daubentonii* have lower cellular immunity than
383 non-reproductive individuals (75).

384 Previous studies have shown that environmental stressors could be critical to HSV
385 transmission in wildlife (76). HSV was, for example, detected in the blood of big brown
386 bats (*Eptesicus fuscus*) arousing from hibernation, but not in non-hibernating individuals,
387 suggesting a reactivation of the virus resulting from the stress associated with arousal
388 (53). Accordingly, our results showed a slight increase of HSV prevalence in adult bats at
389 the end of winter (July to November). However, Reunion free-tailed bats are tropical, and
390 we thus suspect that no strict hibernation occurs (77), supported by observations of bat
391 activity all year long (9). The HSV increase in winter might alternatively result from a
392 misclassification of juveniles in adults, because differentiating those age classes based on
393 the epiphysis fusion is challenging at this period. Since juveniles are less infected than
394 adults, we might have artificially lowered the prevalence in adults.

395 Behavioral and physiological traits are two mechanisms that can produce sex-
396 biased prevalence in several wildlife species (78, 79). Here, males were more frequently
397 shedding HSV than females, as previously reported in rodents (52) or for other DNA bat
398 viruses such as adenoviruses (80). Studies on HSV prevalence in bats are limited and
399 report contrasting results, with no sex difference in *D. rotundus* (12), while Sjodin et al.,
400 (81) suggest that either males or females can be more infected, depending of the
401 herpesvirus strain. Male-biased infection may arise from sex-specific social behaviour like
402 dominance or higher aggressiveness (physical injuries, i.e. bite) (82, 83), that could
403 enhance transmission in males (52, 84). However, this hypothesis is not well-supported
404 in Reunion free-tailed bats, since increased transmission among aggressive males should
405 lead to a higher prevalence during the mating season, which was not observed. Sex
406 hormones can also strongly affect host susceptibility, through the modulation of
407 immunity (85). Testosterone may suppress immune responses in males, increasing
408 susceptibility to pathogen infection (86). The inclusion of hormone and immunity
409 measures should thus be considered in future infection studies to test this hypothesis in
410 bats.

411 Maternal transmission of antibodies conferring specific protection to juveniles is
412 a well-known process in mammals, and has often been suggested and demonstrated in
413 bats (2, 60, 69, 72, 87–89). The level of maternally transferred antibodies to juveniles is
414 thought to reflect the amount of circulating antibodies in females, and could thus
415 positively correlate with infection prevalence (90). Here, although the prevalence in adult
416 females was much higher for HSV than for PMV and LEPTO, up to 20% (in 2019) of
417 juveniles were HSV-positives at the start of the juvenile period (January), while almost all
418 juveniles were non-infected by PMV (with an intermediate pattern observed for LEPTO).
419 This suggests that despite a wider excretion of HSV in the bat population, initial levels of
420 maternal antibodies could be lower for HSV, compared to PMV and LEPTO. If the waning
421 of maternal antibodies in juveniles depends on the initial levels provided by mothers, this
422 can explain the distinct excretion dynamics later observed in juveniles. Indeed, although
423 most juveniles remained PMV and LEPTO negative during the first six months of their life,

424 HSV excretion rapidly increased to reach 80% in July 2020. Contrary to HSV, the protection
425 against PMV and LEPTO seems therefore quasi-total (although lower for LEPTO) and
426 longer (> six months) for these two infections, suggesting different strategies of maternal
427 immunity in bats. Apparent short-term maternal immunity has been proposed in Egyptian
428 fruit bats (*Rousettus aegyptiacus*) infected with Marburg virus, for which juveniles'
429 infection increases by 5-fold within 3 months (2), while a longer protection of about 6-7
430 months has been reported from field and modelling studies on Lagos bat virus and
431 henipaviruses (37, 87, 91). Finally, the rapid and steady HSV infection in juveniles
432 coincides with their initial dispersal all over the island (9) and could thus play a major role
433 in the spatial spread of this virus (2, 23, 69, 72). By contrast, the spread of PMV and LEPTO
434 by juveniles is probably lower (or occurs later in the year), as juveniles remain poorly
435 infected at least during their first six months of life, highlighting that the role of juveniles
436 in infection dynamics is pathogen-dependent (63).

437 Our investigation targeting multiple simultaneous infections revealed a high co-
438 circulation of viruses and bacteria within the bat population, with 59% of bats shedding
439 at least two infectious agents. Our results also showed that bats shedding one virus or
440 bacteria had significantly more chance of shedding another one. This corroborates
441 previous results suggesting positive interactions that could facilitate the excretion of
442 paramyxo- (21, 41) and corona-viruses (16, 25). In the study of Davy et al. (16), the stress
443 of fungal infection by *Pseudogymnoascus destructans* led to a reactivation of coronavirus
444 replication in the little brown bat *Myotis lucifugus*, and co-infection even increased the
445 severity of White Nose Syndrome. Interestingly, in our study, when bats were already
446 shedding two infectious agents, they had less probability of shedding the third one,
447 suggesting that negative interactions can also occur when accumulating infections.
448 Interactions between infectious agents could modulate host susceptibility with
449 contrasting effects (antagonistic and mutualistic) depending on the community of
450 infectious agents considered (27). In a previous investigation on Reunion free-tailed bats
451 we indeed highlighted that coinfection between Paramyxovirus, Coronavirus and
452 Astrovirus was a very rare event (1% of tested samples), without interaction between

453 viruses (23). These studies illustrate how considering simultaneously multiples infections,
454 although complex, is important to better understand drivers of infection in bats (17, 92,
455 93).

456 This study sheds light on the processes driving the spatio-temporal excretion
457 dynamics of RNA and DNA viruses as well as bacteria in bats. In addition to suggested
458 latent herpesvirus infections, our results suggest that bats commonly host persistent
459 paramyxovirus and *Leptospira* infections, with reproduction-related episodic excretion, in
460 both females and males. Surprisingly, transient epidemics and density-dependent
461 transmission do not appear as major drivers of these infections. Moreover, pathogen-
462 dependent waning immunity dynamics likely modulates the role of juveniles in the spatial
463 spread of infections. Testing these hypotheses requires serological data and spatially
464 explicit and mechanistic modelling studies (94). Disruption of balanced host-pathogen
465 relationships due to stressors, such as habitat loss or co-infections, may increase
466 pathogen excretion, and enhance the likelihood of spillovers (3). Further studies
467 connecting multi-infection dynamics to the growing urbanization process that faces
468 Reunion free-tailed bats will help in understanding how human-mediated changes in the
469 ecology of bats may affect natural excretion dynamics and in turn the probability of
470 spillovers (95).

471

472 ## Materials and Methods

473 **Study sites and field sampling**

474 We monitored and sampled 17 roosts (coded with a 3-letter code) from January 2019 to
475 December 2020 (24 months), all over Reunion Island (Fig. 1). Among the 17 studied roosts,
476 12 were monitored across a maximum of 15 successive "sampling periods" occurring
477 every 3–8 weeks, each sampling period lasting for 3–4 weeks to be able to sample
478 multiple roosts (Table 1 and Table S3). An opportunistic sampling was performed for five
479 additional roosts, which were thus monitored only once during the studied period. To
480 investigate within-host dynamics, we used data from recaptured bats, and thus also
481 included previously collected samples from 2018 (referred as the first capture samples).

482 Bat monitoring includes bat capture, biological sampling and roost size estimation
483 and is fully described in Aguillon et al. (9). Captures were carried out using harp traps
484 (Faunatech Ausbat) and Japanese monofilament mist nets (Ecotone) during dusk
485 emergence, with a maximum of 60 individuals captured per night. Bats were hydrated
486 immediately after the capture and kept individually in a clean individual bag hang close
487 to a warm water bottle. For each bat, we measured the forearm length and mass, and
488 determined the sex, age, and reproductive status. Age was established by examining the
489 epiphysis fusion in finger articulations that are not welded for juveniles which was easily
490 identifiable until July, while some juveniles might have been classified as adults in the
491 following months. Active reproductive status was recorded when females were pregnant
492 and lactating, and in males when they had large testes (see 9 for more details). A sterile
493 swab (Puritan Medical Products, USA) was carefully inserted in the corner of the lips to
494 sample saliva and then placed in 250uL of MEM (Minimum Essential Medium Eagle). Using
495 a pipette and a sterile tip, urine droplets were collected in the waterproof bag or directly
496 at the urethral opening when handling bats, and then placed in 50 µL of MEM. All samples
497 were stored in a cooled box in the field before being transferred at -80°C at the laboratory
498 the same night. Finally, each bat was tattooed on the right propatagium with an individual
499 alphanumeric code (96) and released at the capture site.
500 Bats were handled using personal protective equipment regularly changed and all
501 the equipment was disinfected between sites (more details in 9). Bat capture and
502 manipulation protocols were evaluated and approved by the Minister of Higher
503 Education, Research and Innovation (APAFIS#10140-2017030119531267), and conducted
504 under a permit delivered by the Direction de l'Environnement, de l'Aménagement et du
505 Logement (DEAL) of Reunion Island (DEAL/SEB/UBIO/2018-09).
506
507 **Molecular detection of infectious agents**
508 Urine and saliva samples were processed using the Cador Pathogen 96 Qiacube HT kit
509 (Qiagen, Hilden, Germany), using 25 µL and 100 µL of samples, respectively, mixed with
510 175 µL and 100 µL of VXL buffer for the lysis step. Total nucleic acids were extracted using

511 an automated extractor Qiacube with small modifications of the Q Protocol using 350 µL
512 of ACB, 100 µL of AVE, and 30 µL of TopElute. A reverse transcription was then performed
513 on urine extracts (97), followed by the screening of paramyxovirus and *Leptospira*
514 bacteria, using a semi-nested PCR targeting the RNA polymerase gene and a real-time PCR
515 amplifying a fragment of the 16S rDNA gene, respectively (41, 98, 99). For paramyxovirus,
516 amplicons of the first PCR were diluted to 1:10, and 2 µL was then used in the semi-nested
517 PCR.

518 For saliva extracts, a fragment of herpesvirus DNA polymerase was amplified using
519 a nested PCR. During the first PCR, 2 µL of DNA was amplified in a 25 µL mixture containing
520 12.5 µL of GoTaq® Green Master Mix 2X (Promega, Madison, Wisconsin, United States),
521 2.5 µL of each primer at 10 µM DFA (5'-GAYTTYGCNAGYYTNTAYCC-3') and KG1 (5'-
522 GTCTTGCTCACCAGNTCNACNCCYTT-3') (100). We used the following thermal conditions:
523 95°C for 2 min, and 45 cycles at 94°C for 30 sec, 46°C for 1 min and 72°C for 1 min, and a
524 final step at 72°C for 7 min. During the nested PCR, 5 µL of amplificon was amplified in a
525 25 µL mixture containing 12.5 µL of GoTaq® Green Master Mix 2X (Promega, Madison,
526 Wisconsin, United States), 2.5 µL of each primer at 10 µM TGV (5'-
527 TGTAACTCGGTGTAYGGNTTYACNGGNGT-3') and IYG (5'-
528 CACAGAGTCCGTRTCNCCRTADAT-3') (100). We used the following thermal conditions:
529 95°C for 2 min, and 35 cycles at 94°C for 30 sec, 46°C for 30 sec and 72°C for 30 sec, and
530 a final step at 72°C for 7 min. PCR products were revealed using electrophoresis with 2%
531 agarose gel stained with 2% GelRed (Biotium, Fremont, USA).
532

533 **Statistical analyses**

534 To evaluate spatio-temporal dynamics of excretion, we fit binomial generalized additive
535 mixed models (GAMM, 101) via restricted maximum likelihood (REML) using the *mgcv*
536 package (Table S1). We used penalized smoother for the temporal trend variable and
537 controlled that the effective degrees of freedom (EDF) were higher than 1 (non-linear
538 effect). The number of basis functions (k) and smooth parameters (alpha) were adjusted
539 in each model using *gam.check* from the *mgcv* package to avoid overfitting and check for

540 model convergence. Temporal trend was implemented specifying a number for each
541 sampling period starting from 1 (January-February) to 16 (November-December, Table
542 S3). Due to the COVID-19 crisis and the resulting lockdown in France, we did not monitor
543 roosts in April 2020, and do not present data at that time. To properly account for the
544 temporal effect, we decided to code the May-June 2020 sampling period as the middle of
545 March (coded as 10) and July 2020 (coded as 13, Table S3). Final models for mono-, bi-
546 and tri- excretions included the sampling period as smooth function, the roost size, sex-
547 ratio, age and sex (and their interaction) as linear function, and the roost, Julian day and
548 year as random effects. The significance of each modality of age/sex interactions was
549 tested using a Tukey's post hoc test. For mono-excretion models, we included the two
550 other infectious agents and the corresponding bi-excretion as a linear function (details in
551 Tables S2 and S3). Moreover, to determine periods of significant increase/decrease in the
552 prevalence of mono-excretions in adults, we used simplified models that only included
553 sampling period as a smooth function, and identified periods where the 95% confidence
554 interval (CI) of the GAM prediction's derivative did not overlap zero, using the R function
555 *derivSimulCI* (www.gist.github.com/gavinsimpson).

556 To assess the effect of reproductive status on excretion, we focused on adult bats
557 at two specific periods of the year: firstly, in adult females during the pregnancy period
558 (November-December) and then in adult males during the mating period (March-April)
559 (9). We thus used a separate GLMM on a subset of data, for each sex and included
560 reproductive status as a linear effect, keeping roost, Julian day and year as random effects
561 (Table S1 and S2).

562 The influence of sexual spatial segregation patterns in adults was tested on the
563 prevalence of mono-, bi-, and tri-excretions, using the Sexual Segregation and
564 Aggregation Statistic (SSAS, 102) that compares observed aggregation and segregation
565 patterns to a random association of males and females among roosts. We calculated SSAS

values for each sampling period, and tested if the SSAS was correlated to the global monthly prevalence of excretion using a Pearson correlation test.

Then, we analysed the spatial (between roosts) synchrony in prevalence using the same approach as in Meza et al. (13). We selected seven roosts (ESA, MON, PSR, RBL, RPQ, TGI and VSP) that were all monitored during every sampling periods. We used Mantel tests (from *vegan* package) to investigate geographical distances between roosts and calculated pairwise synchrony of prevalence by sampling period for each infectious agent separately, using Spearman correlations tests (Fig. S1).

To investigate within-host excretion dynamics, we tested the probability of changing infectious agent excretion status through time, by fitting a multinomial logistic regression using the *multinom* function in the *nnet* package (103) (Table S4). Because not enough saliva samples were available to analyse the conversion of HSV excretion status, we only modelled conversion status associated with PMV and LEPTO, separately. Conversion status was used as a nominal dependent variable with four levels (negative to negative, negative to positive, positive to negative, and positive to positive), with the following covariates: recapture interval (in days), sex, and reproductive status conversion (active to active, non-active to active, active to non-active and non-active to non-active). Age classes were not included in the models because of the limited number of juveniles compared to adults (n_{JUV} = 29, n_{AD} = 497). This analysis used the non-changing infection, negative to negative, as a baseline outcome against which to compare other infectious status changes. To better visualize the probable continuous excretion over short time windows (<6 months), we calculated and plotted the percentage of bats remaining positive for PMV and LEPTO across different time intervals: less than 2 months, between 2 and 4 months, between 4 and 6 months.

All analyses were performed in Rstudio 1.4.1106 (104) using packages *dplyr*, *effects, emmeans*, *lme4*, *ggeffects*, *ggplot2*, *mgcv, nnet,* and *vegan*.

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Data Availability

Metadata are archived on Zenodo (https://doi.org/10.5281/zenodo.12527877).

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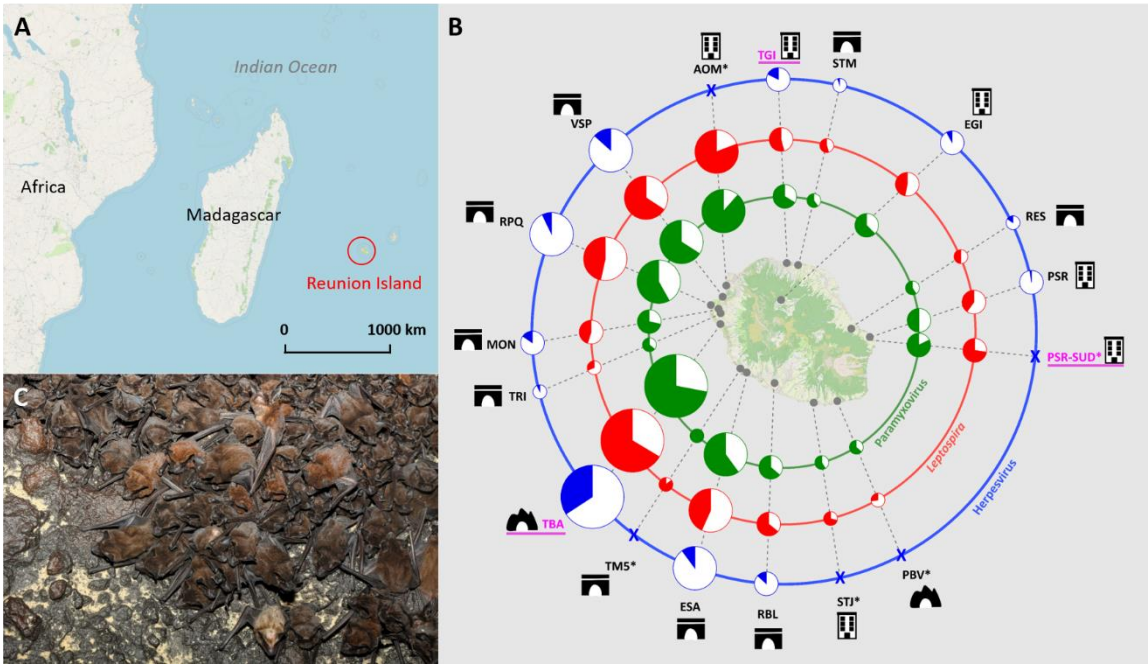
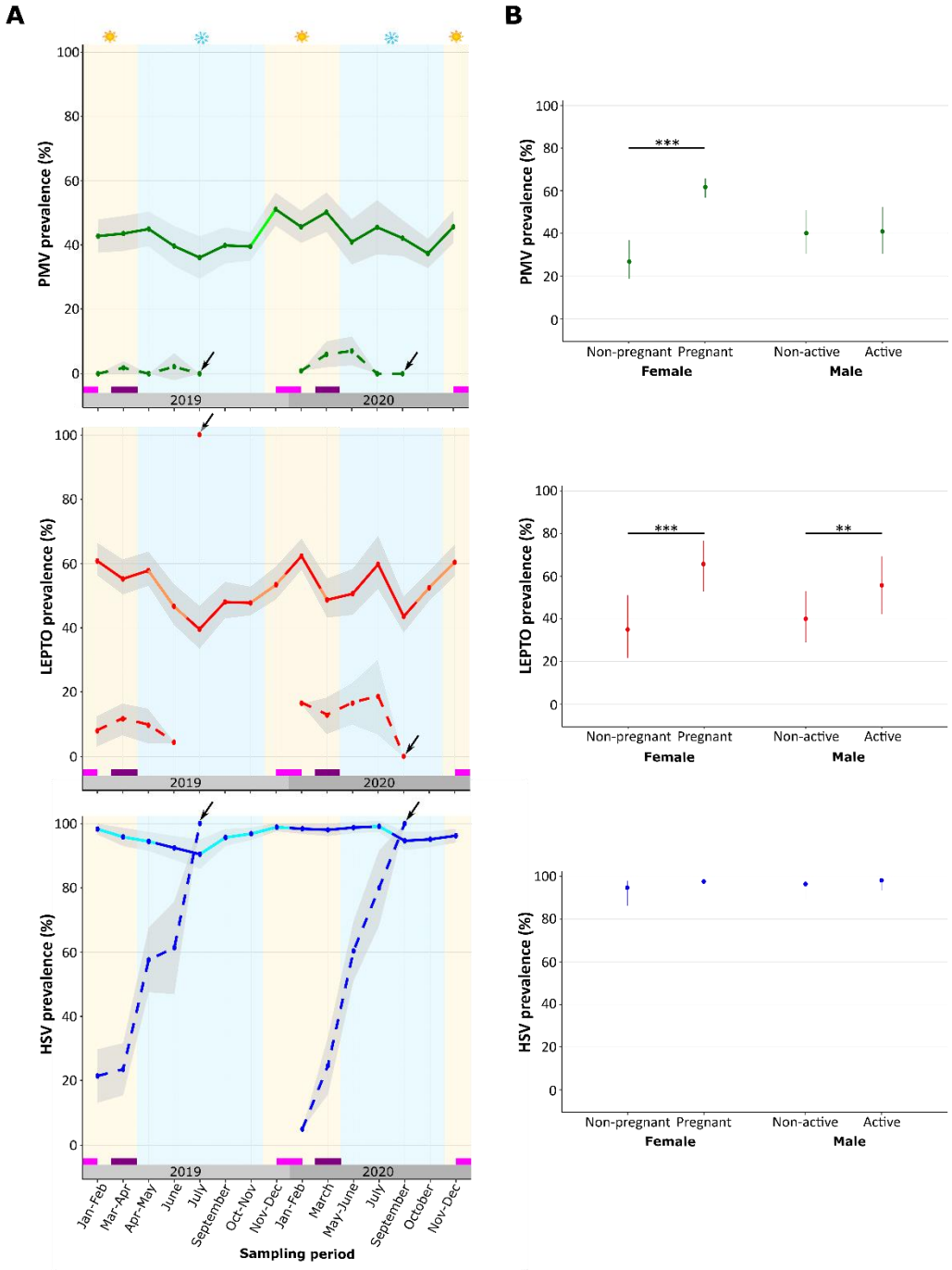
Figures and Tables

Figure 1. Widespread triple infection in *Mormopterus francoismoutoui.* (A) Location of Reunion Island and (B) the studied roosts. (C) A picture of bats aggregated in the maternity roost (TBA site) before parturition. In (B) studied roosts are represented by grey circles on Reunion Island map and prevalence (white section of pie charts) is represented for paramyxovirus (in green), *Leptospira* bacteria (in red) and herpesvirus (in blue). A cross denotes the absence of prevalence data (no tested samples). Pie diameter corresponds to four categories of roost size: small < 500 individuals, medium: 500-2000 individuals, high: 2000-6000 individuals, and exceptional > 100,000 individuals. Accurate roost size estimation was not being performed for EGI and PSR roosts, but they are represented as medium roosts because several hundred/thousands are expected. Maternity roosts are underlined in pink. Roosts that were monitored only once are depicted by an asterisk. Maps were created using Qgis (105) with a background of OpenStreetMap under License CC- BY- S. This figure was inspired by Streicker et al. 2012 (48).



865 **Figure 2. Age and reproduction are the main drivers of paramyxovirus, *Leptospira***
866 **bacteria and herpesvirus excretion dynamics in *Mormopterus francoismoutoui.***
867 (A) Observed prevalence in adults (solid line) and juveniles (dotted line), with grey areas
868 representing 95% confidence intervals. Periods of significant change (where GAM
869 derivative 95% does not overlap zero) are highlighted by light-colored segments (light
870 green for PMV, orange for LEPTO and light blue for HPS). Arrows indicate periods where
871 only one individual was tested. Pink and purple blocks at the bottom indicate the
872 parturition period and the mating period, respectively. Austral summer and winter are
873 indicated in yellow and blue, respectively. (B) GAMM-predicted prevalence in adults
874 according to their reproductive status (active or not). Significant effects of GAMMs are
875 indicated: *** $P < 0.001$ and ** $P < 0.01$.
876

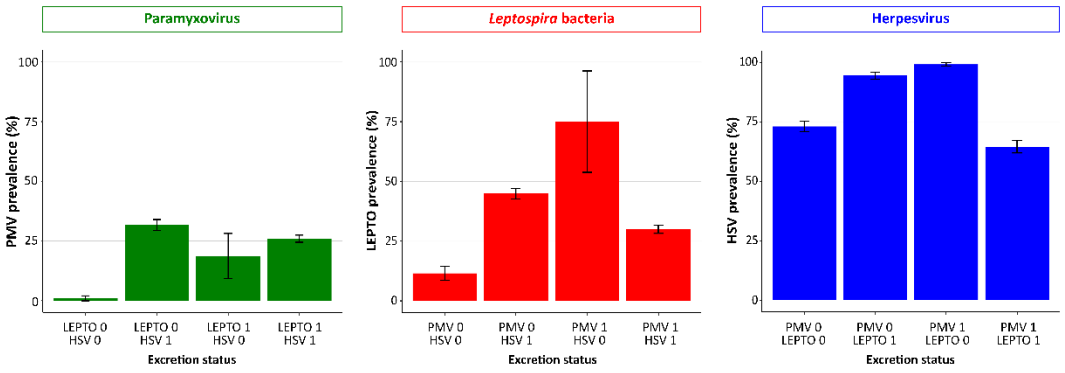

A
B
PMIV prevalence (%)
LEPTO prevalence (%)
HSV prevalence (%)
2019
2020
Jan-Feb
Mar-Apr
Apr-May
June
July
September
Oct-Nov
Nov-Dec
Jan-Feb
March
May-June
July
September
October
Nov-Dec
Sampling period
***
**
Non-pregnant
Pregnant
Female
Non-active
Active
Male

877

878 **Figure 3. The probability of excretion increases with bi- and tri-infections.**

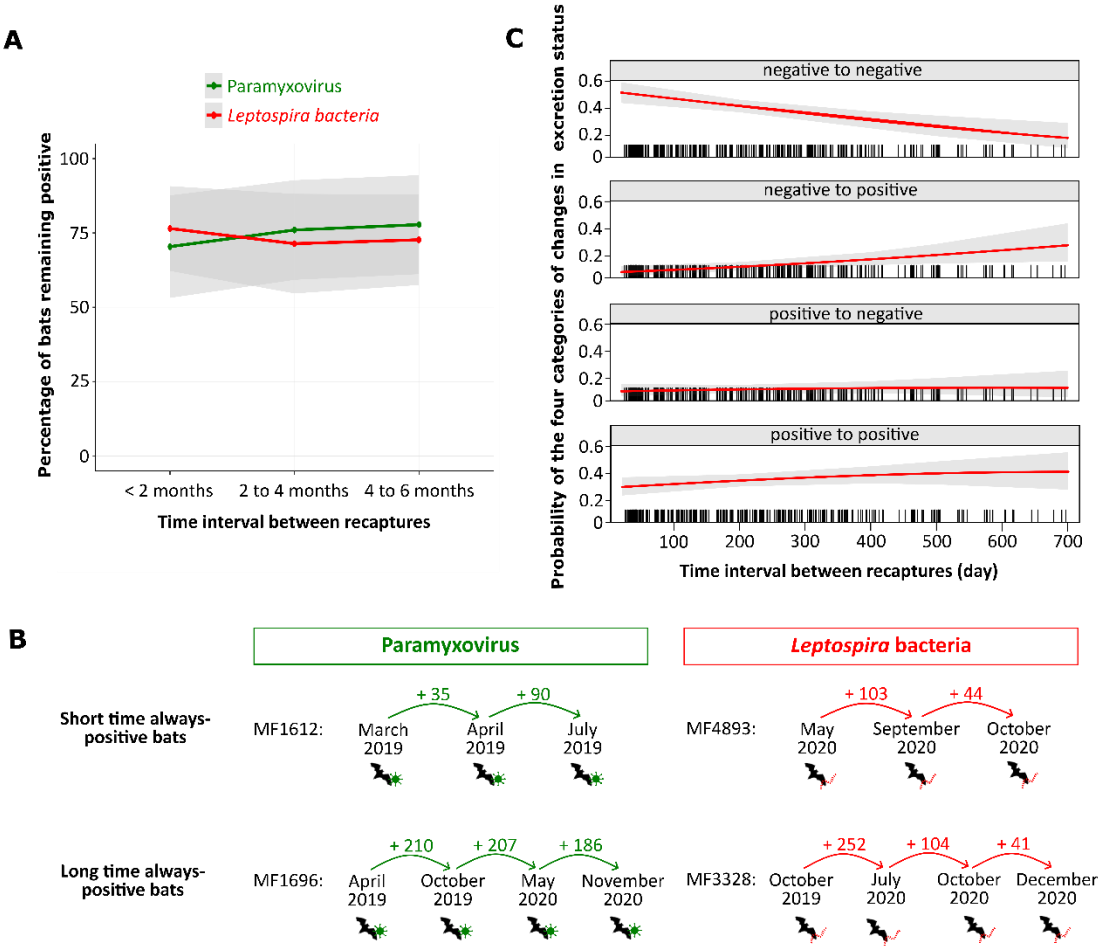
879 Infectious status is coded with 0 for non-shedding bats and 1 for bats shedding infectious

880 agent(s). The 95% confidence interval is indicated by a black bar.



881

882 **Figure 4. Temporal changes of excretion status in recaptured *M. francoismoutoui* bats.**
883 (A) Probability of remaining LEPTO and PMV positive through time. (B) Examples of
884 recaptured bats with always-positive status for PMV and LEPTO. The time interval (in day)
885 between two recaptures is indicated above the arrow. (C) Probability of the four
886 categories of LEPTO excretion status change, with a 95% confidence interval in grey.



887

888 **Table 1. Details on the sampled roosts of *M. francoismoutoui* and the number of tested**

889 **and positive samples.**

890

Roost	Habitat	No. of sampling periods	Roost size estimation (min and max when available)	No. of urine tested	No. of PMV positive (%)	No. of LEPTO positive (%)	No. of saliva tested	No. of HSV positive (%)
AOM	Building	1	2560	52	6 (11.5)	10 (19.2)	0	no data
EGI	Building	3	no data	149	56 (37.6)	79 (53.0)	89	82 (92.1)
ESA	Bridge	15	570 - 3200	634 (PMV) 635 (LEPTO)	254 (40.0)	360 (56.7)	452	406 (89.9)
MON	Bridge	15	210 - 1150	488	144 (28.4)	268 (54.9)	419	355 (84.5)
PBV	Cliff	1	300	11	4 (36.4)	8 (72.7)	0	no data
PSR	Building	15	no data	512 (PMV) 510 (LEPTO)	255 (49.8)	305 (59.8)	396	387 (97.8)
PSR-SUD	Building	1	no data	51	9 (17.6)	14 (27.4)	0	no data
RBL	Bridge	15	350 - 1300	658	238 (36.2)	235 (35.7)	425	369 (86.8)
RES	Bridge	8	35 - 410	223	91 (41.0)	111 (49.8)	212	179 (84.4)
RPQ	Bridge	15	150 - 3000	535 (PMV) 536 (LEPTO)	229 (41.9)	287 (53.4)	416	386 (92.8)
STJ	Building	1	200	34	14 (41.2)	10 (29.4)	0	no data
STM	Bridge	12	27 - 350	364	145 (39.9)	166 (45.6)	281	267 (95.0)
TBA*	Cave	11	0 - 100,000	500	140 (28.0)	168 (33.6)	330	217 (65.8)
TGI*	Building	15	75 - 1270	496	167 (33.7)	229 (46.1)	432	356 (82.4)
TM5	Bridge	1	200	28	0	4 (14.3)	0	no data
TRI	Bridge	4	153 - 251	125	45 (36.0)	86 (68.8)	112	104 (92.9)
VSP	Bridge	15	15 - 6000	658	224 (34.0)	225 (34.2)	417	361 (86.6)
Total	/	/	/	5,518	2,021 (36.6)	2,565 (46.5)	3,981	3,469 (87.1)

891

892