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

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Abstract

Background

Outbreaks of Ebola disease emerge regularly in human populations on the African continent. This highly virulent haemorrhagic fever can be caused by different orthoebolaviruses from the *Filoviridae* family. Several bat species are believed to carry and transmit some of these viruses to people or other animal species. However, clear evidence is still lacking. Natural circulation and zoonotic transmissions of viruses are generally complex as they often involve several host species and are influenced by biological, environmental and anthropogenic factors. A better understanding of the role of bats and other wildlife in the emergence of Ebola disease outbreaks is crucial for their prevention and early detection.

Methods

In this longitudinal study we searched for antibodies against orthoebolaviruses in fruit bats in order to explore some of the factors which might influence their natural circulation. We performed serological tests on populations of 4 bat species (*Eidolon helvum*, *Hypsignathus monstrosus*, *Myonycteris angolensis* and *Rousettus aegyptiacus*) sampled longitudinally for 18 months (2018-2020) in Guinea. The analysis of 1,427 bat samples for antibodies directed against different orthoebolavirus species allowed us to test the influence of biological and environmental variables on seropositivity by using GLMM.

Results

Results showed that the presence of antibodies against one of the orthoebolaviruses antigen is more frequent in the *Eidolon helvum* and *Rousettus aegyptiacus* species, in males, in sexually immature adults, and during the dry season.

Conclusion

Our results suggest that host species, sex, age, reproductive life-cycle and season may play an important role in the circulation of filoviruses. This information can be used to guide

further sampling to specifically characterize the orthoebolavirus species underlying the production of these antibodies and to adjust surveillance protocols at the interface between bats, humans and other animals.

Keywords: Orthoebolavirus, longitudinal studies, serologic tests, Chiroptera, Guinea

Background

Since the first two outbreaks of Ebola disease (EBOD) in 1976 in Yambuku (Democratic Republic of Congo) and Nzara (South Sudan), more than 30 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 a putative animal reservoir or other infected animals (5). The reservoir species of viruses from the *Orthoebolavirus* genus remain unknown, but bats are suspected to play an important role in the ecology of these viruses and transmission mechanisms (6–8). It is hypothesized that bats might infect humans directly when hunted and handled (9), or indirectly through contact with bat excrement or fruit contaminated with saliva or excrement (10). Bats might also be an indirect source by transmitting viruses to bridge hosts which can act as amplifiers and be at the origin of human outbreaks (6,11). This has been suspected in Central Africa and Ivory Coast where apes have been confirmed as the source of human infection in several outbreaks (12,13).

Certain bat species are regarded as potential reservoir of orthoebolaviruses since the discovery of viral RNA and anti-Ebola virus (anti-EBOV) antibodies in 3 fruit bat species

75 (*Epomops franqueti, Hypsignathus monstrosus, Myonycteris torquata*) (6). However, viral
76 RNA was only detected in 13 of 279 tested specimens, and confirmed by sequence analysis in
77 7. Other studies have since shown that other frugivorous species have anti-orthoebolavirus
78 antibodies (*Rousettus aegyptiacus, Eidolon helvum, Epomophorus gambianus, Micropteropus*
79 *pusillus and Myonycteris angolensis*) and that insectivorous species (*Mops condylurus and*
80 *Chaerephon pumilus*) host a recently discovered *Orthoebolavirus* named Bombali virus
81 (7,8,14). This supports the fact that these wild bats are exposed to filoviruses and are able to
82 survive infection, as demonstrated by experimental inoculations for some of these species
83 (15,16). In addition, *Rousettus aegyptiacus* is the reservoir species of Marburg virus, another
84 filovirus (17), and other bat species have been shown to be exposed or infected with other
85 filoviruses such as Lloviu virus detected in Europe (18) and newly described viruses in China
86 (19). Infectious orthoebolaviruses have never been isolated from free ranging bats so far. The
87 viremic period might be short or viruses might end up circulating at a low level and might only
88 be detected for a limited period of time in tissues or oral and rectal swabs, as shown
89 experimentally in *R. aegyptiacus* inoculated with Ebola and Marburg virus (15,20).

90 The aerial lifestyle, gregariousness, longevity as well as immunological and metabolic
91 specificities of bats are traits that may impact the ecology of orthoebolaviruses. Biological and
92 phenological factors, influenced by seasonal or climate changes, are also important, like the
93 migratory nature of certain bat species or reproduction patterns, both influenced by the
94 availability of food resources (21,22). It has been shown that spillover events of Marburg virus
95 to human populations coincide with the birthing season and presence of juveniles in *Rousettus*
96 *aegyptiacus* populations (23). A recent study on an *Eidolon helvum* colony also showed an
97 increase in the presence of antibodies against orthoebolaviruses when juveniles turn into
98 immature adults (24). Other factors can influence the dynamics of orthoebolaviruses in their
99 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 orthoebolavirus research and EBOD surveillance strategies.

The objective of this study was to investigate the effects of biological and environmental factors on the presence of anti-orthoebolavirus antibodies and improve understanding of dynamics of orthoebolavirus circulation in bats. We performed a longitudinal serological survey of orthoebolavirus in four frugivorous bat species in Guinea, a country at risk of EBOD outbreak. Based on the outcomes of the serological investigation, a subset of bats was also screened for filovirus 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 (E. helvum), Hypsignathus monstrosus (H. monstrosus), Myonycteris angolensis (M. angolensis) and Rousettus aegyptiacus (R. aegyptiacus)*, in which anti-orthoebolavirus antibodies have already been detected in the past (8). 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.* (8). 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 (26,27) and combined to genetic confirmation for some specimens, Additional file 1 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. Source of the basemap: https://data.humdata.org/dataset/cod-ab-gin; license: https://data.humdata.org/faqs/licenses.

146 ***Screening for Ebolaviruses Antibodies***

147 We tested dried blood spots (DBS) for anti-orthoebolavirus IgG with a Luminex-
148 based serologic assay adapted for bats as described in De Nys *et al*. (8). The assay included ten
149 recombinant orthoebolavirus proteins, i.e. the glycoprotein (GP), nucleoprotein (NP), and/or
150 viral protein 40 (VP40), for different orthoebolaviruses: Ebola virus Kissidougou and Mayinga
151 strains (NP, GP K, GP M, VP40), Sudan virus (NP, GP, VP40), Bundibugyo virus (GP, VP40)
152 and Reston virus (GP). Recombinant proteins were produced in sf9 insect cell vectors and
153 purchased at Sinobiologicals (Beijing, China) except for REST GP (IBT (Gaithersburg, MD)).
154 The characteristics of the antigens of the different orthoebolavirus species used are as follows:
155 Ebola virus (EBOV) NP (amino acids [aa] 488 to 739 strain Mayinga 1976), VP40 (aa 31 to
156 326, strain Kissidougou-Makona 2014), GP K (aa 1 to 650, Kissidougou-Makona 2014), GP
157 M (aa 1 to 650, Mayinga 1976); Sudan virus (SUDV) NP (aa 361-738, strain Gulu), GP (aa 1
158 to 637, strain Uganda 2000), and VP40 (aa 31 to 326, strain Gulu); Bundibugyo virus (BDBV)
159 GP (aa 1 to 501, strain Uganda 2007), and VP40 (aa 31 to 326, strain Uganda 2007); and Reston
160 virus (RESTV) GP (aa 1 to 650). DBS were eluted and tested as previously described in 200µL
161 of incubation buffer (8,24,28,29). One hundred µL of sample, corresponding to the equivalent
162 of a final plasma dilution of 1/2000, was incubated with 50µL of magnetic beads coated with
163 recombinant protein (2 µg protein/1.25 × 106 beads) in 96-well flat-bottom chimney plates
164 (Greiner bio one, Frickenhausen, Germany) on a plate shaker at 300 rpm for 16 h at 4 °C in the
165 dark. After washing, 0.1 µg/mL of goat anti-bat biotin-labeled IgG (Euromedex,
166 Souffelweyersheim, France) was added to each well and incubated for 30 min at 300 rpm at
167 room temperature. After washing, we added 50 µL of 4 µg/mL streptavidin-R-phycoerythrin
168 (Fisher Scientific/Life Technologies, Illkirch, France) per well and incubated for 10 min at 300
169 rpm at room temperature. Reactions were read with BioPlex-200 (BioRad, Marnes-la-Coquette,
170 France) or MagPix (Luminex, Austin, TX, US). At least 100 events were read for each bead set

171 on a Bioplex-200 instrument, and results were expressed as median fluorescence intensity
172 (MFI) per 100 beads. Samples that showed positive signals were repeated in order to validate
173 the results.

174 To interpret the serological results in the absence of true positive controls, MFI cutoff
175 values for each antigen were determined using four methods in De Nys et al. (8) (See Table S1
176 in Additional file 1). In this study we opted for the cutoff values resulting from the least
177 stringent method, i.e. the mean plus four times the standard deviation of MFIs on negative
178 control samples (M + 4SD) as calculated in De Nys et al. 2018, to search for serological patterns
179 through a multivariate statistical analysis. Negative control samples (n = 145) were from
180 captive-born insectivorous bat species (*Carollia perspicillata*, n=103) hosted at the Parc
181 Zoologique de Montpellier (Montpellier, France) and two frugivorous bat species (*Pteropus*
182 *giganteus*, n=19, *Rousettus aegyptiacus*, n=23) hosted at Wilhelma Zoo and Botanical Garden
183 (Stuttgart, Germany). Samples were considered seropositive for an antigen if the MFI values
184 were above the cutoff value for this antigen. Samples were considered seropositive for an
185 orthoebolavirus if MFI values were above the cutoff value for at least two antigens of this
186 specific orthoebolavirus.

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188 ***Multivariate statistical analyses***

189 Proportions of samples positive to at least one orthoebolavirus antigen or to minimum
190 two antigens of the same orthoebolavirus are presented in a descriptive way to provide a
191 synthetic overview of the data and serological reactivity. Multivariate analyses were performed
192 for orthoebolavirus seropositivity as well as separately for the 3 antigens for which most
193 reactive samples were observed. We developed Generalized Linear Mixed Models (GLMM)
194 to test the impact of several drivers on the probability of a bat being positive by serology for
195 orthoebolavirus antigens (lme4 package in R software). We used results of the serological tests

196 as the response variable with a binomial distribution, and species, sex, age and season as
197 explanatory variables with fixed effects (See Table S2 from Additional file 1). To account for
198 clustered samples collected during the same sampling session at the same site, we included
199 sampling period as a random effect to control for repeated measures from the same trapping
200 session.
201 In order to select the best model, we used the glmulti function (Rsoftware, glmulti package
202 (30)) to rank all possible models combining the explanatory variables by the Akaike
203 Information Criterion (AIC). We selected the models with the lowest AIC (See Table S3 in
204 Additional file 1). The odds ratios were estimated for each explanatory variable. We also used
205 the emmeans and ggeffects functions to obtain estimated marginal means for the response
206 variable and visualize the effect of each explanatory variable. Analysis were carried out on R
207 software 4.1.2.
208
209 ***PCR screening for filoviruses***
210 Oral and rectal swabs from a subset of bats were selected according to the analysis of
211 serological results, extracted and tested for filovirus RNA by a semi-nested PCR following the
212 protocols described in Djomsi et al. 2022 (24). The PCR targeted a 630 bp fragment of the L
213 gene using degenerated primers that detect a wide diversity of filoviruses, i.e., Filo-MOD-
214 FWD: TITTYTCHVTICAAAAICAYTGGG and FiloL.conR:
215 ACCATCATRTTRCTIGGRAAKGCTTT in round 1 and Filo-MOD-FWD: TITTYTCHVTI-
216 CAAAAICAYTGGG, and Filo-MOD-RVS: GCYTCISMIAIIGTTTGIACATT in round 2 (7)
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221 # Results

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223 ***Bat sampling and serological results***

224 We analyzed blood samples collected longitudinally from 1,427 wild frugivorous
225 bats: 196 *E. helvum*, 186 *H. monstrosus*, 380 *M. angolensis* and 665 *R. aegyptiacus* (Table 1
226 and Table S4 from Additional file 1). Descriptive analyses show a total of 336/1427 (24%)
227 samples that are reactive with at least one antigen. Proportions of positive samples per bat
228 species, sex, age class and season are presented in Table 2. A total of 24/1427 (1.7%) bats are
229 reactive with minimum 2 antigens of a specific orthoebolavirus and are thus seropositive for at
230 least one of the orthoebolavirus species tested for (Table 3). Orthoebolavirus seroprevalence is
231 highest in the *R. aegyptiacus* population (3%).

232

233 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)
Myonycteris 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)

234 * UN = undetermined

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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)
M. 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)
M. 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)
M. 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)
M. 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, M. = Myonycteris, R. = Rousettus*, Pos. = positive, Tot. = total, UN =undetermined, CI = Confidence Interval

Table 3. Number and percentage of bats seropositive for orthoebolaviruses (positive for minimum two antigens of the same orthoebolavirus according to the M+4SD cutoff method)

	EBOV			SUDV		BDBV		Seropositive for min. 1 orthoebolavirus	
	Tot.	Pos.	% (95% CI)	Pos.	% (95% CI)	Pos.	% (95% CI)	Pos.	% (95% CI)
*E. helvum**	196	0	**0** (0-1.9)	2	**1** (0.3-3.6)	0	**0** (0-1.9)	2	**1** (0.3-3.6)
H. monstrosus	186	0	**0** (0-2)	0	**0** (0-2)	0	**0** (0-2)	0	**0** (0-2)
M. angolensis	380	0	**0** (0-1)	2	**0.5** (0.1-1.9)	0	**0** (0-1)	2	**0.5** (0.1-1.9)
R. aegyptiacus	665	9	**1.4** (0.7-2.6)	13	**2** (1.1-3.3)	1	**0.2** (0-0.8)	20	**3** (2-4.6)
Total	1427	9	**0.6** (0.3-1.2)	17	**1.2** (0.7-1.9)	1	**0.1** (0-0.4)	24	**1.7** (1.1-2.5)

* *E. =Eidolon, H.= Hypsignathus, M. = Myonycteris, R. = Rousettus*, Pos. = positive, Tot. = total, min. = minimum, CI = Confidence Interval

253 Regarding seroprevalences for each antigen of the different viruses, GPs were the
254 recombinant proteins for which most reactions were observed: 9.8% of the samples showed
255 reactivity against the EBOV Kissidougou strain, 18.8% against SUDV and 9.6% against BDBV
256 versus 0.1 to 3.3 % against other antigens (Table 4). Lower reactivity for VP40 and NP antigens
257 was observed for each bat species. Simultaneous reactivity to GP and NP of the same virus
258 was rare and observed for 0.1% of the samples for EBOV and 0.6% for SUDV. The
259 simultaneous reactivity to the same antigen of different viral lineages was frequent: 53% of the
260 samples were reactive to GP of at least two orthoebolavirus species, 23% to VP40 and no cross-
261 reactivity was observed for NP (See Table S5 from Additional file 1).

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263 Table 4. Number (percentage) of bat blood samples reactive for each orthoebolavirus antigen according to the
264 M+4SD cutoff method

		E. helvum (n=196)		*H. monstrosus* (n=186)		*M. angolensis* (n=380)		*R. aegyptiacus* (n=665)		*Total (n=1427)*	
Orthoebolavirus	Antigen*	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI	no. (%)	95% CI
Ebola											
	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

265 *NP = nucleoprotein; GP = glycoproteine (K = Kissidougou strain; M = Mayinga strain); VP = Viral protein
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267 ***Factors influencing the presence of anti-orthoebolavirus antibodies in bats***

268 We performed analysis on orthoebolavirus seropositivity, as well as on the presence
269 of antibodies against the following 3 antigens: GP Ebola virus Kissidougou (GP EBOV-K), GP

Sudan virus (GP SUDV) and GP Bundibugyo virus (GP BDBV). The GLMM was not converging for orthoebolavirus seropositivity, due to the low seroprevalence rates. However, for each separate GP antigen we obtained fairly similar best fitted models (Table 5), i.e. the Species, Sex and Age variables were consistently selected in the best models for each antigen. The variable Season was also selected in the best models for GP EBOV-K and GP BDBV and models with or without Season were near to equivalent for GP SUDV (Table 5).

Table 5. Best models obtained for the presence of antibodies against GP EBOV Kissidougou, GP BDBV and the 2 best models for GP SUDV

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 6. Interestingly, *H. monstrosus* and *M. angolensis* had consistently and significantly lower GP 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 GP 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 GP seroprevalences than females for all the models (OR ranging from 1.56 to 1.72, $p < 0.034$).

294 Table 6. Regression table representing the odds ratios of the best statistical models for GP EBOV-K, GP BDBV
295 and GP SUDV
296

	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
M. 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			

297 *significant <0.05; GP = glycoprotein; EBOV-K = Ebola virus Kissidougou strain; BDBV = Bundibugyo virus; SUDV = Sudan virus;
298 OR = Odds Ratio; CI = Confidence Interval

299

300 Similarly, the estimated marginal means of GP seroprevalences were significantly
301 higher for *R. aegyptiacus* and *E. helvum* compared to *H. monstrosus* and *M. angolensis* (Figure
302 2 and Additional file 1 Table S6) and for males compared to females (Figure 3), whatever the
303 antigen. GP seroprevalences were significantly higher for immature adults compared to
304 juveniles for GP EBOV-K and compared to juveniles and adults for GP SUDV (Figure 4). The
305 GP seroprevalences were also significantly higher in the dry season compared to the rainy
306 season for GP EBOV-K and GP BDBV (Figure 5).

307

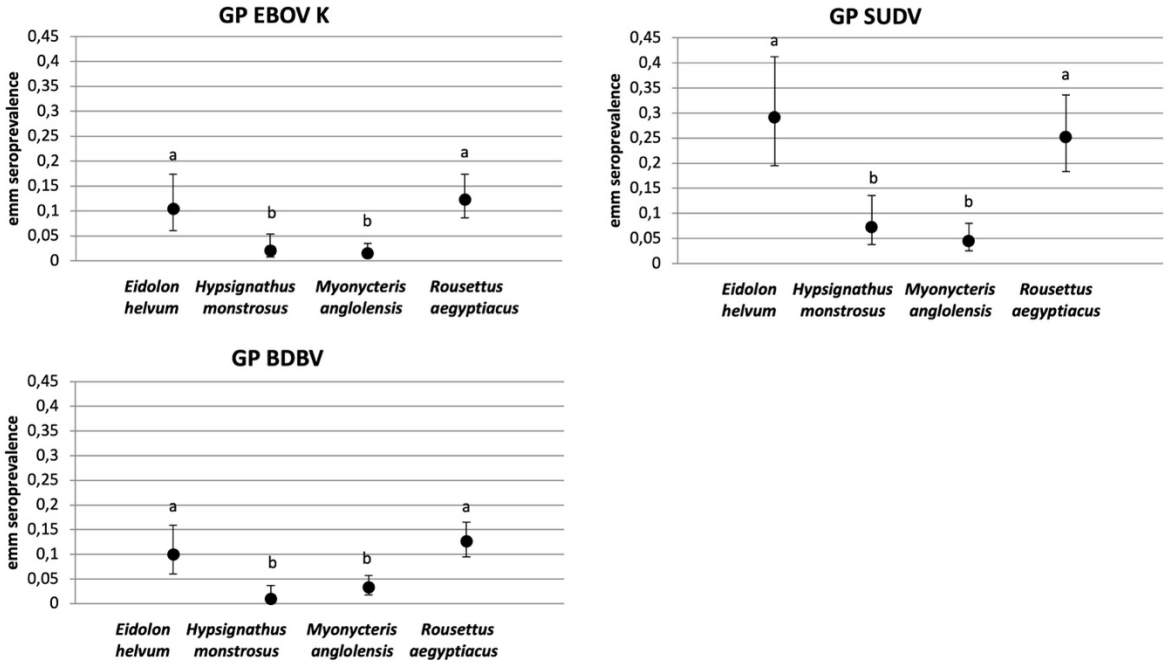

Figure 2. Estimated marginal means (emm) of bat seroprevalence depending on the species and the antigens (estimated marginal means annotated with different letters are significantly different ($p < 0.05$))

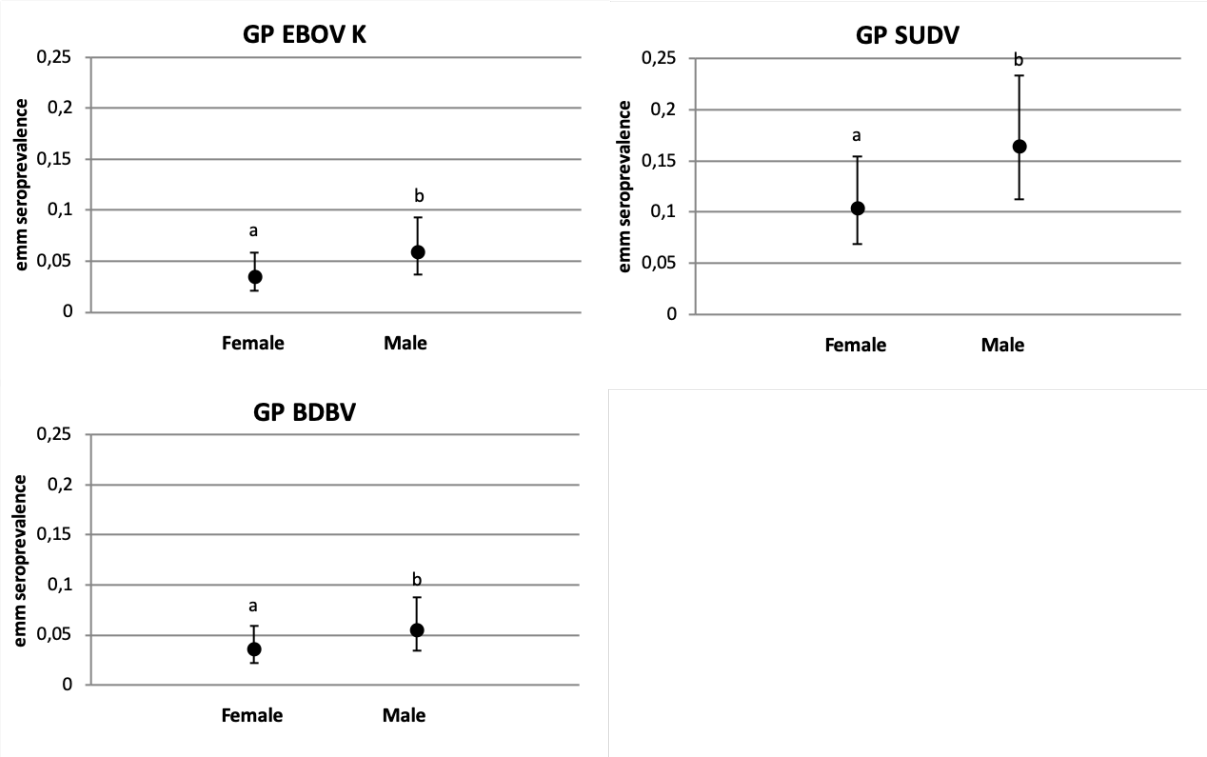

Figure 3. Estimated marginal means of bat seroprevalence depending on the sex and the antigens (estimated marginal means annotated with different letters are significantly different ($p < 0.05$))

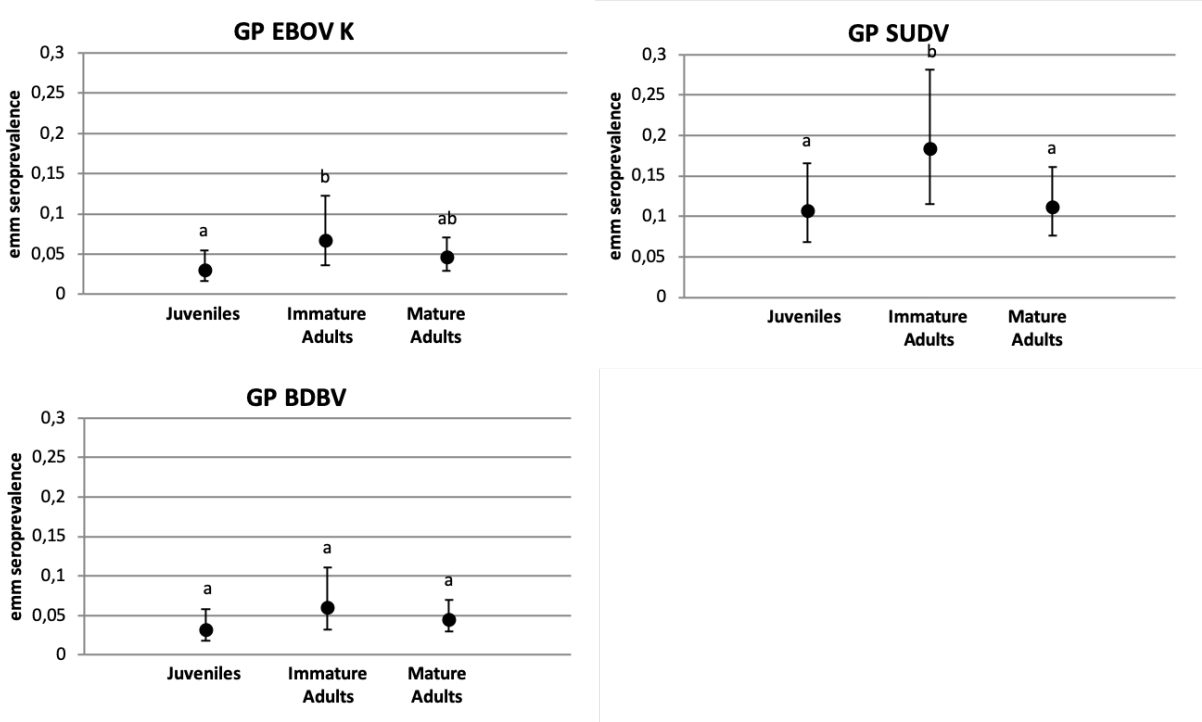

Figure 4. Estimated marginal means of bat seroprevalence depending on the age class and the antigens (estimated marginal means annotated with different letters are significantly different ($p < 0.05$))

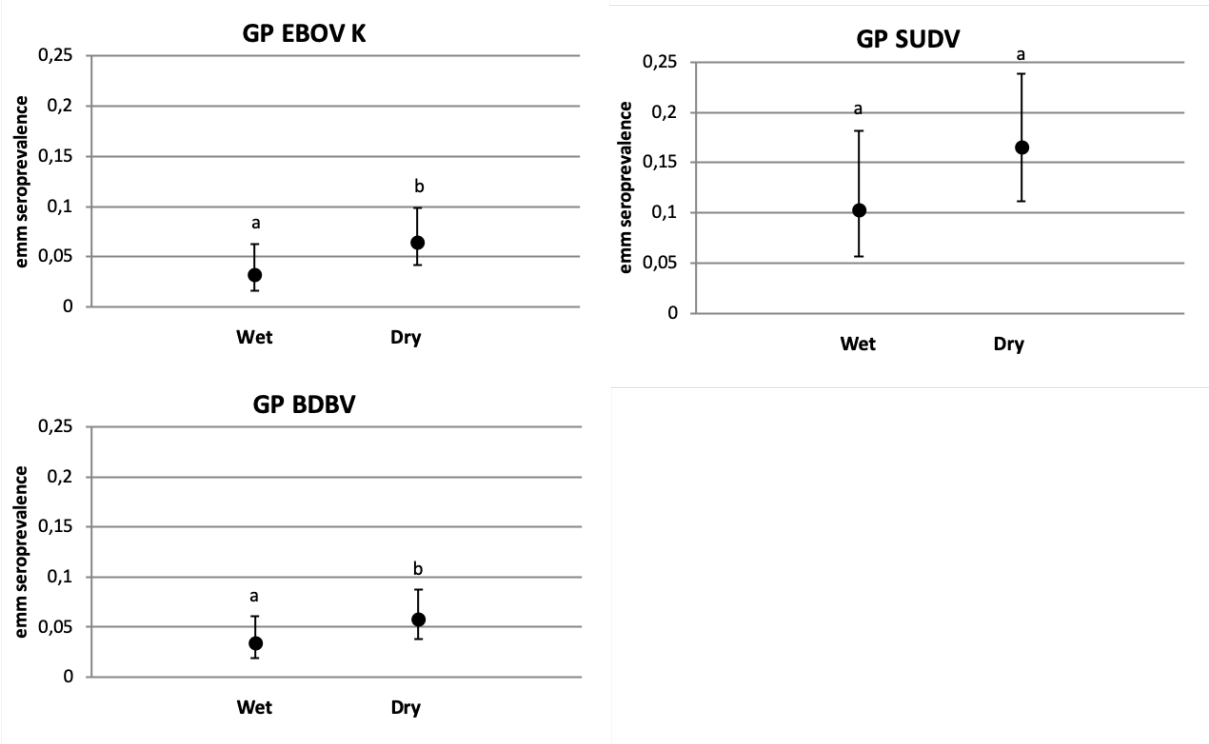

Figure 5. Estimated marginal means of bat seroprevalence depending on the season and the antigens (estimated marginal means annotated with different letters are significantly different ($p < 0.05$))

328 ***Filovirus RNA detection***

329 Given the results of the serological and statistical analysis, oral (195) and rectal (141)
330 swabs from 142 *R. aegyptiacus* and 53 *E. helvum* were screened by PCR. Juveniles and
331 immature adults, as well as a subset of adults which were serologically positive to at least one
332 antigen were selected (see Table S7 from Additional file 1). In addition, 64 *H. monstrosus* (64
333 oral and 63 rectal swabs) as well as 75 *M. angolensis* (75 oral and 75 rectal swabs) were also
334 tested given that *H. monstrosus* is one of the 3 African fruit bat species in which viral EBOV
335 RNA has been detected in previous studies (6) and that antibodies against orthoebolaviruses
336 were detected in both species. All samples were negative.

337

338 **Discussion**

339

340 Although a number of cross-sectional studies and significant sampling efforts show
341 the presence of antibodies against orthoebolaviruses in several bat species (8,14,28), only one
342 reported detection of Ebola virus RNA in three fruit bats species (6). This low detection rate of
343 viral genetic material makes it difficult to understand the dynamics of orthoebolavirus
344 circulation in bats, the role of bats in orthoebolavirus maintenance and inter-species
345 transmissions and related zoonotic risks. Antibody detection remains therefore a valuable
346 indirect indicator of orthoebolavirus circulation in bats even if its interpretation remains
347 challenging (8,31). Lack of positive controls, standardized cut-off determination methods,
348 serological cross-reactivity, knowledge on antibody response dynamics in bats and viral
349 molecular evidence generally complicate interpretation of results.

350 Here we opted for a longitudinal sampling strategy which allowed us to gain further
351 insight into dynamics of orthoebolaviruses or closely related filoviruses in fruit bats, which is
352 valuable for guiding further research efforts and surveillance strategies. Serological results are

353 in line with cross-sectional studies carried out in the same region (8,32), confirming
354 seropositivity for several orthoebolavirus antigens in the four bat species of interest, the
355 proportion of samples reacting with at least one antigen (23,5% versus 18,2% and 18,9% for
356 the least stringent cut-off) and simultaneous reactivity against same antigens of different
357 orthoebolavirus species. Antibody kinetics in human EBOD survivors have been shown to vary
358 for GP and NP antigens, with NP-antibodies declining faster (33). This might contribute to the
359 higher seroprevalence observed here for antibodies against GP antigens, and the rarity of
360 simultaneous reactivity to GP and NP antigens, also found in other studies (8,32). The lack of
361 specificity due to antibody cross-reactivity between filoviruses and the use of single GP
362 antigens per virus for statistical analysis might constitute limitations of this study. However,
363 clear and biologically meaningful patterns are observed with the methodology used. This
364 suggests that the serological results for the GP antigens as interpreted here do reflect past or
365 present infection with orthoebolavirus species tested for in this study or with one or several
366 other, perhaps not yet identified, filoviruses which induce antibodies that cross-react with
367 known orthoebolavirus antigens.

368 Our results show that the presence of antibodies against orthoebolavirus GP antigens
369 in fruit bats is best explained by bat species, sex, age as well as season. Best fitted models were
370 almost identical regardless of the orthoebolavirus tested for (GP EBOV-K, GP SUDV or GP
371 BDBV). We opted for interpretation of results in terms of orthoebolavirus GP in general and
372 not specific to orthoebolavirus species as the homogeneity of the model outputs for the different
373 orthoebolavirus GP antigens might be linked to cross-reactivity between GP antigens, as has
374 been described in previous studies (8,29,31).

375 *E. helvum* and *R. aegyptiacus* consistently showed significantly higher
376 orthoebolavirus GP seroprevalences than *H. monstrosus* and *M. angolensis* and *R. aegyptiacus*
377 had the highest seroprevalence when taking into account minimum 2 antigens per virus. *R.*

378 *aegyptiacus* are reservoir hosts of Marburg virus, a filovirus also known to circulate in West
379 Africa (17,34), and have been experimentally shown to be less likely to maintain
380 orthoebolaviruses (35). One explanation for our serological findings in *R. aegyptiacus* might
381 be that they reflect Marburg virus infection through IgG cross-reactivity with orthoebolavirus
382 antigens rather than infection with an orthoebolavirus. Serological IgG cross-reactivity between
383 orthoebolaviruses and Marburg virus has experimentally been shown to be very limited in bats
384 using indirect ELISA with infectious-based filovirus antigens (31). However, we used
385 recombinant viral protein antigens for our multiplex serological assays, hence cross-reaction of
386 anti-Marburg virus IgG might be higher compared to assays based on infectious-based antigens
387 (31). An alternative explanation for our serological results in *R. aegytpiacus* could be regular
388 exposure of *R. aegyptiacus* to known or unknown orthoebolaviruses through other infected
389 animals, leading to antibody response, and potential low-level replication followed by rapid
390 viral clearance. *R. aegyptiacus* is actually accountable for the highest prevalence of
391 orthoebolavirus seropositive bats (minimum 2 antigens) of our study. Despite the fact that *R.*
392 *aegyptiacus* might not be able to maintain known orthoebolaviruses infection, they have
393 actually been shown to produce virus-specific IgG when challenged experimentally with
394 orthoebolaviruses (35). Moreover, inoculation experiments on *R. aegyptiacus* use viral strains
395 originating from human epidemics, which might have been subject to mutations during
396 passages on cells. The extent to which other potentially circulating orthoebolaviruses are able
397 to cause more or less persistent infection in this species is unknown. Our results for *E. helvum*
398 are in line with other serological surveys which have detected antibodies against
399 orthoebolavirus GP in colonies from various geographical sites (8,24,36). An experimental
400 study has shown reduced susceptibility of Eidolon cells to EBOV (37). However, susceptibility
401 to other filoviruses might vary. In the same way as for *R. aegyptiacus*, the detected antibody
402 response might reflect exposure to orthoebolavirus(es) followed by limited replication and

403 rapid viral clearance or a certain degree of infection by one or several other closely related
404 filoviruses.
405 Beyond species-specific physiology and immunity, bat behaviour and ecology certainly also
406 contribute to inter-species variability in antibody responses. Large social groups have been
407 shown to allow higher rates of intra-species contacts which, for some pathogens, might facilitate
408 transmission and increase prevalence (38). *E. helvum* and *R. aegyptiacus* are social species
409 living in large colonies (tens or hundreds of thousands of individuals), therefore offering a large
410 potential for viral transmission, while *M. angolensis* and *H. monstrosus* live in smaller groups
411 (less than fifty for *M. angolensis* and twenty for *H. monstrosus*) (26). *H. monstrosus* is known
412 to form leks which gather thousands of individuals during the mating season in central Africa
413 (39), but leks seem to be smaller and sparse in forested Guinea (field observation). *E. helvum*
414 migrate long distances and form a panmictic population across most of the African continent
415 (40), which might also favor repeated viral reintroductions in colonies. Specific feeding and
416 roosting habits might lead to different degrees of inter-species contacts with other bat or non-
417 bat hosts of orthoebolaviruses and thus viral exposure. As a matter of fact, *R. aegyptiacus* and
418 *H. monstrosus* were observed sharing the same feeding sites in Macenta (T. Pouliquen, pers.
419 comm.), forested Guinea, hence exposure of one of these species to filoviruses hosted by the
420 other followed by immune reaction cannot be excluded. Actually, specific Marburg antibodies
421 have been documented in *H. monstrosus* in Gabon (41). Understanding the role of bats in
422 orthoebolavirus maintenance and transmission requires a perspective at the community level
423 rather than species level. Further studies on bat demographics, ecology, behaviour as well as
424 identification of the viruses responsible for the detected antibody responses are still needed to
425 elucidate these species differences in seroprevalence and their role in viral maintenance and
426 transmission, particularly in Guinea.

427 This study suggests for the first time that male fruit bats are more likely to be
428 seropositive against orthoebolavirus GP than females despite species-specific sampling
429 limitations. This gender difference was however not observed in a longitudinal study on *E.*
430 *helvum* in Cameroon (24). Further longitudinal species-specific studies focusing on this
431 question are needed to investigate this in different species, and to explore the explanatory
432 hypothesis based on observations made on humans. Studies on human populations have shown
433 that EBOV frequently persists in male survivors' semen, sometimes for several years as
434 suggested by the recent epidemic recrudescence in Guinea (2,42). This might result in repeated
435 antigenic stimulation and slower decrease of antibodies (33,43). We cannot exclude that this
436 scenario also occurs in some bat species, perhaps at specific times, and underlies higher GP
437 seroprevalences in males (under the assumption that antibodies decline over time when there is
438 no persistence). Behaviour such as mating and aggressive contacts might also increase the
439 frequency of close interactions of males with conspecifics compared to females and favor
440 microbial transmissions, as has been observed for other host species (44). In the same line, a
441 recent study on astro- and paramyxovirus circulation in bats has shown higher viral detection
442 rates in males (45).

443 The effect of age on the probability of detecting anti-orthoebolavirus GP antibodies,
444 with peak seroprevalences in the age class of immature adults, is in line with Marburg virus
445 infection dynamics where viral infection peaks in older juvenile (6 months old) *R. aegyptiacus*
446 (23) as well as with orthoebolavirus serological profiles described in *E. helvum* in Cameroun
447 (24,32). Passive immunity, made up of maternal antibodies to Marburg, wanes in juvenile *R.*
448 *aegyptiacus* aged 4 to 5 months, making them vulnerable to infection with the virus (46). This
449 could also be the case for other filoviruses in fruit bats and explain increases in orthoebolavirus
450 GP seroprevalence in immature adults following peaks of viral infections once older juveniles
451 (4 to 6 months) are losing their passive immunity. Juvenile bats sampled in the present study

452 were at least several months old as they were flying independently, which makes this hypothesis
453 plausible in explaining the observed pattern of increased GP seroprevalence in immature adults
454 compared to these older juveniles. Regardless of the underlying mechanisms, and similarly to
455 what has been observed in *E. helvum* (24), results suggest that a seroconversion against
456 orthoebolavirus or closely related filovirus takes place during the transition between juvenile
457 and immature adults. The duration of immunity against orthoebolaviruses in bats being a
458 knowledge gap, the lower antibody prevalence observed here in mature adults compared to
459 immature adults, especially for GP SUDV, could suggest a waning in time of the antibody
460 response, with potential re-stimulation in certain individuals, as suggested in humans (43).

461 Finally, orthoebolavirus GP seroprevalences seem to be generally higher during the dry
462 season (November to April) compared to the rainy season. *E. helvum,* which have relatively
463 high seroprevalence rates, are almost absent of the study site during the rainy season due to
464 their migratory behaviour. Reported seroprevalences may thus also reflect bat population
465 dynamics. Moreover, this seasonal pattern is most probably also related to other factors which
466 we couldn't measure in this study hence caution is needed with interpretation of this result.
467 Food abundance such as fruits can be limited during the dry season. Consequently, this could
468 lead to an exacerbated intra and interspecific competition for food (47) and increased viral
469 transmission followed by increased seroprevalences. Moreover, bat species studied here present
470 one or several birth pulses during this period, and for instance new *E. helvum* recruits reach
471 about 6-7 months of age at the beginning of the dry season. Rainfall and bat reproduction are
472 probably strongly interlinked (22,48), hence seasonal effects detected here are likely to reflect
473 the effect of reproductive life-cycle (and thus also age) on seroprevalence. The data per bat
474 species was insufficient to identify temporal patterns at a finer scale or assess the effect of
475 parameters such as period of copulation, parturition, lactation and weaning for each bat species.

476 However, other studies show that these periods can correspond to increased risks of viral
477 transmissions or impact viral maintenance (41,49,50).

478 Despite important sampling efforts over time, sample size remains small in relation to
479 the size of bat populations, and species-specific sample size was too limited to go into further
480 analysis at the bat species level and at a finer temporal scale. Nevertheless, this study provides
481 valuable information to guide further research on filovirus circulation in wildlife as well as
482 surveillance in the study region. Due to potential serological cross-reactivity between
483 orthoebolavirus species (29,31,33), serological observations may reflect past infections with
484 various orthoebolaviruses or closely related filoviruses, including undiscovered viruses. The
485 regular discovery of novel filovirus species across continents such as new filoviruses in China
486 (51,52), Bombali virus in four African countries (7,53–55), Lloviu virus in three European
487 countries (18,56,57), suggests that the full filovirus diversity has only been partly explored so
488 far. Molecular data which could help determine the filovirus species responsible for the
489 antibodies detected in our study and confirm serological interpretation remains limited. Future
490 identification of the virus(es) underlying these serological signals requires sequencing of viral
491 genetic material and virus isolation, and is essential to understand filovirus maintenance,
492 transmission dynamics and risks of inter-species spillover or emergence. Our results suggest
493 that intensive sampling efforts to search for evidence of and characterize active viral infection
494 should include a strong focus on older juveniles as well as male bats. A special attention should
495 be given to *E. helvum* and *R. aegyptiacus* which present significantly higher seroprevalences
496 compared to the other bat species, without excluding research on other species. In the same
497 way, surveillance of spillover events related to filovirus circulation in bats should be reinforced
498 where and when circulation and risks might be enhanced: at the interface with bat colonies
499 when older juveniles are highly prevalent, especially *E. helvum* (during the dry season for
500 forested Guinea) and *R. aegyptiacus* (already demonstrated for Marburg virus in *R.*

501 *aegyptiacus*). Based on these hypotheses, we screened a selection of individuals for filovirus
502 RNA, but all samples were negative. Given the observed GP seroprevalence rates, we assume
503 that the failure to detect filovirus RNA might be explained by either very low viral shedding at
504 time of sampling, the circulation of unknown filoviruses which might not be detected by the
505 PCR protocol used, or inappropriate sample type. Viral load might indeed be generally higher
506 in internal organs than in rectal and oral swabs and it might thus be necessary to use the above-
507 mentioned criteria to target a subset of individuals for more invasive sampling. Screening of *H.*
508 *monstrosus* and *M. angolensis* which showed lower GP seroprevalence rates also revealed
509 negative results. Nevertheless, EBOV RNA has been detected in organ samples from *H.*
510 *monstrosus* in the past (6). Isolating filovirus nucleic acid from bats remains a challenge and
511 when detected, prevalence rates based on rectal and oral swabs are often low (e.g. 1, 6 % for
512 BOBV in Goldstein et al. 2018 (7) and 0,7% for Marburg virus in Paweska et al. 2020 (58)).
513 Better targeted and larger sample sizes, combined with metagenomic approaches might be
514 necessary to increase probability of filovirus RNA detection. Further experimental studies can
515 also provide a clear insight into host competence for maintenance and transmission of known
516 viruses and be complementary to field studies (59).

517 **Conclusions**

518 This study improved the understanding of filovirus dynamics in frugivorous bats by
519 using a longitudinal approach that helps to test the role of potentially influential factors. It
520 emphasizes the importance of host reproductive seasonality as a driver of pathogen circulation
521 and thus spillover risks, which should be considered when designing sampling protocols for
522 surveillance and research. Data can for instance be used to update orthoebolavirus spillover risk
523 maps already developed for forested Guinea (60). The study was implemented in an ecosystem
524 exposed to intensive human transformation influencing viral ecology. Substantial sampling

525 efforts need to be pursued and combined with data from ecological and behavioural studies on
526 bats and inter-species relations to allow for a broader comprehension of the ecology of
527 filoviruses at the community level. These efforts should also be replicated in different social-
528 ecological ecosystems with different exposure to human footprint for a comparative analysis.
529

530 **List of abbreviations**

531 AIC: Akaike Information Criterion
532 BDBV: Bundibugyo Ebola virus
533 bp: base pairs
534 EBOD: Ebola disease
535 EBOV: Ebolavirus
536 ELISA: Enzyme-Linked Immuno-Sorbent Assay
537 emm: Estimated Marginal Means
538 GLMM: Generalized Linear Mixed Models
539 GP: Glycoprotein
540 GP-K or GP EBOV-K: Glycoprotein Ebola virus Kissidougou strain
541 GP-M or GP EBOV-M: Glycoprotein Ebola virus Mayinga strain
542 IgG: Immunoglobulin G
543 MFI: Median Fluorescence Intensity
544 M + 4SD: mean plus four times the Standard Deviation
545 NP: Nucleoprotein
546 OR: Odds Ratios
547 p: p value (significant < 0.05)
548 PCR: Polymerase Chain Reaction
549 RESTV: Reston Ebola virus
550 RNA: Ribonucleic acid
551 SUDV: Sudan Ebola virus
552 VP40: Viral protein 40
553

Declarations

Ethics approval and consent to participate

This study was approved by the Comité National d'Ethique pour la Recherche en Santé (CNERS) in Guinea (permit number 025/CNERS/20), 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 (permit 017OGUIPAR/MEEF/2017).

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MC has contributed to the design, acquisition, analysis, interpretation of the data, and drafting of the manuscript. AC, MB have contributed to the conception, design, analysis, interpretation and revision of the manuscript. AA, MP, AKK have contributed to the design, acquisition, analysis, interpretation of the data and revision of the manuscript. AS and MID have contributed to the data acquisition, interpretation and revision of the manuscript, TP and GT have contributed to the acquisition of the data and revision of the manuscript. JC and HDN have contributed to the conception, design, data acquisition, analysis, interpretation and drafting of the manuscript. All authors read and approved the final manuscript.

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Additional file 1

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Methods S1. Molecular confirmation of bat species

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

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

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

844 **Table S4**. Longitudinal representation of the number of bats sampled per month for
845 orthoebolavirus serology (October 2018 - July 2020)

846 **Table S5**. Simultaneous reactivity of bat antibodies against antigens of different
847 orthoebolavirus lineages (M+4SD cutoff)

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

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