Effects of biological and environmental factors on filovirus serology in bats in Guinea

Maëliss Champagne, [1,2] Julien Cappelle, [1] Alexandre Caron, [1,3] Thibault Pouliquen, [1,4] Aboubacar Samoura, [5] Mohamed Idriss Doumbouya, [6] Guillaume Thaurignac, [2] Ahidjo Ayouba, [2] Alpha Kabinet Keita, [2,4] Martine Peeters, [2] Mathieu Bourgarel, [1,7] Hélène M. De Nys [1,7]

1. ASTRE, CIRAD, INRAE, Université de Montpellier, Montpellier, France
2. TransVIHMI, Institut de Recherche pour le Développement (IRD), Université de Montpellier, INSERM, Montpellier, France
3. Faculdade de Veterinária, Universidade Eduardo Mondlane, Maputo, Mozambique
4. Centre de Recherche et de Formation en Infectiologie de Guinée (CERFIG), Université Gamal Abdel Nasser de Conakry, Conakry, Guinea
5. Office Guinéen des Parcs Nationaux et Réserves de Faune (OGPRF), Ministère de l'Environnement, et du Développement Durable (MEDD), Conakry, Guinea
6. Direction Nationale des Services Vétérinaires, Ministère de l'Elevage et des Productions Animales, Conakry, Guinea
7. CIRAD, UMR ASTRE, Harare, Zimbabwe

Correspondence should be addressed to Hélène M. De Nys; helene.de_nys@cirad.fr

Abstract

We investigated factors which might influence the circulation of ebolaviruses in fruit bats in Guinea in order to better understand their role in the ecology of ebolaviruses and evaluate risks of spill-over to other animals or humans. We performed serological tests on populations of 4 bat species (*Eidolon helvum*, *Hypsignathus monstrosus*, *Lissonycteris angolensis* and *Rousettus aegyptiacus*) sampled longitudinally for 18 months (2018-2020). The analysis of 1,427 bat samples for antibodies directed against different ebolavirus species allowed to test the influence of biological and environmental variables on seropositivity. Results showed that species (*Eidolon helvum and Rousettus aegyptiacus*), sex (males), age (sexually immature adults, and thus reproductive life-cycle), as well as season (dry) have a significant positive effect on the presence of antibodies against ebolaviruses in bats. These factors may play an important role in the circulation of filoviruses. Search for viral evidence and surveillance strategies should take these into consideration.

Introduction

Since the first two outbreaks of Ebola virus disease (EVD) in 1976 in Yambuku (DRC) and Nzara (South Sudan), 32 outbreaks have been officially identified in humans across Central and West Africa (1). The frequency of these outbreaks seems to have recently increased (1).

Despite recent epidemics which originated from viral resurgence in human populations (2–4), most outbreaks are believed to have resulted from independent zoonotic transmissions, through direct or indirect contact with the animal reservoir or other infected animals (5). The reservoir species of ebolaviruses remain unknown, but bats are suspected to play an important role in the ecology of the virus and transmission mechanisms (6–8). It is hypothesized that bats

51 might infect humans directly when hunted and handled (9), or indirectly through contact with
52 bat excrements or fruits contaminated with saliva or excrements (10). Bats might also be an
53 indirect source by transmitting viruses to bridge hosts which can act as amplifiers and be at the
54 origin of human outbreaks (6,11). This has been suspected in Central Africa and Ivory Coast
55 where apes have been confirmed as the source of infection in several outbreaks (12,13).

56 Certain bat species are regarded as potential reservoir of ebolaviruses since the
57 discovery of viral RNA and anti-Ebolavirus (anti-EBV) antibodies specific to the Zaire
58 ebolavirus in 3 fruit bat species (*Epomops franqueti, Hypsignathus monstrosus, Myonycteris*
59 *torquata*) (6). However, viral RNA was only detected in 13 of 279 tested specimens, and
60 confirmed by sequence analysis in 7. Other studies have since shown that other frugivorous
61 species have anti-EBV antibodies (*Rousettus aegyptiacus, Eidolon helvum, Epomophorus*
62 *gambianus, Micropteropus pusillus and Lissonycteris angolensis*) and that an insectivorous
63 species (*Mops sp.*) hosts a recently discovered ebolavirus named Bombali virus (7,8,14). This
64 supports the fact that these wild bats are exposed to filoviruses and are able to survive infection,
65 as demonstrated by experimental inoculations for some of these species (15,16). In addition,
66 *Rousettus aegyptiacus* are the reservoir species of Marburg virus, another filovirus (17), and
67 other bat species have been shown to be exposed or infected with other filoviruses such as
68 Lloviucuevavirus detected in Europe (18) and newly described viruses in China (19). Until
69 now, infectious ebolaviruses have never been isolated from bats. The viremic period might be
70 short or viruses might end up circulating at very low level and could therefore only be detected
71 for a limited period of time, as shown experimentally in *R. aegyptiacus* inoculated with
72 Marburg virus (15,20).

73 The aerial lifestyle, gregariousness, longevity as well as immunological and metabolic
74 specificities of bats are traits that may impact the ecology of ebolaviruses. Biological and
75 phenological factors, influenced by seasonal or climate changes, are also important, like the

migratory nature of certain bat species or reproduction patterns, both influenced by the availability of food resources (21,22). It has been shown that spillover events of Marburg virus to human populations coincide with the birthing season and presence of juveniles in *Rousettus aegyptiacus* populations (23). A recent study on an *Eidolon helvum* colony also showed an increase in the presence of antibodies against ebolaviruses when juveniles turn into immature adults (24). Other factors can influence the dynamics of ebolaviruses in their natural hosts, for instance population size and geographic distribution seem to impact viral richness within bat populations (25). All these factors influence to some extent viral circulation, risk of spillover events and emergence and are important to understand to adjust ebolaviruses research and EVD surveillance strategies.

The objective of this study was to investigate the effects of biological and environmental factors on the presence of anti-Ebola antibodies and improve understanding of dynamics of ebolaviruses circulation in bats. We performed a longitudinal serological survey of ebolaviruses in four frugivorous bat species in Guinea, a country at risk of EVD outbreak. Based on the outcomes of the serological investigation, a subset of bats was also screened for filoviruses RNA.

Methods

Sample and data collection

We collected blood samples as well as oral and rectal swabs from four frugivorous bat species, i.e. *Eidolon helvum, Hypsignathus monstrosus, Lissonycteris angolensis and Rousettus aegyptiacus*, already detected with anti-EBV antibodies (7). Capture and sampling were carried out longitudinally over 13 sampling periods (i.e. 16 to 28 consecutive days of sampling per period) between November 2018 and July 2020, following the protocols already

described in De Nys *et al.* (7). Bats were released directly after sampling. They were captured in feeding and roosting areas located mainly in four study sites of the prefecture of Macenta (Figure 1), in forested Guinea, the region where the 2013-2016 Ebola epidemic emerged. Sites were visited every 2 to 4 weeks during the course of the study. The environment was mainly constituted of highly transformed habitat, with secondary and degraded forests being gradually converted into a mosaic of forests and crops over the last 20 years (L. Lee-Cruz, pers. Comm.). It varied from secondary forest, fields, villages to more urbanised areas. Climate is characterized by a wet (May to October) and a dry (November to April) season.

Capture site (geographic coordinates, ecological environment), morphology, sex, age class, status of lactation and late gestation, and species (determined according to morphological criteria combined to genetic confirmation for some specimens, Supporting information Methods S1) were also recorded. Age was categorised as follows: juvenile (articular epiphyses not yet fully ossified), sexually immature adult (articular epiphyses ossified, but underdeveloped sexual organs), sexually mature adult (articular epiphyses ossified and clearly developed sexual organs).

This study was approved by the Comité National d'Ethique pour la Recherche en Santé of Guinea (permits 011/CNERS/19; 025/CNERS/20; 019/CNERS/20) and the Office Guinéen des Parcs et Réserves (permit 017/OGUIPAR/MEEF/2017).

Figure 1. Sampling sites, A: Map of Guinea with the prefecture of Macenta in grey; B: prefecture of Macenta with sampling sites (dots) and number of sampled bats

Screening for Ebolaviruses Antibodies

We tested dried blood spots (DBS) for anti-EBVs IgG with a Luminex-based serologic assay adapted for bats as described in De Nys *et al*. (7). The assay included ten recombinant Ebola virus proteins, i.e. the glycoprotein (GP), nucleoprotein (NP), and/or viral protein 40 (VP40), for different ebolaviruses: Ebola Zaire Kissidougou and Mayinga strains (NP, GP K, GP M, VP40), Sudan (NP, GP, VP40), Bundibugyo (GP, VP40) and Reston (GP). Recombinant proteins were produced in sf9 insect cell vectors and purchased at Sinobiologicals (Beijing, China) except for REST GP (IBT (Gaithersburg, MD)). The characteristics of the antigens of the different ebolavirus species used are as follows: EBOV, previously called Zaire (NP, amino acids [aa] 488 to 739 strain Mayinga 1976; VP40, aa 31 to 326 strain Kissidougou-Makona 2014; GP-k (aa 1 to 650, Kissidougou-Makona 2014) GP-m (aa 1 to 650, Mayinga 1976); SUDV (NP (aa 361-738, strain Gulu), GP, aa 1 to 637, strain Uganda 2000; and VP40, aa 31 to 326, strain Gulu,); BDBV, Bundibugyo (GP, aa 1 to 501, strain Uganda 2007, and VP40, aa 31 to 326, strain Uganda 2007) and RESTV, Reston (GP, aa 1 to

141 650). DBS were eluted and tested as previously described in 200µL of incubation buffer
142 (7,24,26,27). One hundred µL of sample, corresponding to the equivalent of a final plasma
143 dilution of 1/2000, was incubated with 50µL of magnetic beads coated with recombinant
144 protein (2 µg protein/1.25 × 106 beads) in 96-well flat-bottom chimney plates (Greiner bio one,
145 Frickenhausen, Germany) on a plate shaker at 300 rpm for 16 h at 4 °C in the dark. After
146 washing, 0.1 µg/mL of goat anti-bat biotin-labeled IgG (Euromedex, Souffelweyersheim,
147 France) was added to each well and incubated for 30 min at 300 rpm at room temperature. After
148 washing, we added 50 µL of 4 µg/mL streptavidin-R-phycoerythrin (Fisher Scientific/Life
149 Technologies, Illkirch, France) per well and incubated for 10 min at 300 rpm at room
150 temperature. Reactions were read with BioPlex-200 (BioRad, Marnes-la-Coquette, France) or
151 MagPix (Luminex, Austin, TX, US). At least 100 events were read for each bead set on a
152 Bioplex-200 instrument, and results were expressed as median fluorescence intensity (MFI) per
153 100 beads. Samples that showed positive signals were repeated in order to validate the results.
154 To interpret the serological results in the absence of true positive controls, MFI cutoff
155 values for each antigen were determined using four methods in De Nys et al. (7) (See Table
156 S1in the Supporting information). In this study we opted for the cutoff values resulting from
157 the least stringent method, i.e. the mean plus four times the standard deviation of MFIs on
158 negative control samples (M + 4SD) as calculated in De Nys et al. 2018, to search for
159 serological patterns through a multivariate statistical analysis. Negative control samples (n =
160 145) were from captive-born insectivorous bat species (*Carollia perspicillata*, n=103) hosted
161 at the Parc Zoologique de Montpellier (Montpellier, France) and two frugivorous bat species
162 (*Pteropus giganteus*, n=19, *Rousettus aegyptiacus*, n=23) hosted at Wilhelma Zoo and
163 Botanical Garden (Stuttgart, Germany). Samples were considered positive for the presence of
164 antibodies against an antigen if the MFI values were above the cutoff value.
165

166 ***Multivariate statistical analyses***

167 Proportions of samples positive to at least one antigen are presented in a descriptive way
168 to provide a synthetic overview of the data and serological reactivity. Multivariate analyses
169 were performed separately for the 3 antigens for which most reactive samples were observed.
170 We developed Generalized Linear Mixed Models (GLMM) to test the impact of several drivers
171 on the probability of a bat being positive by serology for EBV antigens (lme4 package in R
172 software). We used results of the serological tests as the response variable with a binomial
173 distribution, and species, sex, age and season as explanatory variables with fixed effects (See
174 Table S2 from Supporting information). To account for clustered samples collected during the
175 same sampling session at the same site, we included sampling period as a random effect to
176 control for repeated measures from the same trapping session.
177 In order to select the best model, we used the glmulti function (Rsoftware, glmulti package
178 (28)) to rank all possible models combining the explanatory variables by the Akaike
179 Information Criterion (AIC). We selected the models with the lowest AIC (See Table S3 in
180 Supporting information). The odds ratios were estimated for each explanatory variable. We also
181 used the emmeans and ggeffects functions to obtain estimated marginal means for the response
182 variable and visualize the effect of each explanatory variable. Analysis were carried out on R
183 software 4.1.2.
184

185 ***PCR screening for filoviruses***

186 Oral and rectal swabs from a subset of bats were selected according to the analysis of serological
187 results, extracted and tested for filoviruses RNA by a semi-nested PCR following the protocols
188 described in Djomsi et al. 2022 (24). The PCR targeted a 630 bp fragment of the L gene using
189 degenerated primers that detect a wide diversity of filoviruses, i.e., Filo-MOD-FWD:
190 TITTYTCHVTICAAAAICAYTGGG and FiloL.conR:

ACCATCATRTTRCTIGGRAAKGCTTT in round 1 and Filo-MOD-FWD: TITTYTCHVTI-CAAAAICAYTGGG, and Filo-MOD-RVS: GCYTCISMIAIIGTTTGIACATT in round 2 (29)

Results

Bat sampling and serological results

We analyzed blood samples collected longitudinally from 1,427 wild frugivorous bats: 196 *E. helvum*, 186 *H. monstrosus*, 380 *L. angolensis* and 665 *R. aegyptiacus* (Table 1 and Table S4 from Supporting information). Descriptive analyses show a total of 336/1427 (24%) samples that are reactive with at least one antigen. Proportions of positive samples per bat species, sex, age class and season are presented in Table 2.

Table 1. Bats sampled for ebolavirus serology by species, sex and age class

Species	**Total no. sampled**	**Sex (%)**			**Age (%)**			
		Male	Female	UN*	Juvenile	Immature adult	Mature adult	UN
Eidolon helvum	**196**	91 (46.4)	104 (53.1)	1 (0.5)	17 (8.7)	36 (18.4)	117 (59.7)	26 (13.3)
Hypsignathus monstrosus	**186**	97 (52.1)	86 (46.2)	3 (1.6)	42 (22.6)	8 (4.3)	126 (67.7)	10 (5.4)
Lissonycteris angolensis	**380**	176 (46.3)	203 (53.4)	1 (0.3)	86 (22.6)	41 (10.8)	244 (64.2)	9 (2.4)
Rousettus aegyptiacus	**665**	287 (43.2)	366 (55)	12 (1.8)	223 (33.5)	54 (8.2)	362(54.4)	26 (3.9)
Total	**1427**	651 (45.6)	759 (53.2)	17 (1.2)	368 (25.8)	139 (9.7)	849 (59.5)	71 (5)

* UN = undetermined

Table 2. Number and percentage of samples positive for at least 1 of the ebolavirus antigens according to the M+4SD cutoff method

Species	Total		
	Tot.	Pos.	% (95% CI)
*E. helvum**	196	69	**35** (29-42)
H. monstrosus	186	17	**9** (6-14)
L. angolensis	380	37	**10** (7-13)
R. aegyptiacus	665	213	**32** (29-36)
Total	1427	336	**24** (21-26)

Season	Dry			Wet		
	Tot.	Pos.	% (95% CI)	Tot.	Pos.	% (95% CI)
E. helvum	195	69	**35** (29-42)	1	0	**0** (0-95)
H. monstrosus	173	14	**8** (5-13)	13	3	**23** (8-50)
L. angolensis	183	8	**4** (2-8)	197	29	**15** (10-20)
R. aegyptiacus	388	148	**38** (33-43)	277	65	**23** (19-29)
Total	939	239	**25** (23-28)	488	97	**20** (17-24)

Sex	Male			Female			UN		
	Tot.	Pos.	% (95% CI)	Tot.	Pos.	% (95% CI)	Tot.	Pos.	% (95% CI)
E. helvum	91	43	**47** (37-57)	104	25	**24** (17-33)	1	1	**100** (5-100)
H. monstrosus	97	9	**9** (5-17)	86	8	**9** (5-17)	3	0	**0** (0-56)
L. angolensis	176	16	**9** (6-14)	203	21	**10** (7-15)	1	0	**0** (0-95)
R. aegyptiacus	287	100	**35** (30-41)	366	107	**29** (25-34)	12	6	**50** (25-75)
Total	651	168	**26** (23-29)	759	161	**21** (18-24)	17	7	**40** (22-64)

Age	Juvenile			Immature adult			Mature adult			UN		
	Tot.	Pos.	% (95% CI)	Tot.	Pos.	% (95% CI)	Tot.	Pos.	% (95% CI)	Tot.	Pos.	% (95% CI)
E. helvum	17	8	**47** (26-69)	36	15	**42** (27-58)	117	34	**29** (22-38)	26	12	**46** (29-65)
H. monstrosus	42	2	**5** (1-16)	8	1	**13** (1-47)	126	14	**11** (7-18)	10	0	**0** (0-28)
L. angolensis	86	7	**8** (4-16)	41	8	**20** (10-34)	244	19	**8** (5-12)	9	3	**33** (12-65)
R. aegyptiacus	223	58	**26** (21-32)	54	21	**39** (27-52)	362	121	**33** (29-38)	26	13	**50** (32-68)
Total	368	75	**20** (17-25)	139	45	**32** (25-41)	849	188	**22** (19-25)	71	28	**39** (29-51)

* *E. =Eidolon, H.= Hypsignathus, L. = Lissonycteris, R. = Rousettus*, Pos. = positive, Tot. = total, UN =undetermined

Regarding seroprevalences for each antigen of the different viruses, GPs were the recombinant proteins for which most reactions were observed: 9.8% of the samples showed reactivity against the Zaire Kissidougou strain, 18.8% against Sudan and 9.6% against Bundibugyo versus 0.1 to 3.3 % against other antigens (Table 3). Lower reactivity for VP40 and NP antigens was observed for each bat species. Simultaneous reactivity to GP and NP of the same virus was rare and observed for 0.1% of the samples for EBOV and 0.6% for SUDV.

232 The simultaneous reactivity to the same antigen of different viral lineages was frequent: 53%
233 of the samples were reactive to GP of at least two ebolavirus species, 23% to VP40 and no
234 cross-reactivity was observed for NP (See Table S5 from Supporting information).

235
236 Table 3. Number (percentage) of bat blood samples reactive for each ebolavirus antigen according to the M+4SD
237 cutoff method

		E. helvum (n=196)		*H. monstrosus* (n=186)		*L. angolensis* (n=380)		*R. aegyptiacus* (n=665)		*Total (n=1427)*	
Ebolavirus	Antigen*	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI
Zaïre											
	NP	**1 (0.5)**	0.03-3	**1 (0.5)**	0.03-3	**1 (0.3)**	0.01-1	**11 (2)**	0.9-3	**14 (1)**	0.6-2
	GP K	**35 (18)**	13-24	**5 (3)**	1-6	**7 (2)**	0.9-4	**93 (14)**	12-17	**140 (10)**	8-11
	GP M	**10 (5)**	3-9	**0**	0-2	**0**	0-1	**37 (6)**	4-8	**47 (3)**	2-4
	VP40	**1 (0.5)**	0.3-3	**0**	0-2	**1 (0.2)**	0.01-1	**17 (3)**	2-4	**19 (1)**	0.9-2
	NP+GP	**0**	0-2	**0**	0-2	**0**	0-1	**2 (0.3)**	0.08-1	**2 (0.1)**	0.04-0.5
Sudan											
	NP	**1 (0.5)**	0.03-3	**1 (0.5)**	0.03-3	**7 (2)**	0.9-4	**19 (3)**	2-4	**28 (2)**	1-3
	GP	**66 (34)**	27-41	**14 (8)**	5-12	**18 (5)**	3-7	**171 (26)**	23-29	**269 (19)**	17-21
	VP40	**3 (2)**	0.5-4	**0**	0-2	**3 (0.8)**	0.3-2	**18 (3)**	2-4	**24 (2)**	1-2
	NP+GP	**1 (0.5)**	0.03-3	**0**	0-2	**1 (0.3)**	0.01-1	**7 (1)**	0,5-2	**9 (0.6)**	0.3-1
Bundibugyo											
	GP	**32 (16)**	12-22	**2 (1)**	0.3-4	**15 (4)**	2-6	**88 (13)**	11-16	**137 (10)**	8-10
	VP40	**0**	0-2	**0**	0-2	**1 (0.3)**	0.01-2	**7 (1)**	0.5-2	**8 (0.6)**	0.3-1
Reston											
	GP	**0**	0-2	**1 (0.5)**	0.03-3	**0**	0-2	**1 (0.2)**	0.008-0.8	**2 (0.1)**	0.04-0.5

238 *NP = nucleoprotein; GP = glycoproteine (K = Kissidougou strain; M = Mayinga strain); VP = Viral protein
239
240

241 ***Factors influencing the presence of anti-EBV antibodies in bats***

242 We performed analysis on the presence of antibodies against the following 3 antigens:
243 GP Zaïre Kissidougou (GP EBOV-K), GP Sudan (GP SUDV) and GP Bundibugyo (GP
244 BDBV). For each antigen we obtained fairly similar best fitted models (Table 4), i.e. the
245 Species, Sex and Age variables were consistently selected in the best models for each antigen.
246 The variable Season was also selected in the best models for GP EBOV-K and GP BDBV and
247 models with or without Season were near to equivalent for GP SUDV (Table 4).

248

Table 4. Best models obtained for the presence of antibodies against GP Zaire Kissidougou, GP Bundibugyo and the 2 best models for GP Sudan

Ranking	Model	AIC
1	GP_EBOV-K ~ 1 + Species + Age + Sex + Season	736.2752
1	GP_BDBV ~ 1 + Species + Age + Sex + Season	740.2046
1	GP_SUDV ~ 1 + Species + Age + Sex	1136.707
2	GP_SUDV ~ 1 + Species + Age + Sex + Season	1136.824

Odds ratios (OR) for each explanatory variable selected in the best models are displayed with Confidence Intervals in Table 5. Interestingly, *H. monstrosus* and *L. angolensis* had consistently and significantly lower seroprevalences than *E. helvum* (OR ranging from 0.08 to 0.19 and 0.11 to 0.30 respectively, $p < 0.002$). Immature adults had consistently higher seroprevalences than juveniles in all models (OR ranging from 1.84 to 2.33), although it was only significant for GP EBOV-K and GP-SUDV models ($p < 0.021$) but not for GP-BDBV ($p = 0.069$). Males had consistently and significantly higher seroprevalences than females for all the models (OR ranging from 1.56 to 1.72, $p < 0.034$).

Table 5. Regression table representing the odds ratios of the best statistical models for GP EBOV-K, GP BDBV and GP SUDV

	GP EBOV-K* AIC=736.3			GP BDBV AIC=740.2			GP SUDV AIC=1136.7		
Variables	OR	95% CI	p-value*	OR	95% IC	p-value*	OR	95% IC	p-value*
Species									
E. helvum	—	—		—	—		—	—	
H. monstrosus	0.18	0.07, 0.49	<0.001	0.08	0.02, 0.36	<0.001	0.19	0.10, 0.37	<0.001
L. angolensis	0.13	0.05, 0.34	<0.001	0.30	0.14, 0.64	0.002	0.11	0.06, 0.20	<0.001
R. aegyptiacus	1.21	0.67, 2.17	0.5	1.31	0.73, 2.32	0.4	0.76	0.48, 1.21	0.3
Sex									
Female	—	—		—	—		—	—	
Male	1.72	1.15, 2.57	0.008	1.56	1.03, 2.36	0.034	1.70	1.10, 2.30	<0.001
Age									
Juvenile	—	—		—	—		—	—	
Immature	2.33	1.17, 4.62	0.016	1.91	0.95, 3.83	0.069	1.84	1.10, 3.10	0.021
Mature	1.55	0.94, 2.54	0.085	1.40	0.82, 2.39	0.2	1.03	0.72, 1.48	0.9
Season									
Wet	—	—		—	—				
Dry	2.10	1.01, 4.36	0.046	1.72	1.02, 2.92	0.043			

*significant <0.05; GP = glycoprotein; EBOV-K = Zaire Ebolavirus Kissidougou strain; BDBV = Bundibugyo Ebolavirus; SUDV = Sudan Ebolavirus; OR = Odds Ratio; CI = Confidence Interval

Similarly, the estimated marginal means of seroprevalences were significantly higher for *R. aegyptiacus* and *E. helvum* compared to *H. monstrosus* and *L. angolensis* (Figure 2 and Supporting information Table S6) and for males compared to females (Figure 3), whatever the antigen. Seroprevalences were significantly higher for immature adults compared to juveniles for GP EBOV-K and compared to juveniles and adults for GP SUDV (Figure 4). The seroprevalences were also significantly higher in the dry season compared to the rainy season for GP EBOV-K and GP BDBV (Figure 5).

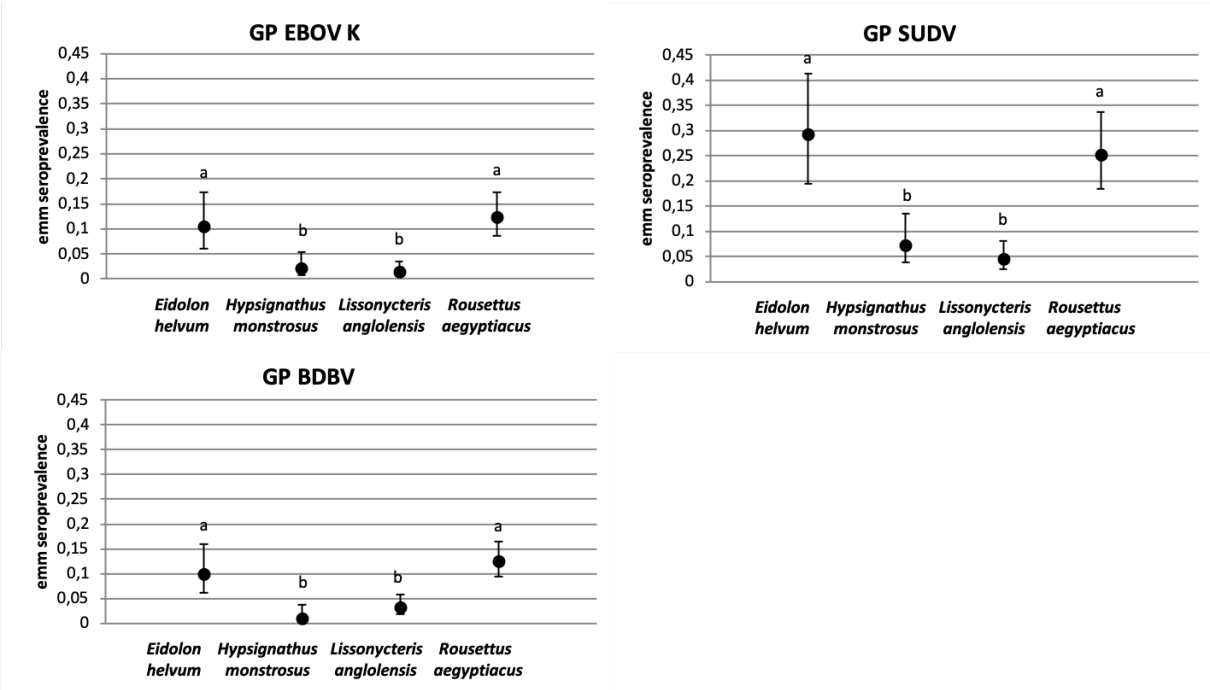

Figure 2. Estimated marginal means (emm) of bat seroprevalence depending on the species and the antigens (estimated marginal means annotated with different letters are significantly different ($p < 0.05$))

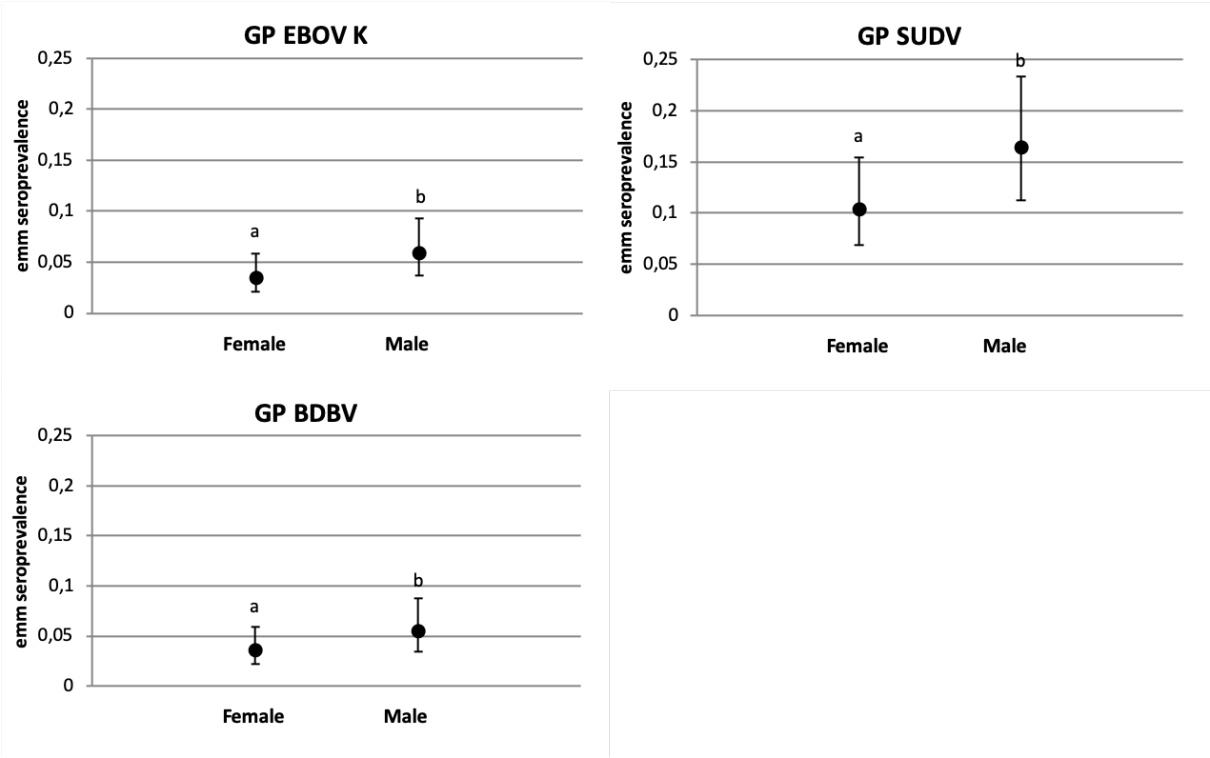

Figure 3. Estimated marginal means of bat seroprevalence depending on the sex and the antigens (estimated marginal means annotated with different letters are significantly different ($p < 0.05$))

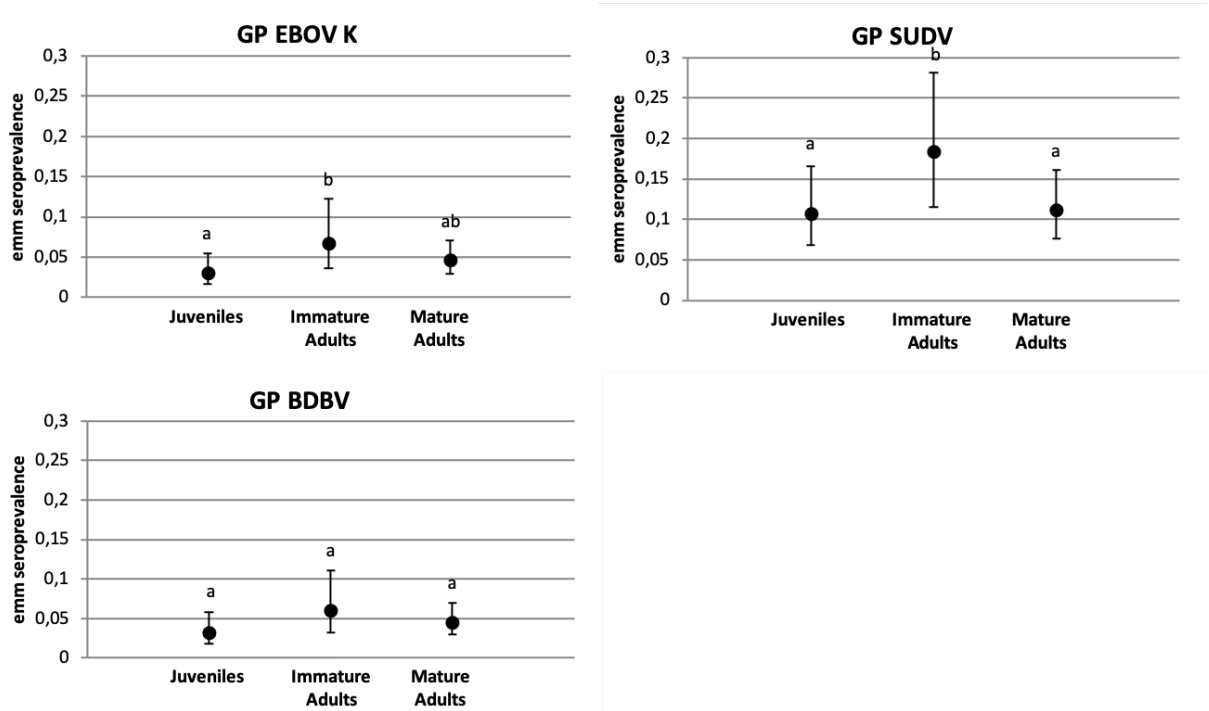

Figure 4. Estimated marginal means of bat seroprevalence depending on the age class and the antigens (estimated marginal means annotated with different letters are significantly different ($p < 0.05$))

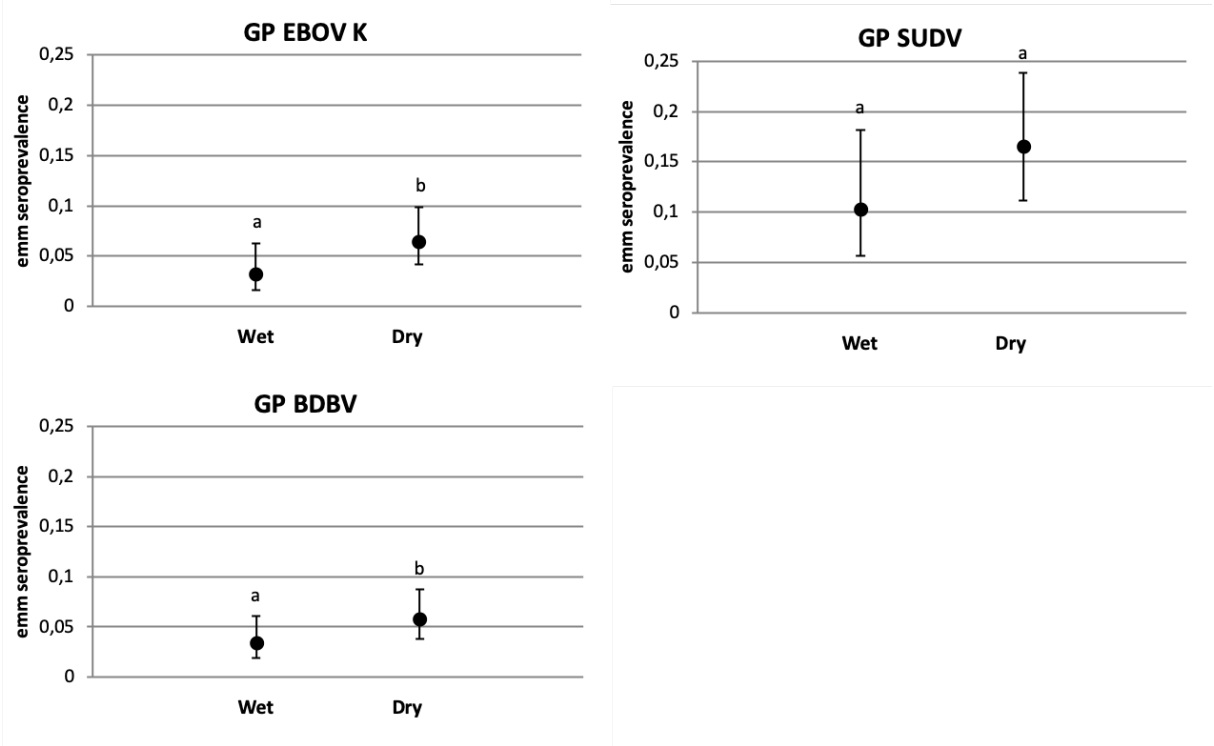

Figure 5. Estimated marginal means of bat seroprevalence depending on the season and the antigens (estimated marginal means annotated with different letters are significantly different ($p < 0.05$))

323 ***Filoviruses RNA detection***

324 Given the results of the serological and statistical analysis, oral (195) and rectal (141) swabs
325 from 142 *R. aegyptiacus* and 53 *E. helvum* were screened by PCR. Juveniles and immature
326 adults, as well as a subset of adults which were serologically positive to at least one antigen
327 were selected (see Table S7 from Supporting information). All samples were negative.

328

329 **Discussion**

330

331 Although a number of cross-sectional studies and significant sampling efforts show
332 the presence of antibodies against ebolavirus in several bat species (7,14,26), only one reported
333 detection of Zaire Ebola virus RNA in three fruit bats species (6). This makes it difficult to
334 understand the dynamics of ebolavirus circulation in bats, the role of bats in ebolavirus
335 maintenance and inter-species transmissions and related zoonotic risks. Here we opted for a
336 longitudinal sampling strategy which allowed to gain further insight into dynamics of
337 ebolaviruses or closely related filoviruses in fruit bats, which is valuable for guiding further
338 research efforts and surveillance strategies. Serological results are in line with a previous cross-
339 sectional study carried out in the same region (7), confirming seropositivity for ebolavirus
340 antigens in the four bat species of interest, the proportion of samples reacting with at least one
341 antigen (23,5% versus 18,2% for the least stringent cut-off in (7)) and simultaneous reactivity
342 against same antigens of different ebolavirus lineages. Antibody kinetics in human ebolavirus
343 disease survivors have been shown to vary for GP and NP antigens, with NP-antibodies
344 declining faster (30). This might contribute to the higher prevalence observed here for
345 antibodies against GP antigens, and the rarity of simultaneous reactivity to GP and NP antigens.
346 Lack of positive controls, standardized cut-off determination methods and knowledge on
347 antibody response dynamics in bats complicate interpretation of results. We are aware that the

348 risk of false positives might constitute a limitation of this study. However, clear and biologically
349 meaningful patterns are observed with the methodology used in this study, which suggests that
350 the serological results for the GP antigens as interpreted here do reflect past or present infection
351 with one of the ebolavirus species tested in this study or cross-reactivity with another filovirus
352 not yet identified.

353 Our results show that the presence of antibodies against ebolaviruses in fruit bats is
354 best explained by bat species, sex, age as well as season. Best fitted models were almost
355 identical regardless of the ebolavirus tested for (GP EBOV-K, GP SUDV or GP BDBV). We
356 opted for interpretation of results in terms of ebolaviruses in general and not specific to
357 ebolavirus species as the homogeneity of the model outputs for the different ebolaviruses might
358 be linked to cross-reactivity between GP antigens, as has been described in previous studies
359 (7,27,31). *E. helvum* and *R. aegyptiacus* consistently showed significantly higher
360 seroprevalences than *H. monstrosus* and *L. angolensis*. Large social groups have been shown
361 to allow higher rates of inter individual contacts which, for some pathogens, might facilitate
362 transmission and increase prevalence (32). *E. helvum* and *R. aegyptiacus* are social species
363 living in large colonies (tens or hundreds of thousands of individuals), therefore offering a large
364 potential for viral transmission, while *L. angolensis* and *H. monstrosus* live in smaller groups
365 (less than fifty for *L. angolensis* and twenty for *H. monstrosus*) (33). *H. monstrosus* is known
366 to form leks which gather thousands of individuals during the mating season in central Africa
367 (34), but leks seem to be smaller and sparse in forested Guinea (field observation). *E. helvum*
368 migrate long distances and form a panmictic population across most of the African continent
369 (35), which might also favor repeated viral reintroductions in colonies. Beyond these factors,
370 species-specific physiology and immunity certainly also contribute to these differences.
371 Important to note is that *R. aegyptiacus* are reservoir hosts of Marburg virus, a filovirus also
372 known to circulate in West Africa (17,36), and have been experimentally shown to be less likely

373 to maintain ebolavirus (37). One explanation for our serological findings in *R. aegyptiacus*
374 might be that they reflect Marburg virus circulation through IgG cross-reactivity with
375 ebolaviruses rather than infection with an ebolavirus. However, this remains questionable as
376 serological IgG cross-reactivity between ebolaviruses and Marburg virus has experimentally
377 been shown to be very limited in bats using indirect ELISA with infectious-based filovirus
378 antigens (31). To bear in mind is that specific feeding and roosting habits might lead to different
379 degrees of inter-species contacts with other bat or non-bat hosts of ebolaviruses and thus viral
380 exposure. As a matter of fact, *R. aegyptiacus* and *H. monstrosus* were observed sharing the
381 same feeding sites in Macenta (T. Pouliquen, pers. comm.), forested Guinea, hence exposure of
382 one of these species to filoviruses hosted by the other followed by immune reaction cannot be
383 excluded. Actually, specific Marburg antibodies have been documented in *H. monstrosus* in
384 Gabon (38). Understanding the role of bats in ebolavirus maintenance and transmission requires
385 a perspective at the community level rather than species level. Further studies on bat
386 demographics, ecology and behavior are still needed to elucidate these differences in
387 seroprevalence, particularly in Guinea.

388 This study suggests for the first time that male fruit bats are more likely to be
389 seropositive against ebolaviruses than females. Given species specific sampling limitations,
390 species-specific studies focusing on this question should be done to confirm this for different
391 species, and to explore the explanatory hypothesis based on observations made on humans.
392 Studies on human populations have shown that ebolavirus frequently persists in male survivors'
393 semen, sometimes for several years as suggested the recent epidemic recrudescence in Guinea
394 (2,39). This might result in repeated antigenic stimulation and slower decrease of antibodies
395 (30,40). We cannot exclude that this scenario also occurs in some bat species, perhaps at
396 specific times, and underlies higher seroprevalences in males (under the assumption that
397 antibodies decline over time when there is no persistence). Behaviour such as mating and

398 aggressive contacts might also increase the frequency of close interactions of males with
399 conspecifics compared to females and favor viral transmissions. In the same line, a recent study
400 on astro- and paramyxovirus circulation in bats has shown higher viral detection rates in males
401 (41).

402 The effect of age on the probability of detecting anti-EBV antibodies, with peak
403 seroprevalences in the age class of immature adults, is in line with Marburg virus infection
404 dynamics where viral infection peaks in older juvenile (6 months old) *R. aegyptiacus* (23) as
405 well as with ebolavirus serological profiles described in *E. helvum* in Cameroun (24). Passive
406 immunity, made up of maternal antibodies to Marburg, wanes in juvenile *R. aegyptiacus* aged
407 4 to 5 months, making them vulnerable to infection with the virus (42). This could also be the
408 case for other filoviruses in fruit bats and explain increases in ebolavirus antibody
409 seroprevalence in immature adults following peaks of viral infections once older juveniles (4
410 to 6 months) are losing their passive immunity. Juvenile bats sampled in the present study were
411 at least several months old as they were flying independently, which makes this hypothesis
412 plausible in explaining the observed pattern of increased seroprevalence in immature adults
413 compared to these older juveniles. Regardless of the underlying mechanisms, and similarly to
414 what has been observed in *E. helvum* (24), results suggest that a seroconversion against
415 ebolavirus or closely related filoviruses takes place during the transition between juvenile and
416 immature adults. The duration of immunity against ebolaviruses in bats being a knowledge gap,
417 the lower antibody prevalence observed here in mature adults compared to immature adults,
418 especially for GP SUDV, could suggest a waning in time of the antibody response, with
419 potential re-stimulation in certain individuals, as suggested in humans (40).

420 Finally, seroprevalences seem to be generally higher during the dry season (November
421 to April) compared to the rainy season. *E. helvum,* which have relatively high sero-prevalence
422 rates, are almost absent of the study site during the rainy season due to their migratory

423 behaviour. Reported seroprevalences may thus also reflect bat population dynamics. Moreover,
424 this seasonal pattern is most probably also related to other factors which we couldn't measure
425 in this study hence caution is needed with interpretation of this result. Food abundance such as
426 fruits can be limited during the dry season. Consequently, this could lead to an exacerbated
427 intra and interspecific competition for food (43) and increased viral transmission followed by
428 increased seroprevalences. Moreover, bat species studied here present one or several birth
429 pulses during this period, and for instance new *E. helvum* recruits reach about 6-7 months of
430 age at the beginning of the dry season. Rainfall and bat reproduction are probably strongly
431 interlinked (22,44), hence seasonal effects detected here are likely to reflect the effect of
432 reproductive life-cycle (and thus also age) on seroprevalence. The data per bat species was
433 insufficient to identify temporal patterns at a finer scale or assess the effect of parameters such
434 as period of copulation, parturition, lactation and weaning for each bat species. However, other
435 studies show that these periods can correspond to increased risks of viral transmissions or
436 impact viral maintenance (38,45,46).

437 Despite important sampling efforts over time, sample size remains small given large bat
438 population sizes, and species-specific sample size was too limited to go into further analysis at
439 the bat species level and at a finer temporal scale. Nevertheless, this study provides valuable
440 information to guide further research on ebolavirus circulation in wildlife as well as surveillance
441 in the study region. Due to potential serological cross-reactivity between ebolavirus species
442 (27,30,31), serological observations may reflect past infections with various ebolaviruses or
443 closely related filoviruses, including undiscovered viruses, and unfortunately virologic data
444 which could help with serological interpretation remains limited. Future identification of the
445 virus(es) underlying these serological signals is essential to understand filovirus maintenance,
446 transmission dynamics and risks of inter-species spillover or emergence. Our results suggest
447 that intensive sampling efforts to search for evidence of and characterize active viral infection

448 should include a strong focus on older juveniles as well as male bats. A special attention should
449 be given to *E. helvum* and *R. aegyptiacus* which present significantly higher seroprevalences
450 compared to the other bat species, without excluding research on other species. In the same
451 way, surveillance of ebolavirus spillover events related to ebolavirus circulation in bats should
452 be reinforced where and when circulation and risks might be enhanced: at the interface with bat
453 colonies when older juveniles are highly prevalent, especially *E. helvum* (during the dry season
454 for forested Guinea) and *R. aegyptiacus* (already demonstrated for Marburg virus in *R.*
455 *aegyptiacus*). Based on these hypotheses, we screened a selection of individuals for filoviruses
456 RNA, but all samples were negative. Given the observed sero-prevalence rates, we assume that
457 the failure to detect filovirus RNA might be explained by either very low viral shedding at time
458 of sampling, the circulation of unknown filoviruses which might not be detected by the PCR
459 protocol used, or inappropriate sample type. Viral load might indeed be generally higher in
460 internal organs than in rectal and oral swabs and it might thus be necessary to use the above-
461 mentioned criteria to target a subset of individuals for more invasive sampling.

462 In conclusion, this study improved the understanding of filovirus dynamics in
463 frugivorous bats by using a longitudinal approach that helps to test the role of potentially
464 influential factors. It emphasizes the importance of host reproductive seasonality as a driver of
465 pathogen circulation and thus spillover risks, which should be considered when designing
466 sampling protocols for surveillance and research. Data can for instance be used to update
467 ebolavirus spillover risk maps already developed for forested Guinea (47). The study was
468 implemented in an ecosystem exposed to intensive human transformation influencing viral
469 ecology. Substantial sampling efforts need to be pursued and combined with data from
470 ecological and behavioural studies on bats and inter-species relations to allow for a broader
471 comprehension of the ecology of filoviruses at the community level. These efforts should also

be replicated in different social-ecological ecosystems with different exposure to human footprint for a comparative analysis.

Acknowledgments

We thank the Comité National d'Ethique pour la Recherche en Santé (CNERS) and the Office Guinéen des Parcs Nationaux et Réserves de Faune (OGPRF) of the Ministère de l'Environnement, et du Développement de la République de Guinée for authorising this study. We thank the Direction Nationale des Services Vétérinaires of the Ministère de l'Agriculture et de l'Elevage of Guinea, the OGPFR, and the CERFIG for their constant support and facilitation of the project, as well as all the field staff (Aboubacar Diallo, Pierre Kabinet Kamano, Mamadou Aliou Bah, Jean Paul Guilavogui, Mamadou Aliou Sow, Aboubacar Camara Bountouraby, Aboubacar Camara, Fassou Kourouma , KoïKoï Sakouvogui).

Data availability

The datasets generated and analysed during the current study are available in the Supporting information or in the following repository: https://doi.org/10.18167/DVN1/KMKUQ8

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper

Funding Statement

This research was carried out as part of the EBO-SURSY project (https://rr-africa.oie.int/en/projects/ebo-sursy-en/), funded by the European Union Delegation under the

496 Agreement [FOOD/2016/379-660], and the EbOHealth project, publicly funded by I-SITE
497 MUSE (Montpellier Université d'Excellence https://muse.edu.umontpellier.fr/en/muse-isite -
498 MUSE2018-EbOHEALTH) through the French National Research Agency (ANR) under the
499 "Investissements d'avenir" program (grant number ANR-16-IDEX-0006).
500 The funders had no role in study design, data collection and analysis, decision to publish, or
501 preparation of the manuscript.
502

503 Supporting information

504 **Methods S1**. Molecular confirmation of bat species

505 **Table S1.** Mean fluorescence intensity cutoff values for different viral antigens, by Ebola
506 virus lineage and statistical method used to determine cutoff according to De Nys et al, 2018.

507 **Table S2**. Characteristics of the variables used in the GLMM statistical models.

508 **Table S3**. Ranking of the top 5 best statistical models for the 3 glycoproteins by increasing
509 AIC value carried out on Rstudio software (version 1.4.1106)

510 **Table S4**. Longitudinal representation of the number of bats sampled per month for
511 ebolavirus serology (October 2018 - July 2020)

512 **Table S5**. Simultaneous reactivity of bat antibodies against antigens of different ebolavirus
513 lineages (M+4SD cutoff)

514 **Table S6**. p-values calculated with the estimated marginal means methods to compare
515 differences in antibody positivity according to age, sex, season and species (with the M+4SD
516 method) (significant p-value <0.05)

517 **Table S7**. Samples tested by PCR for filovirus RNA

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