

# Leptospira and paramyxovirus infection dynamics in a bat maternity enlightens pathogen maintenance in wildlife

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1 ***Leptospira* and Paramyxovirus infection dynamics in a bat maternity**  
2 **enlightens pathogen maintenance in wildlife**

3

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16

17 **Short title:** Dual infection dynamics in a bat maternity

18 **Summary**

19 Bats are reservoirs for several zoonotic pathogens of medical importance; however infection  
20 dynamics of pathogens in wild bat populations remain poorly understood. Here, we examine  
21 the influence of host crowding and population age structure on pathogen transmission and  
22 diversity in bat populations. Focusing on two pathogen taxa of medical importance,  
23 *Leptospira* bacteria and paramyxoviruses, we monitored host population and pathogen  
24 shedding dynamics within a maternity colony of the tropical bat species *Mormopterus*  
25 *francoismoutoui*, endemic to Réunion Island. Our data reveal astonishingly similar infection  
26 dynamics for *Leptospira* and paramyxoviruses, with infection peaks during late-pregnancy  
27 and two-months after the initial birth pulse. Furthermore, though co-infection occurs  
28 frequently during the peaks of transmission, the patterns do not suggest any interaction  
29 between the two pathogens. Partial sequencing reveals a unique bat-specific *Leptospira* strain  
30 contrasting with the co-circulation of four separate Paramyxovirus lineages along the whole  
31 breeding period. Patterns of infection highlight the importance of host crowding in pathogen  
32 transmission and suggest that most bats developed immune response and stop excreting  
33 pathogens. Our results support that bat maternity colonies may represent hotspots of  
34 transmission for bacterial and viral infectious agents and highlight how seasonality can be an  
35 important determinant of host-parasite interactions and disease emergence.

36

37 **Introduction**

38 Seasonal changes can have important effects on infectious disease dynamics (Altizer et al.,  
39 2006). Of the many driving factors of infection that demonstrate seasonal cycles, annual  
40 reproduction has been identified as having a significant impact on the dynamics of various  
41 host-parasite interactions, particularly in animal species that are gregarious during the  
42 reproductive season (Altizer et al., 2006). Indeed, during this period, aggregation within  
43 colony, increased metabolic activity and the highly synchronized birth of newborns (i.e. birth  
44 pulse) lead to increased numbers of susceptible hosts and hence boost the potential for  
45 transmission of infectious agents (e.g. Hosseini *et al.* 2004). Investigating the infection  
46 dynamics during host breeding seasons may therefore help to better identify major  
47 mechanisms of pathogen maintenance within wild animal populations.

48 Bats play important ecological roles in prey and predator cycles, arthropod suppression,  
49 seed dispersal, pollination, as well as nutrient distribution and recycling (Kunz et al., 2011).  
50 Additionally, bats have been identified as natural reservoirs of many microbial agents,  
51 including pathogens of threat to human health (Wibbelt et al., 2010). Their ability to enter  
52 torpor or hibernation, the large sizes of their gregarious groups and possibly some  
53 peculiarities in their immune system, have been suggested to facilitate maintenance of  
54 infections in these unique long-lived flying mammals (Calisher et al., 2006). The role of  
55 seasonal breeding in bat infection dynamics has been the subject of several recent studies  
56 (Amengual et al., 2007; Turmelle et al., 2010; Drexler et al., 2011; Field et al., 2011;  
57 Mühldorfer et al., 2011), mostly focusing on viruses. For example, lyssaviruses and  
58 henipaviruses have been observed to exhibit robust and specific amplification in maternity  
59 colonies (Drexler et al., 2011; George et al., 2011). Furthermore, although multi-pathogen  
60 interactions have been shown to drive infection dynamics in other host-parasite models (e.g.  
61 Telfer *et al.* 2010), very few studies have considered the dynamics of multiple infections in

62 natural bat populations (Drexler et al., 2011; Mühldorfer et al., 2011).

63 Leptospirosis is considered as the most common bacterial zoonosis in the world and is  
64 caused by pathogenic spirochetes of the genus *Leptospira* (Adler and de la Peña Moctezuma,  
65 2010). The incidence of human leptospirosis is high in tropical islands, particularly in the  
66 Indian Ocean Islands where some of the highest human incidences have been reported to date  
67 (Pappas et al., 2008). Chronic carrier animals are characterized by persistent leptospire  
68 colonization of renal tubules, which are then shed for months or years in urine and  
69 contaminate the environment. Human infection occurs either directly from contact with  
70 animal reservoirs or indirectly from contaminated soil or water (Adler and de la Peña  
71 Moctezuma, 2010). Rodents are classically recognized as the most significant reservoirs for  
72 the maintenance and dissemination of leptospire worldwide (Bharti et al., 2003). More  
73 recently, bats have also been identified as potential reservoirs of leptospire in a number of  
74 locations (e.g. Matthias *et al.* 2005; Tulsiani *et al.* 2011), with sometimes very high infection  
75 rates (Lagadec et al., 2012). Mathematical modeling has suggested seasonality of host  
76 reproduction as an important factor affecting *Leptospira* infection dynamics in rodent  
77 populations (Holt et al., 2006), however, no published information is currently available  
78 concerning *Leptospira* infection dynamics and maintenance in bat populations.

79 The *Paramyxoviridae* form a large virals family including the causative agents of many  
80 human and animal diseases, and some that have recently been linked to emerging and re-  
81 emerging epidemics (e.g. Hendra and Nipah viruses; Aguilar & Lee, 2011). Rodents and bats  
82 have been recently shown to host a broad spectrum of paramyxoviruses (Drexler et al., 2012;  
83 Wilkinson et al., 2012; Baker, Todd, et al., 2013). Phylogenetic reconstruction of host/virus  
84 associations suggests frequent spillover events between different hosts, with a predominance  
85 of host shifts from bats to other animal species (Drexler et al., 2012; Wilkinson et al., 2014),  
86 which has elsewhere been linked with the bat reproduction period (Plowright et al., 2008). For

87 instance, Plowright *et al.* (2008) have identified reproduction as a key factor in the risk of  
88 Hendra virus infection in Australian fruit bats and especially in the emergence of these viruses  
89 in horse populations.

90 In this study, we investigated the dynamics of two pathogens of medical importance, the  
91 bacteria *Leptospira* and Paramyxoviruses, which have previously been described in  
92 insectivorous bats (Lagadec *et al.*, 2012; Wilkinson *et al.*, 2012). The tropical and endemic bat  
93 species *Mormopterus francoismoutoui* is the most abundant bat species on Réunion Island,  
94 and roosts in large colonies in a variety of sites such as bridges, houses, churches and caves.  
95 We chose to study the largest known cave-based maternity colony of these bats in order to (i)  
96 determine whether the infection rate and/or the genetic diversity of infectious agents are  
97 influenced by host crowding and the succession of different developmental stages (adults,  
98 newborns and juveniles) over the breeding season, and (ii) compare the temporal infection  
99 profiles of both infectious agents. We hypothesize that transmission is host-density dependent  
100 favored by colony-induced crowding and fueled by the input of immunologically naïve  
101 juveniles (Drexler *et al.*, 2011). Results are expected to reveal specific characteristics of the  
102 ecology and evolution of *Leptospira* and paramyxoviruses and to highlight general patterns in  
103 pathogen maintenance in bat populations.

104

## 105 **Results**

### 106 ***Bat colony dynamics***

107 The bat population studied was strictly composed of adults at the beginning of the survey in  
108 late November (Fig. 1a). A large number of newborns were observed in early January,  
109 together with the first juveniles (~3 weeks old), indicating that the birth pulse occurred from  
110 mid-December to mid-January. We estimated that the parturition period lasted up to mid-  
111 February as the last observation of newborns was made on February 8<sup>th</sup>.

112 Over all outings, a total of 2652 urine droplets were counted on the 16 plastic trays. The  
113 spatial distribution of the urine droplets suggested that bats use a main route for exiting the  
114 cave: those trays facing the center of the entrance were more exposed to bat passage than the  
115 ones placed on each side of the main flyway ( $\chi^2_{15} = 49.26$ ,  $p < 0.001$ ; model 1 in Table S3).  
116 Trays lying directly underneath the main bat flyway (yellow-red in Fig. S1b) showed  
117 statistically significant variations in the number of urine droplets collected over time ( $\chi^2_7 =$   
118  $48.43$ ,  $p < 0.001$ ; Fig. 1b and model 1 in Table S3). Indeed, at the first sampling session (i.e.  
119 November 20<sup>th</sup>), the number of exiting adult bats reached a maximum: 46 500 individuals as  
120 estimated by the video. Following this maximum, the number of exiting bats declined  
121 drastically to reach a minimum on January and remained very low for about three additional  
122 weeks. This corresponds to the parturition period where females gave birth and thus remained  
123 with newborns leading to this reduced pattern of exit. Then, the number of exiting bats  
124 increased to reach a second peak on mid-February, coinciding with the first flights of juvenile  
125 bats. Beyond that time point, the number of exiting bats declined progressively towards the  
126 end of the season corresponding to the definitive emptying of the cave; the number of exiting  
127 bats estimated by video analysis was still 13 400 individuals at the end of the sampling period  
128 (i.e. March 21<sup>st</sup>). In late May, a last visual inspection showed that the cave was unoccupied.

129

### 130 *Leptospira and paramyxovirus infection dynamics*

131 Altogether, 420 individual urine droplets were screened by PCR for *Leptospira* and  
132 paramyxoviruses during the 2012-2013 breeding season. The bacterial and viral excretion  
133 positively identified individual bats as being currently infected with either of the two  
134 infectious agents. Hereafter, ‘infection rate’ refers to the proportion of PCR-positive samples.  
135 Both *Leptospira* and paramyxovirus infection rates varied significantly over the breeding  
136 season (*Leptospira*:  $\chi^2_7 = 25.209$ ,  $p < 0.001$ ; paramyxovirus:  $\chi^2_7 = 59.879$ ,  $p < 0.001$ , models 2

137 and 3 in Table S3) and revealed similar patterns of temporal variation (Fig. 1c). The relative  
138 intensity of *Leptospira* excretion also showed significant variation over time, despite the large  
139 variation in the Ct values ( $\chi^2_7 = 148.06$ ,  $p = 0.003$ , fig. 1d and model 4 in Table S3). Overall,  
140 infection dynamics were characterized by two peaks, both in the rates of infection (Fig. 1c)  
141 and the intensity of *Leptospira* excretion (Fig. 1d). Notably, the first peak occurred at the  
142 beginning of the breeding season, during the late-pregnancy period of females, with infection  
143 rates ( $\pm 95\%$  confidence interval) reaching 45% ( $\pm 12\%$ ) and 61% ( $\pm 11\%$ ) for *Leptospira* and  
144 paramyxovirus, respectively. Later, during the parturition period, both infection rates (as well  
145 as the intensity of *Leptospira* excretion) decreased drastically down to a minimum of 6%  
146 ( $\pm 6\%$ ) and 4% ( $\pm 5\%$ ) for *Leptospira* and paramyxovirus respectively (Table S4). The second  
147 infection peak (as well as the intensity of *Leptospira* excretion) occurred roughly two months  
148 after the beginning of the parturition period. Towards the end of the season, infection rates  
149 slightly decreased, with *Leptospira* and paramyxovirus detection rates reaching 21% ( $\pm 11\%$ )  
150 and 31% ( $\pm 13\%$ ), respectively. Infection rates were also monitored at the start of the  
151 following season in order to collect data on the early stages of colony formation. Infection  
152 rates were 28% ( $\pm 16\%$ ) and 21% ( $\pm 16\%$ ) on October 18<sup>th</sup> 2013 for *Leptospira* and  
153 paramyxovirus respectively, and increased within 2 weeks, to reach 62% ( $\pm 18\%$ ) and 52%  
154 ( $\pm 18\%$ ) respectively (Table S4).

155 Our data showed that bats were frequently co-infected (Fig. 2a). Statistically, the  
156 presence of one infectious agent was strongly correlated to the presence of the other agent ( $\chi^2_1$   
157 = 22.170,  $p < 0.001$ , models 2 and 3 in Table S3). Moreover, the rate of co-infection  
158 significantly varied over the breeding season ( $\chi^2_7 = 41.761$ ,  $p < 0.001$ ) and followed the same  
159 dynamics as mono-infections (Fig. 2b and model 5 in Table S3). However, the expected  
160 probability of being co-infected at random for each sampling date was not significantly  
161 different from the observed values (G-test:  $p > 0.1$  for all tests; Fig. 2b). Finally, the intensity



162 of *Leptospira* excretion was not affected by co-infection with paramyxovirus ( $\chi^2_1 = 0.020$ ,  $p =$   
163 0.957, model 4 in Table S3).

164

### 165 ***Genetic diversity of Leptospira and paramyxovirus***

166 Phylogenetic analyses of *Leptospira* provided similar results for *secY* and *rrs2* genes (Fig. 3a  
167 and Fig. S4 respectively). Both phylogenetic trees revealed the circulation of a single  
168 haplotype in the maternity colony, closely related to *Leptospira borgpetersenii*. This  
169 haplotype was distinct from those previously described in wild animals and humans in the  
170 western Indian Ocean. In contrast, paramyxoviruses detected in the cave were much more  
171 diverse as 4 different genetic groups were observed (Fig. 3b). The first group corresponds to  
172 virus sequences associated with three different bat families of the western Indian Ocean  
173 region (Molossidae, Miniopteridae and Vespertilionidae). The second group includes virus  
174 sequences detected in the very closely related bat species *M. acetabulosus* (Mauritius) and *M.*  
175 *jugularis* (Madagascar). Virus sequence in the third group were initially identified in kidney  
176 tissue from a rat caught in Réunion Island near the studied cave (Wilkinson et al., 2014).  
177 Finally, one sequence grouped with viruses largely associated with rats in the region (group  
178 4). There was no evidence of any correlation between the occurrence of the different  
179 Paramyxovirus genetic groups and sampling date ( $p = 0.467$ ), suggesting that the 4 genetic  
180 groups were likely co-circulating within the colony.

181

### 182 **Discussion**

183 Previous studies have demonstrated that dynamics of bat-hosted infectious agents exhibit a  
184 strong seasonal pattern, with infection peaks being recorded during the breeding period  
185 (Turmelle et al., 2010; Drexler et al., 2011). Here, using a non-invasive sampling strategy in a  
186 natural maternity colony, we monitored the excretion of both a bacterial and a viral agent,

187 namely *Leptospira* and paramyxovirus, by a tropical insectivorous bat species endemic to  
188 Réunion Island. Our data provide evidence that infection rates express strong temporal  
189 fluctuations within the time scale of the breeding season. We find that *Leptospira* and  
190 paramyxoviruses display remarkably comparable infection dynamics, a feature suggestive of  
191 synchronized host susceptibility and a common transmission route within the colony, i.e.  
192 urine. Data from two successive breeding seasons showed epidemic bursts occurring during  
193 colony formation and two-months after the birth pulse. These findings strongly suggest the  
194 occurrence of a vigorous horizontal transmission within the maternity colony and support that  
195 seasonal coloniality and synchronized bat behavior is a major determinant of infection  
196 dynamics at the population level.

197         Synchronized host susceptibility in females may have resulted from a transient  
198 pregnancy-related depressed immunity. Increased susceptibility to infectious agents during  
199 pregnancy has been demonstrated in different mammalian systems (e.g. Sheldon & Verhulst  
200 1996; Cattadori *et al.* 2005) and short-term trade-offs between immune activity and  
201 reproduction have been suggested to explain such patterns. In the greater mouse-eared bat  
202 (*Myotis myotis*), for instance, weaker cell-mediated responses and heavier ectoparasite  
203 infestations have been observed in pregnant females compared to non-reproductive females  
204 from the same roost (Christe *et al.*, 2000). Our sampling design does not give access to the  
205 immunological status of the population, and thus does not allow verification of this  
206 hypothesis. However, such undulating patterns of immunomodulation and viral replication in  
207 females during late-pregnancy have already been reported in other field studies investigating  
208 Hendra or filo-viruses dynamics in frugivorous bats (e.g. Plowright *et al.* 2008; Breed *et al.*  
209 2011).

210         Following the infection burst that occurs early-on in colony formation, a rapid and  
211 highly synchronous decline in *Leptospira* and paramyxovirus excretion was observed,

212 suggesting that a large majority of adult bats rapidly control the acute infection and that  
213 excretion of infectious agents is only transitory. This pattern is most likely the consequence of  
214 the synchronous development of a robust immune response in bats against the two infectious  
215 agents. Such an immune response would not only protect parturient females, but also  
216 newborns through the passive transfer of specific antibodies from mother to offspring as  
217 demonstrated experimentally in frugivorous bat species (Baker, Suu-Ire, et al., 2013; Epstein  
218 et al., 2013), and largely suggested by several field studies (Plowright et al., 2008; Breed et  
219 al., 2011), including one carried out on insectivorous bats (Drexler et al., 2011). Though  
220 transitory, the conferred passive protection may shape age related patterns of infection  
221 (Boulinier and Staszewski, 2008) and has been suggested to last between one and six months  
222 after maternal severing in insectivorous (Drexler et al., 2011) and frugivorous bats (Baker,  
223 Suu-Ire, et al., 2013; Epstein et al., 2013), respectively. Thus, the influx of juveniles in the  
224 group of susceptible bats is delayed until the disappearance of passively transferred maternal  
225 antibodies. Indeed, the two-months delay observed before appearance of the second infection  
226 peak is consistent with such waning of passively transferred maternal antibodies, as  
227 previously suggested by a field study on *Myotis* bats (Drexler et al., 2011). In addition to an  
228 immunization of the mothers, the emergence of uninfected juveniles may contribute to the  
229 prevalence decrease measured in February. However, considering that females give birth to a  
230 single pup, this decrease should be at maximum of 50%, which is consistently lower than the  
231 actual observed drop (i.e. xx% for *Leptospira* and xx% for paramyxovirus, respectively).  
232 Thus we propose that the dramatic decrease in prevalence results from both immunization of  
233 the mothers and the emergence of transiently immunized juveniles. Interestingly, some of the  
234 expected positive relationship between host density and infection prevalence (Kallio et al.,  
235 2010) may be lacking at the end of the season when host density has increased by twice with  
236 the birth of newborns. The number of immune females may explain why infection rates, and

237 especially that of paramyxovirus, did not return to the peak levels measured at the beginning  
238 of the season. Further investigations addressing the serological status of pregnant mothers,  
239 newborns and juveniles towards *Leptospira* and paramyxovirus need to be carried out in order  
240 to properly address these major points.

241         Seasonality of reproduction might play a role in the evolution of infectious agents by  
242 causing alternating periods of potential mixing of different lineages with high transmission  
243 and population bottlenecks that simultaneously limit strain diversity and cause rapid genetic  
244 shifts (e.g. Ferguson *et al.*, 2003). In the present study, we observed that one single haplotype  
245 of *Leptospira* co-circulated with multiple paramyxovirus lineages, though no apparent  
246 evolution of the viral diversity could be recorded over the timescale of our observations.  
247 Multiple circulating virus strains may affect within-host and within population dynamics by  
248 conferring cross-immunity (Hayman *et al.*, 2013). Interestingly, we also found evidence of  
249 paramyxovirus sharing between bat and rodent populations, suggesting that host shift between  
250 rats and bats may contribute to the paramyxovirus diversity observed within the maternity  
251 colony.

252         The presence of multiple infectious agents within a host, i.e. co-infection, can critically  
253 impact infection dynamics (Telfer *et al.*, 2010). Interactions may be synergistic or  
254 antagonistic and infectious agents may interact either directly or indirectly via ‘bottom-up’  
255 (e.g. competition for shared host resources) or ‘top-down’ processes (e.g. immune-mediated  
256 competition or facilitation) (Pedersen and Fenton, 2007). Here, although bats were frequently  
257 co-infected with *Leptospira* and paramyxovirus, the observed rate of co-infection was not  
258 different from what is expected at random and we found that the presence of paramyxovirus  
259 infection had no effect on the intensity of *Leptospira* excretion. The possibility that the  
260 intensity of paramyxovirus infection could be positively influenced by *Leptospira* infection  
261 could not be tested with our experimental setup and, thus, cannot be ruled out. The large

262 confidence intervals for pathogen prevalence and intensity of *Leptospira* infection preclude  
263 any solid conclusion but our study suggest that co-infection most likely appears as the mere  
264 consequence of the temporal conjunction between the infection dynamics and did not result in  
265 any apparent interaction between the two pathogens.

266 In conclusion, our study has focused on infection dynamics within the time frame of the  
267 bat breeding season. Although it is difficult to distinguish between episodic shedding from  
268 persistently infected bats and transient epidemics (Plowright et al., 2015), our result suggest  
269 that following epidemic bursts, the vast majority of infected bats likely develop immunity  
270 efficiently and stop excreting pathogens; however, the question of infection maintenance  
271 beyond the breeding season has not been addressed. A recent theoretical study, using  
272 stochastic epidemiological models with a seasonal birth pulse, suggests that pre-existing  
273 immunity is critical for pathogen maintenance in bat populations (Peel et al., 2014). Here,  
274 rates of infection at the beginning and end of the breeding season were roughly similar  
275 (~25%) implying that the bat maternity *in fine* serves as an ecological “vaccination center”,  
276 providing potential antibody immunity to large proportions of the population outside of the  
277 breeding season. Transmission outside of maternity colonies may thus be maintained by a  
278 small fraction of individuals chronically excreting the two pathogens, waning immunity  
279 and/or immigration events between colonies (Breed et al., 2011; Field et al., 2011; Sohayati et  
280 al., 2011; Peel et al., 2012). As fission–fusion social structures are being increasingly  
281 recognized in bats (Kerth et al., 2011), and because the bat species studied herein tends to  
282 split into small populations during non-reproductive period, it is possible that a complex  
283 meta-population structure will influence seasonal infection dynamics within the community  
284 (Plowright et al., 2011). Multidisciplinary research on the interplay between population  
285 structures and temporal dynamics in the context of their associated pathogens are important  
286 for the prediction of possible emergence events in specific geographic regions (Restif et al.,

287 2012), such as the western Indian Ocean, where leptospirosis and paramyxoviruses are widely  
288 prevalent (Bourhy et al., 2010; Lagadec et al., 2012; Wilkinson et al., 2012; Desvars et al.,  
289 2013; Dietrich et al., 2014).

290

## 291 **Experimental procedures**

### 292 *Urine collection*

293 A colony of the insectivorous bat *Mormopterus francoismoutoui* was monitored during the  
294 2012-2013 breeding season, in Réunion Island, a tropical oceanic Island located 800km East  
295 of Madagascar. The maternity colony is sited in a natural cave of approximately 30m<sup>3</sup> and is  
296 occupied by one single bat species, i.e. *M. francoismoutoui*, from October to May. The  
297 maternity colony is mostly composed of adult females at the early stages of the breeding  
298 season. A total of eight field outings were carried out from November 2012 to April 2013, a  
299 timing that did not allow monitoring the first weeks of the colony formation. We hence  
300 conducted additional monitoring in the same cave during the next season in the very early  
301 stages of colony formation, with four field outings during October 2013. A non-invasive  
302 sampling strategy was implemented in order to avoid direct manipulation of bats thus limiting  
303 disturbance of the colony. This strategy is based on the demonstration that infected bats  
304 excrete both *Leptospira* and paramyxovirus in their urine (Baker et al., 2012; Desvars et al.,  
305 2013). In addition, urine droplets are spontaneously shed by flying bats during their daily  
306 emergence at dusk and could then be collected on plastic films layered on the ground. For  
307 each sampling date, 16 plastic film (Saran wrap) -covered cardboard rectangles (20x15cm)  
308 were placed inside larger plastic trays and positioned at the entrance of the cave following a  
309 referenced map. This allowed positioning each tray at the exact same place throughout the  
310 whole survey. As shown on the overview of the sampling design provided in Fig. S1 trays  
311 were separated by at least 1 meter in order to minimize the possibility of sampling several

312 urine droplets from a single bat specimen. After the emergence of bats, up to 5 distant (about  
313 5cm) urine droplets assumed to correspond to 5 independent bat specimens, were collected  
314 from each of the 16 plastic trays and buffered in micro-tubes containing 300  $\mu$ L of Minimum  
315 Essential Medium Eagle buffer. Samples were then kept in the field in a cool box filled with  
316 ice packs and brought back to the lab where they were immediately stored at  $-80^{\circ}\text{C}$  until  
317 tested.

318

### 319 *Bat colony dynamics*

320 The colony was visually monitored throughout the whole study in order to estimate the  
321 population age structure and timing of parturition. The presence of adults (brown), newborns  
322 (pink colored) and juveniles (dark grey) was thus checked by visual inspection at each  
323 sampling date (see photos in Fig. S2).

324 We attempted to characterize changes in bat behavior during the breeding season by  
325 monitoring the numbers of bats at emergence. Relative estimates were obtained by counting  
326 the number of individual urine droplets landing on the plastic films, as we showed that the  
327 number of urine droplets correlates with the number of exiting bats (see details in Fig. S3).  
328 Thus, collected figures allowed comparative analysis between sampling field missions.

329 Video analyses were also used to estimate the absolute size of the exiting population.  
330 When lightness and weather conditions permitted, videos of bats leaving the cave were  
331 acquired using the sky as a backdrop. Custom MATLAB-encoded algorithms were then used  
332 to define a line within the video-images and video frames were segmented based on the  
333 contrast of each image (dark bats on a clear sky background). A binary image was then  
334 constructed using mask data along the defined line (X-dimension), and for each frame of the  
335 video (Y-dimension). Non-touching objects were then counted in the obtained image mask to  
336 generate a minimum-number estimate of the number of bats that had left the cave.

337

338 ***Screening and genetic diversity of Leptospira and Paramyxovirus***

339 Paramyxoviruses are single stranded negative sense RNA viruses, and thus their nucleic acids  
340 require reverse transcription before detection via PCR. Therefore, detection was carried out  
341 on a single cDNA preparation for both bacterial and viral detection. Using QIAamp Mini spin  
342 columns (Qiagen, Valencia, CA), total nucleic acids were extracted following manufacturer's  
343 instructions from 140µl of each buffered urine sample. A negative control was added for each  
344 extraction run. Following extraction, 10 µL of total nucleic acids were submitted to reverse  
345 transcription using the Promega cDNA kit (Promega, Madison, USA) with 1.25 µL of  
346 Hexamers (Promega, Madison, USA), following manufacturer's instructions.

347 Five microliters of cDNA were then used as templates for *Leptospira* and paramyxovirus  
348 detections using previously described detection methods (Smythe et al., 2002; Tong et al.,  
349 2008). Briefly, the presence of *Leptospira* was detected using a probe-specific real-time PCR,  
350 targeting a fragment of the *16S rRNA* gene, specific of all known pathogenic *Leptospira*  
351 species. The threshold cycle (Ct) of positive samples was noted to infer the relative bacterial  
352 load and thus the relative intensity of *Leptospira* excretion. In order to compare Ct values  
353 among different PCR runs, we verified the consistency of the Ct value for the positive control  
354 in each run. For positive samples, a partial fragment of the *16S rRNA* and *secY* genes was then  
355 amplified, as described in Dietrich *et al.* (2014), and sequenced (Genoscreen, Lille, France)  
356 for subsequent phylogenetic analyses. For the detection of paramyxoviruses, a semi-nested  
357 PCR targeting the polymerase-encoding gene was performed as previously described  
358 (Wilkinson et al., 2012). After electrophoresis on a 2% agarose gel stained with GelRed  
359 (Biotium Inc.), PCR products of the approximate anticipated size (450-500 bp) were purified  
360 using the QIAGEN PCR purification kit, cloned into the pGEMt vector system (Promega),  
361 and submitted to direct Sanger sequencing (Genoscreen, Lille, France). DNA sequences of



362 paramyxoviruses obtained from independent bacterial clones were aligned to generate  
363 consensus sequences for each sample in order to correct for the majority of sequencing or  
364 PCR-introduced errors. These sequence data have been submitted GenBank database under  
365 accession number KJ607934-KJ607957 for *Leptospira* and KJ748495-KJ748520 for  
366 paramyxoviruses

367 Nucleotide sequences were assembled and edited manually using ChromasLite 2.01  
368 (Technelysium Pty Ltd, South Brisbane, Australia), and aligned using CLC Sequence Viewer  
369 6.8 (CLC bio, Aarhus, Denmark) with references sequences including samples from bats and  
370 terrestrial small-mammals of the western Indian Ocean Islands (see details in Tables S1 and  
371 S2). Phylogenetic trees were constructed using PHYML (Guindon and Gascuel, 2003) under  
372 the AIC best-fitted model of evolution (*Leptospira*: TPM3uf+I and HKY+I for *16S rRNA* and  
373 *secY*; Paramyxoviruses: TIM3+I+G) selected by JMODELTEST v.0.1.1 (Posada, 2008), a  
374 BIONJ as starting tree and NNI tree search. The robustness of branches was evaluated  
375 performing bootstrap analysis with 1000 repetitions.

376

### 377 ***Statistical analyses***

378 We examined changes in the number of urine droplets (as a correlate of the number of bats  
379 emerging at dusk) over the breeding season and among the trays by using a Generalized  
380 Linear Model (GLM) with a Poisson distribution and a log link function. “Sampling date” and  
381 “tray number” were included as explanatory variables.

382 We investigated the dynamic of infections (both mono- and co-infections) by using  
383 GLMs with binomial error and a logit link function, with the “sampling date” as an  
384 explanatory variable. We aimed to determine whether the presence of one infectious agent  
385 was associated with the presence of the alternative infectious agent, adding “infection status”  
386 for *Leptospira* and paramyxoviruses as explanatory variables in models testing mono-

387 infections. A G-test was used to test differences between the co-infection rates, as observed or  
388 as expected at random. Expected co-infection rates at each sampling date were calculated as  
389 the product of the rates of infection with each single pathogen measured at the same date  
390 (Nieto and Foley, 2009).

391 The temporal variation in the relative intensity of pathogen excretion in urine was  
392 analyzed for *Leptospira* only, as no accurate quantitative information on viral load could be  
393 obtained from the semi-nested PCR used for paramyxovirus detection. We used a GLM with  
394 a Gaussian distribution and a logit link function, using the threshold cycle (Ct) as a proxy for  
395 the relative intensity of *Leptospira* excretion and “sampling date”, “tray number” and  
396 “paramyxovirus infection status” as explanatory variables.

397 All starting models were simplified by backward stepwise elimination of non-significant  
398 terms ( $p > 0.05$ ) beginning with interactions, to obtain the minimum adequate model  
399 (Crawley, 2007) (see models in Table S3). In models using a binomial error structure, we  
400 systematically checked for over dispersion by calculating that the ratio of *residual deviance*  
401 *over residual degrees of freedom* was  $<2$ . Curves were produced using a loess smoother.  
402 Evolution of pathogen genetic diversity (proportion of the different lineages over time) was  
403 analyzed using a Fisher’s exact test. All analyses were carried out using the R software  
404 package v.3.0.2.

405

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419

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- 566

567 **Figure legends**

568 **Fig. 1.** Seasonal dynamics of (a, b) the bat population and (c, d) *Leptospira* and  
569 paramyxovirus infections in the maternity colony of *M. francoismoutoui*. (a) Estimated  
570 presence of adults, newborns and juveniles within the colony (black rectangles). (b)  
571 Emergence of bats at dusk. The continuous line represents the number of urine droplets lying  
572 along the main flight-path of bats exiting the cave at dusk, and the shaded area the standard  
573 deviation. (c) *Leptospira* and paramyxovirus infection rates. The continuous lines represent  
574 the proportion of PCR-positive samples for *Leptospira* (green) and paramyxovirus (purple),  
575 and the shaded area the 95% CI. (d) Intensity of *Leptospira* excretion. The dashed line  
576 represents the mean Ct values for positive *Leptospira* samples (represented in its negative  
577 form) and the shaded area the standard deviation. In each panel, the x-axis depicts the  
578 seasonal time scale in month and the white dots correspond to sampling dates.

579

580 **Fig. 2.** (a) Proportion of mono- and co-infections among infected individuals and (b) seasonal  
581 dynamics of co-infections in the *M. francoismoutoui* maternity colony. (a) White bars  
582 represent mono-infected individuals and grey bars individuals with co-infection. Error bars  
583 represent 95% confidence intervals. (b) The continuous line represents the observed rate of  
584 co-infection, while the dashed line corresponds to the expected probability at random. 95% CI  
585 are represented by shaded areas. The x-axis depicts the seasonal time scale in month and the  
586 white dots correspond to sampling dates.

587

588 **Fig. 3.** Phylogenetic relationships of (a) *Leptospira* and (b) paramyxovirus detected in the  
589 maternity colony of *M. francoismoutoui*. Samples from this study are represented in green for  
590 *Leptospira* and purple for paramyxovirus and are coded with sample ID and date (month-day-  
591 year). Reference sequence labels refer to the host species and geographic location. Genbank



592 accession numbers are indicated in parentheses. Bootstrap values higher than 80% are  
593 represented by a dark circle.

594