Received: 8 March 2018 | Accepted: 28 May 2019

DOI: 10.1111/mec.15145

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Tropical rainforest flies carrying pathogens form stable associations with social nonhuman primates

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Funding information

This study was supported in part by the DFG Research Group "Sociality and Health in Primates" (FOR2136). J.F.G. was supported by the Canadian Institutes of Health Research's Strategic Training Initiative in Health Research's Systems Biology Training Program, an NSERC Vanier Canada Graduate Scholarship (CGS), a long-term Research Grant from the German Academic Exchange Service (DAAD-91525837-57048249), and also by the DAAD with funds from the German Federal Ministry of Education and Research (BMBF) and the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme (FP7/2007-2013) under REA grant agreement 605728 (P.R.I.M.E. – Postdoctoral Researchers International Mobility Experience). Core-funding for the Taï Chimpanzee Project is provided by the Max Planck Society.

Abstract

Living in groups provides benefits but also incurs costs such as attracting disease vectors. For example, synanthropic flies associate with human settlements, and higher fly densities increase pathogen transmission. We investigated whether such associations also exist in highly mobile nonhuman primate (NHP) Groups. We studied flies in a group of wild sooty mangabeys (*Cercocebus atys atys*) and three communities of wild chimpanzees (*Pan troglodytes verus*) in Taï National Park, Côte d'Ivoire. We observed markedly higher fly densities within both mangabey and chimpanzee groups. Using a mark–recapture experiment, we showed that flies stayed with the sooty mangabey group for up to 12 days and for up to 1.3 km. We also tested mangabey-associated flies for pathogens infecting mangabeys in this ecosystem, *Bacillus cereus* biovar *anthracis* (*Bcbva*), causing sylvatic anthrax, and *Treponema pallidum pertenue*, causing yaws. Flies contained treponemal (6/103) and *Bcbva* (7/103) DNA. We cultured *Bcbva* from all PCR-positive flies, confirming bacterial viability and suggesting

that this bacterium might be transmitted and disseminated by flies. Whole genome sequences of *Bcbva* isolates revealed a diversity of *Bcbva*, probably derived from several sources. We conclude that flies actively track mangabeys and carry infectious bacterial pathogens; these associations represent an understudied cost of sociality and potentially expose many social animals to a diversity of pathogens.

KEYWORDS
disease vector, polyspecific associations, sociality

1 | INTRODUCTION

Living in social groups provides organisms with a number of benefits but can also incur costs such as increasing the attraction of disease vectors. Possibly the strongest links between sociality and the risk of vector-borne disease in social living animals have been identified from comparative studies showing that malaria prevalence increases with group size across Neotropical primate species (Davies, Ayres, Dye, & Deane, 1991; Nunn & Heymann, 2005). Synanthropic flies, which form close associations with human settlements and their livestock, have also been implicated in increasing disease risk in human settlements. A number of synanthropic fly species have been shown to serve as mechanical vectors for human pathogens (i.e., where a vector moves a parasite but is otherwise not necessary for the pathogen to complete its life cycle), and increasing fly densities have been shown to increase human disease risk (Banjo, Lawal, & Adeduji, 2005; Förster et al., 2007; Graczyk, Knight, Gilman, & Cranfield, 2001; Greenberg, 1971).

Synanthropic flies are involved in transmitting a broad array of pathogens. For example, they play a role in the transmission of many protozoan parasites (e.g., *Toxoplasma gondii*, Wallace, 1971; *Giardia* spp., Markus, 1980; *Cryptosporidium parvum*, Graczyk et al., 1999), bacteria (e.g., *Chlamydia trachoma*, Emerson, Bailey, Mahdi, Walraven, & Lindsay, 2000; *Escherichia coli*, Iwasa, Makino, Asakura, Kobori, & Morimoto, 1999; *Vibrio cholerae*, Echeverria, Harrison, Tirapat, & McFarland, 1983), viruses (e.g., enteroviruses, Gregorio, Nakao, & Beran, 1972; Rift Valley fever virus, Turell, Dohm, Geden, Hogsette, & Linthicum, 2010) and helminth eggs (e.g., *Ascaris* spp. and *Trichuris trichiura*, Monzon et al., 1991). Whether the associations observed between synanthropic flies and humans are a product of the development of agriculture and a more sedentary human lifestyle (Graczyk et al., 2001; Hassell, Begon, Ward, & Fèvre, 2017), or whether such associations exist more broadly in hunter-gatherer populations or wild nonsedentary, nonhuman primate (NHP) social groups and influence disease ecology is, to our knowledge, currently unknown.

To address this knowledge gap, we examined the density of flies inside and outside a group of sooty mangabeys (*Cercocebus atys atys*) and two communities of chimpanzees (*Pan troglodytes verus*) living in Taï National Park (TNP), Côte d'Ivoire, and conducted a mark–recapture experiment in the mangabey group to examine how a high density of flies is maintained as the group moves several kilometres each day through the tropical rainforest. Concurrently, we captured flies in the syntopic community of wild chimpanzees that came into proximity of the mangabey group to examine whether flies marked in the mangabey group might change association to a primate species. We tested flies found in association with the mangabey group for mammal DNA to see whether they had contact with other species and might move pathogens between species. We molecularly characterized the fly species present and examined whether specific fly species captured in association with these social groups might play a role in disease epidemiology by testing these flies for the DNA of pathogens known to infect wildlife in this ecosystem. Specifically, we tested for bacteria causing sylvatic anthrax (*Bacillus cereus* biovar *anthracis*: *Bcbva*; Hoffmann et al., 2017; Leendertz et al., 2004) and yaws (*Treponema pallidum pertenue*: *TPE*; Gogarten et al., 2016; Knauf et al.., 2018). To confirm that these flies containing *Bcbva* DNA could be involved in pathogen transmission and contained viable bacteria, we attempted to culture *Bcbva* from these flies and then sequenced the full genome of these isolates. To examine a potential epidemiological link between the *Bcbva* in flies and that killing mangabeys, we also cultured *Bcbva* from a mangabey that died during the study period in the same area and sequenced its full genome.

Sylvatic anthrax, caused by *Bcbva*, is a persistent and widespread cause of death in a broad range of mammalian hosts in this ecosystem and was responsible for more than 38% of wildlife mortality observed over 26 years (Hoffmann et al., 2017; Leendertz et al., 2004). *Bcbva* was the probable cause of death for 11 of 23 mangabeys and 31 of 55 chimpanzees for which necropsies were performed in TNP, and seroprevalence rates are extremely low in these species, suggesting *Bcbva* is highly lethal (Hoffmann et al., 2017; Zimmermann et al., 2017). Furthermore, *Bcbva* DNA was detected in more than 5% of flies sampled throughout TNP and many of these flies were shown to contain viable spores (Hoffmann et al., 2017). In nonsylvatic ecosystems, it has been suggested that flies mechanically spread spores from *Bacillus anthracis*, the closely related causative agent of classical anthrax, from carcasses through the environment, potentially leading to subsequent transmission (Blackburn, Ert, Mullins, Hadfield, & Hugh-Jones, 2014; Fasanella et al., 2010). Little is known about the transmission and persistence of sylvatic anthrax.

NHPs in TNP are also infected with *TPE* and present with severe lesions (Figure 1a; Gogarten et al., 2016; Knauf et al., 2018). Many mangabeys in the study group (referred to as the Audrenisrou group) presented with symptoms of yaws during the study period

FIGURE 1 (a) An adult mangabey in the study group with a severe *Treponema pallidum pertenue* infection showing extensive facial tissue destruction, including damage to bone and cartilage, and a poor general condition. (b) Flies feeding on mangabey faeces. (c) A duiker carcass in TNP covered in flies and their larva. (c) Schematic showing the timing of *Bcbva* isolate collection: the fly isolate with only one variant position with the dead mangabey isolate, which are thus either epidemiologically linked or stemmed from the same source, are highlighted in bold

(16% of individuals; data not shown) and full *TPE* genomes generated from lesion samples confirmed this pathogen is present in animals collected in the study group with these symptoms (Knauf et al., 2018). Chimpanzees in this ecosystem also appear to be infected with *TPE*, with next generation sequencing data from chimpanzee bones confirming a *T. pallidum* pathogen is present in these communities, although no samples from lesions are yet available to confirm infections (Gogarten et al., 2016). Flies have long been hypothesized to play a role in the epidemiology of yaws, with studies showing that flies can carry treponemes from lesions (Kumm, 1935; Satchell & Harrison, 1953) and, in experimental conditions, that flies transmitted the parasite from one host to another when feeding on lesions (Kumm & Turner, 1936). Furthermore, a high proportion of flies captured in two national parks in Tanzania, where wild olive baboons (*Papio anubis*) are infected with *TPE*, were found to contain *T. pallidum* DNA (Knauf et al., 2016); based on the low variability of the genomic regions of *T. pallidum* examined in their study, it was not possible to definitively determine which subspecies of *T. pallidum* was present in these flies, although the authors argue their results suggest that flies often come into contact with the spirochete on these baboons as there is no evidence for other *T. pallidum* subspecies circulating in this ecosystem (Knauf et al., 2016).

2 | MATERIALS AND METHODS

2.1 | Study site

This study was conducted on flies that associate with wild primates in TNP, Côte d'Ivoire (6°20′–5°10′N, 4°20′–6°50′W). TNP represents the largest remaining primary forest in West Africa and the wild NHP populations present in this ecosystem represent some of the best-studied in the world; studies on the chimpanzees and monkeys of TNP were initiated in 1979 and 1989 respectively (Boesch & Achermann, 2000; McGraw, Zuberbühler, & Noë, 2007) and a veterinary programme that started in 2001 has targeted a broad array of pathogens associated with these populations (Gogarten et al., 2014; Hoffmann et al., 2017; Leendertz et al., 2006; Rich et al., 2009). We focused on a group of sooty mangabeys habituated to human observers in November 2012 (the Audrenisrou group), which consisted of ~60 individuals during the study period (Gogarten et al., 2018;

Mielke et al., 2017). We also captured flies near a habituated chimpanzee community (the North group) with 17 individuals (in August 2013), whose territory overlaps that of the mangabey group, and two neighbouring habituated chimpanzee communities (the South and East groups, containing 41 and 36 individuals respectively in December 2018).

2.2 | Fly trapping to assess density inside and outside the primate groups

A large diversity of fly species is found in TNP (Hoffmann et al., 2017). Genera present contain species that are known to be necrophagous, coprophagous, haematophagous or myiatic, and their diets can be flexible and opportunistic, including different food types depending on what is available, although the life cycles and ecology of these sylvatic fly communities are poorly described. Flies were caught using either custom-made traps (described in Hoffmann et al., 2017) placed over a commercial attractant based on animal proteins that mimic a decaying carcass (hereafter: "synthetic carcass-baited traps"; Unkonventionelle Produkte Feldner) or using faeces as an attractant. Flies were not attracted to faecal samples once cooled; to circumvent this limitation and ensure sampling conditions were similar at all distances from the primate group, a thermos full of hot water was used to warm faecal samples in a plastic bag placed on the top of the thermos (Figure S1 in Appendix S1). Flies were trapped using a clear plastic bag lowered over faeces and startled flies would then fly and walk upwards in the bag (hereafter: "faeces-baited traps"). Both types of traps were left open for 20 min and flies were euthanized with ether. Flies from a given trap were stored at ambient temperature on silica in 50-ml Falcon tubes containing up to 20 flies until they were transported back to the Robert Koch Institute and subsequently stored at 4°C. Flies caught with synthetic carcass-baited traps did not have contact with the bait, while flies captured with faeces-baited traps were in contact with faeces prior to capture. A researcher remained next to the traps throughout the entire experiment to discourage curious NHPs from coming into contact with the traps. Our aim was to avoid any potential increase in disease risk for the primates through exposure to flies in the traps or through exposure to human microorganisms on the traps themselves. Had NHPs come into contact with the traps, we would have immediately stopped the experiment. Throughout these experiments no mangabeys or chimpanzees came into contact with the fly traps.

Over 5 days between July 30 and August 3, 2013, six fresh faecal samples were collected in the morning at the mangabey sleeping tree. Several hours after the mangabeys left the sleeping site, trapping of flies using faeces-baited traps was conducted at 1 km from the group, 500 m from the group and within the mangabey group. These trapping distances were selected because individuals are usually within a range of 100 m of one another; we hoped that 500 and 1,000 m were sufficiently far from the study group that we would be clearly outside of it, while enabling us to travel the distance quickly so we could find the group again after sampling. On each day, two independent traps were set at different locations at each of the three distances, for a total of six trapping events per day. To avoid biasing our sampling, the order of sampling each day was randomized. We repeated this experiment using synthetic carcass-baited traps on 10 additional days between May 13 and May 29, 2014.

We repeated these fly density experiments with two communities of chimpanzees (South and East groups) using faeces-baited traps in December 2018. Based on our results with the mangabeys and because of the larger home-range sizes of chimpanzees that makes finding the group after you leave it difficult, we were not able to also set traps at 1,000 m from the group and traps were almost always first set in the chimpanzee group and then 500 m from the group.

2.3 | Mark and recapture of flies

Flies were collected in the morning beneath the mangabey sleeping tree using a clear plastic bag placed over fresh faeces (Figure 1b). Flies were placed in a cooler with ice packs to anaesthetize them. Based on studies marking lice on lemurs (Zohdy, Kemp, Durden, Wright, & Jernvall, 2012), we marked flies on the posterior abdomen or thorax with a small amount of nail polish from a pipet tip, with a distinct colour used at each marking location. GPS coordinates were taken at the point of marking. Flies were marked with five different colours on five days (N_{marked} = 1,591 flies; July 19, 21, 22, 23^{r} and 27). Flies captured after the initial marking day were checked for nail polish, and the GPS point, number of marked and number of unmarked flies were recorded during each capture event ($N_{recapture\ effort}$ = 3,164 flies). On August 1, 11 chimpanzees from the North group moved through the mangabey group's territory at the same time we were conducting the mark–recapture experiment; the North group has a much larger but overlapping territory with the sooty mangabey Audrenisrou group (Boesch & Achermann, 2000). On the day after they passed close to the mangabey group, we examined whether flies marked in the mangabey group might have found their way into the chimpanzee party by opportunistically collecting flies (N = 166) with faeces-baited traps.

2.4 | Flies in the social group after sleeping near a decaying carcass

On May 2, 2014 the mangabey group slept in a tree near a dead duiker carcass (Figure 1c); to examine whether flies on the carcass might pick up pathogens from carcasses and then travel with the group, on May 3, flies were captured over faeces at 9:00 a.m. as the mangabeys were leaving the tree, and again at 10:00 a.m., 12:00 a.m., 1:17 p.m. and lastly at 5:30 p.m. when the mangabeys entered their sleeping tree. A chimpanzee died elsewhere in the forest on the same day that the duiker carcass was found with the mangabeys and the veterinarian on site prioritized performing the necropsy on the chimpanzee and so no necropsy was performed on this dead duiker carcass. However, we did perform necropsies from duikers in the study area on April 11, 17 and 28 and May 13, 2014, for which *Bcbva* was confirmed as the cause of death (Hoffmann et

al., 2017), demonstrating that anthrax was causing mortality in this duiker population. A necropsy on a mangabey in the study group that died on May 10, 2014 confirmed that *Bcbva* was the probable cause of death, showing that mangabeys were being exposed to anthrax through some route (Hoffmann et al., 2017). This sample set allowed for an examination of a potential epidemiological link between *Bcbva* in flies present in the group before the mangabey's death (*N* = 3) and the *Bcbva* that killed the mangabey, but also with flies that were captured in the group after this mangabey's death (*N* = 4).

2.5 | Molecular analyses

2.5.1 | DNA extraction

DNA was extracted from a subset of individual flies captured using the synthetic carcass-baited traps (*N* = 45) and faeces-baited traps (*N* = 33) in the mangabey group. DNA was also extracted from a subset of flies captured over fresh mangabey faeces on the morning after the group slept near a duiker carcass (*N* = 5 flies/capture events, total 25 flies). DNA was extracted from flies using the GeneMATRIX Stool DNA Purification Kit (Roboklon). Prior to homogenization with a Fast Prep (MP Biomedicals) each fly was cut into smaller pieces with sterilized scissors, but otherwise extraction followed the manufacturer's instructions. DNA was extracted from the spleen of the dead mangabey using the DNeasy Blood and Tissue Kit (Qiagen). DNA concentration was measured using a Nanodrop device (Thermo Scientific) and extracts were stored at −20°C.

2.5.2 | Fly species identification

To determine the fly species for this subset of flies we Sanger-sequenced the *COI* fragment of these 103 flies captured in the sooty mangabey group following Folmer, Black, Hoeh, Lutz, and Vrijenhoek (1994; details in Appendix S1). We aligned the resulting 96 sequences using MUSCLE (Edgar, 2004) as implemented in SEAVIEW version 4 (Gouy, Guindon, & Gascuel, 2009). This alignment was used for phylogenetic analyses in both maximum likelihood (ML) and Bayesian frameworks. We generated an ML tree using PHYML version 3 with smart model selection (PHYML-SMS; Guindon et al., 2010; Lefort, Longueville, & Gascuel, 2017). We used a full optimization approach, and the tree search used subtree pruning and regrafting and the Bayesian information criterion for model selection. Branch robustness was assessed with Shimodaira–Hasegawa-like approximate likelihood ratio tests (SH-like aLRTs: Anisimova, Gil, Dufayard, Dessimoz, & Gascuel, 2011). We also ran Bayesian Monte Carlo Markov chain (BMCMC) analyses with BEAST version 1.8.2 (Drummond, Suchard, Xie, & Rambaut, 2012) using the nucleotide substitution model identified by PHYML-SMS, a lognormal relaxed clock (uncorrelated) and a birth–death speciation model. Multiple BMCMC runs were performed; we checked that runs converged and that the posterior set was properly sampled using TRACER version 1.6 (http://tree.bio.ed.ac.uk/software/tracer/). We combined posterior sets of trees using LOGCOMBINER version 1.8.2 (distributed with BEAST) and identified the maximum clade credibility tree using TREEANNOTATOR version 1.8.2 (distributed with BEAST). Branch robustness was assessed with posterior probabilities. We also performed an ML analysis on a reduced data set only comprising unique sequences (*N* = 44), which we identified using FABOX version 1.41 (Villesen, 2007). The resulting tree was used for a species delimitation analysis using a Bayesian Poisson tree processes (bPTP) model (Zhang, Kapli, Pavlidis, & Stamatakis, 2013). We performed taxonomic assignment of the respective molecular operational taxonomic unit (MOTU) using BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) and the nonredundant nucleotide database of NCBI.

2.5.3 | Mammal testing

To determine whether flies associated with this social group were exposed to mammal DNA, we used a pan-mammal polymerase chain reaction (PCR) targeting a 130-bp region of the mitochondrial 16S DNA (with primers and blockers described in Boessenkool et al., 2012; Taylor, 1996; full protocol described in Calvignac-Spencer et al., 2013). Chromatograms were evaluated using GENEIOUS PRO version 8.1.3 and cleaned sequences were assigned to mammalian species using BLAST.

2.5.4 | Pathogen screening

Bcbva

Following Hoffmann et al. (2017) all DNA extracts were first tested for *Bcbva* in duplicate with a real-time PCR targeting *pag* (a gene coding for the protective antigen [PA]) located on the pXO1 plasmid. Positive samples were then tested in duplicate using real-time PCRs targeting *capB* (a gene coding for capsule synthesis) located on PXO2 and Island IV, a chromosomal marker unique to *Bcbva*, which can be used to discriminate it from *Bacillus anthracis*. Standards of a known concentration were available for all three assays, allowing us to estimate template copy numbers and all assays were consistently able to detect 10 template copies. All real-time PCR runs were conducted using a Stratagene qPCR MX3000 cycler (Stratagene) and fluorescence signals were quantified with the software MXPRO (www.genomics.agilent.com). Conservatively, only samples positive in duplicate in all three *Bcbva* assays were considered positive for *Bcbva* DNA for the analyses presented below.

From all *Bcbva*-positive flies, culture was attempted under biosafety level 3 conditions. Half of the fly mush that remained after DNA extraction was plated onto the following plates: Columbia blood agar (Oxoid), blood-trimethoprim agar (1.6 mg trimethoprim, 6.4 mg sulfamethoxazole, 20 mg polymyxin B per litre agar medium) and Cereus Ident agar (Heipha Diagnostica) with the chromogenic substrate 5-bromo-4-chloro-3-indoxyl-myoinositol-1-phosphate (Klee et al., 2006). In addition, a 10-µl aliquot of the mush was diluted 1:10 in sterile NaCl, heat treated for 30 min at 65°C and then plated on the plates described above. Cultures were incubated at 37°C and monitored daily and any morphologically suspicious colonies were subcultured, heat-inactivated and tested in real-time PCR

as described above. We followed the same procedure for culturing *Bcbva* from the mangabey that died on May 10, 2014. *Bcbva* was retrieved from all native and heat-treated samples, indicating the presence of heat-resistant spores.

Libraries for whole-genome sequencing were prepared using the Nextera XT Sample Preparation Kit (Illumina) and Nextera XT Index Kit (Illumina) using 1 ng of heat-extracted DNA from the isolates as input. We used AMpure XP beads (Beckman Coulter) for PCR clean-up. Final concentrations were assessed using a Qubit High Sense Double Stranded DNA Assay kit (Invitrogen) and equimolar amounts of libraries were pooled and sequenced in two runs using the Illumina MiSeq platform with v3 chemistry (2 × 300 bp) and the Illumina NextSeq 500 platform (2 × 150 bp). Reads from both runs were combined, and quality filtered using TRIMMOMATIC, setting the quality score to 30 over a sliding window of four bases, with a minimum read length of 40 bp for the surviving reads. Filtered reads were then mapped to a *Bcbva* chromosomal reference genome (NC_014335) using BWA-MEM (Li, 2013), and deduplicated using Picard's MARKDUPLICATES. Alignments with an MAPQ smaller than 30 and a mapping length lower than 30 were removed using SAMTOOLS (Li et al., 2009) and the BBMap reformat script (https://github.com/BioInfoTools/BBMap/blob/master/sh/reformat.sh). Variants were called using the Variant Calling Pipeline (https://gitlab.com/RKIBioinformaticsPipelines/VariantCalling/) that uses GATK (McKenna et al., 2010) and SNPEFF (Cingolani et al., 2012) A summary of the reads generated and the coverage for each isolate are available in Table S1. We considered a variant as present if it was present in 95% of reads and at a minimum 10× coverage. To compare the dead mangabey *Bcbva* isolate with the *Bcbva* isolates from flies and accommodate uneven depth across isolates, we took all variants that were confidently assigned (present in 95% of reads and at a minimum 10× coverage) in the mangabey and assessed whether they were also present at those positions in the fly isolates (present in 95% of reads and at a minimum 10× coverage). We also looked at all other variants that were confidently assigned (present in 95% of reads and at a minimum 10× coverage) in the fly isolates and determined whether they were present in the mangabey isolate (present in 95% of reads and at a minimum 10× coverage). For each fly, we then had an assessment of the variants shared with the dead mangabey, the variants confidently not shared with the dead mangabey, and unknown positions where sequencing depth was not sufficient to confidently call a variant in either the fly or the dead mangabey isolate. We previously described *Bcbva* diversity within carcasses and found that isolates differed by a maximum of two chromosomal single nucleotide polymorphisms (SNPs; Hoffmann et al., 2017), suggesting flies differing from the dead mangabey isolate by two or less variant positions are either epidemiologically linked or stemmed from the same source.

Treponemal DNA

DNA extracts were tested using a PCR amplifying a 67-bp DNA fragment, including primers from the DNA *polA* gene (described in Leslie, Azzato, Karapanagiotidis, Leydon, & Fyfe, 2007). An additional PCR using fusion primers was performed to append M13F/R sequences to first-round amplicons and thereby enable their sequencing (as described in Gogarten et al., 2016). We tested the sensitivity of this assay and found it was able to detect as few as 10 template molecules spiked into fly extracts (see Appendix S1 for details). To add further support that *Treponema pallidum* was present, we performed a seminested PCR amplifying a 189-bp sequence of the *T. pallidum cfpA* gene (Harper et al., 2012; see Appendix S1 for details; Harper et al., 2008). Products were Sanger-sequenced and sequences were compared to publicly available sequences in NCBI through BLAST (Altschul et al., 1990). We assessed the sensitivity of this assay and found it was able to detect 1,000 template molecules spiked into fly extract, but not able to consistently detect 100 template molecules spiked into fly extracts (see Appendix S1 for details). Even tissue samples positive for *T. pallidum* have been positive in the short *polA* assay and then negative in the *cfpA* assay, despite having observed the spirochete in electron micrographs and ultimately being able to capture the whole genome using hybridization capture methods, further highlighting the low sensitivity of the seminested *cfpA* assay (unpublished data: S.C.S., J.F.G., F.H.L.; Knauf et al., 2018).

The regions amplified by both the *polA* and the seminested *cfpA* assays are identical in closely related *Treponema paraluisleporidarum*, the closest relative of *T. pallidum* described to date (Šmajs et al., 2011), as well as other *T. pallidum* subspecies. To date, *T. paraluisleporidarum* has only been found in rabbits and hares, and no rabbits or hares have been detected in flies in TNP despite extensive sampling of flies (unpublished data: C.H., C.S.C., F.H.L.; Hoffmann et al., 2017), suggesting rabbits and hares are unlikely to be sources of treponemal bacteria in these flies. Similarly, no other *T. pallidum* subspecies have been described in the wildlife in this ecosystem. Because of the variation in sensitivity of the two assays used here and the fact that no hosts for *T. paraluisleporidarum* have been detected in flies in TNP, samples that yielded a *Treponema* sequence in the *polA* assay were tentatively considered *TPE* positive for the analyses presented below. *TPE* is microaerophilic and cannot grow on standard culture media (Edmondson, Hu, & Norris, 2018), requiring direct culture of the treponemes in laboratory animals. We were unable to perform such culture experiments, so it was not possible to determine the viability of the *TPE* and these results should be interpreted with caution.

2.5.5 | Statistical analyses

To test whether the number of flies captured at synthetic carcass-baited or faeces-baited traps was influenced by the distance from the mangabey group, we used a generalized linear mixed effect models using a Poisson error structure and log link function (Baayen, 2008), with the number of flies caught at each trap as the response variable. Models were fitted separately for synthetic carcass-baited traps and faeces-baited traps. We included in these models the distance from the social group as a categorically coded fixed effect and trapping day as a random effect. To control for the effect of the ID of the person setting the trap and the hour the trap was set, these were included as further fixed effects. The hour the trap was set

was approximately symmetrically distributed and *z*-transformed. To model the overdispersion that was evident in these models, we included trap ID as an observation-level random effect (Harrison, 2014). Models were fitted in R using the function glmer in the R package *lme4*, using 1,000 parametric bootstraps. Samples used for the faeces-baited trap model consisted of 30 traps set over 5 days, while for the synthetic carcass-baited traps this consisted of 60 traps set over 11 days.

As an overall test of the effect of the distance from the mangabey group on the number of flies caught by a trap, we compared the full model with a null model lacking this fixed effect but comprising the same random effects structure and control fixed effects (Forstmeier & Schielzeth, 2011) using a likelihood ratio test (Dobson, 2002). We checked whether the assumptions of normally distributed and homogeneous residuals were fulfilled by visually inspecting a qqplot and residuals plotted against fitted values; both indicated no obvious deviations from these assumptions for either faeces-baited traps or synthetic carcass-baited traps.

To rule out problems of collinearity we determined variance inflation factors (VIFs: Field, 2005) for the standard linear model excluding random effects, which for faeces-baited traps revealed a VIF of 1.02 for distance from the mangabey group, 1.00 for ID of capturer and 1.02 for the hour the trap was set. For the synthetic carcass-baited traps this revealed a VIF of 1.01 for distance from the mangabey group, 1.02 for ID of capturer and 1.03 for the hour the trap was set, suggesting there were no issues with collinearity for either model.

To examine whether the number of flies captured with faeces-baited traps was higher in the chimpanzee groups than 500 m away from the group, we ran a separate paired *t*-tests for each of the two groups. We log-transformed the number of flies captured to improve normality of this variable. Small sample sizes (10 and nine traps in the East and South groups respectively, along with 10 and nine paired traps 500 m away from the East and South groups respectively) precluded the use of generalized linear mixed effect models including time of trap set and date as random effects or an analysis of the impact of variation in party size on fly density.

To test for an association between character states (i.e., the type of attractant used to capture the fly, whether the fly contained mammal DNA, *Bcbva* positivity or *T. pallidum* positivity) and the fly phylogeny, we used Bayesian tip-association significance testing (BaTS). This approach tests whether any given taxon on the tree is more likely to share a character state with a sister taxon than expected by chance. BaTS uses the posterior sets of trees generated through the BMCMC analysis described above and incorporates the phylogenetic uncertainty arising from the data into its test of phylogeny–trait associations (Parker, Rambaut, & Pybus, 2008).

3 | RESULTS

3.1 | Fly density

Overall there was a clear impact of the distance from the mangabey group on the number of flies captured for both synthetic carcass-baited traps (likelihood ratio test comparing full and null model: χ^2 = 19.929, *df* = 2, $p < 0.001$; Table S2) and faeces-baited traps (χ^2 = 41.443, *df* = 2, $p < 0.001$; Table S3). More specifically, the number of flies captured at each trap type was higher within the group than outside the group (Figure 2; Table 1). Similarly, in both chimpanzee groups, there were more flies in the group than 500 m away from the group (East group: $\bar{x}_{0m}$ = 19.1 flies, $\bar{x}_{500m}$ = 3.8 flies, paired *t*-test, $p < 0.001$, t = 12.974, *df* = 9; South group: $\bar{x}_{0m}$ = 17.7 flies, $\bar{x}_{500m}$ = 2.4 flies, paired *t*-test, $p < 0.001$, t = 6.556, *df* = 8; Figure 3; Table S4).

3.2 | Mark–recapture

Of the 1,591 flies marked in the mangabey group, 51 (3.2% of flies that were marked) were recaptured using a recapture effort of 3,164 in the mangabey group (Table S5). These flies were captured up to 1.3 km from the point of marking (mean distance travelled = 703 m, *SD* = 297 m) and were recaptured with the group up to 12 days after marking (mean days since marking = 3.4 days, *SD* = 2.6 days; Figure 4). The fly density experiment using faeces as a bait described above was carried out shortly after the marking for the mark–recapture experiment had finished. Specifically, the fly density experiment using a faeces bait experiment was carried out 3–7 days after the last fly was marked in the mangabey group. During this fly density experiment using faeces as a bait, 0% of flies (0/92) captured at 500 and

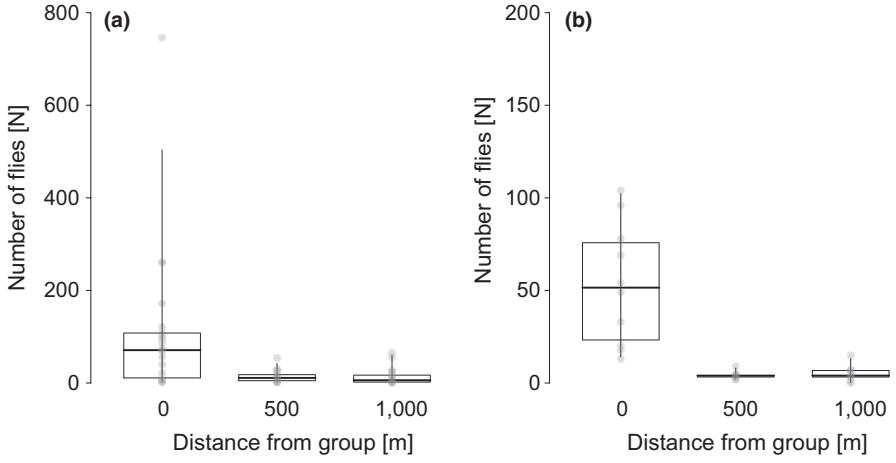

FIGURE 2 The number of flies captured at traps set at different distances from the mangabey social group using: (a) synthetic carcass-baited traps and (b) faeces-baited traps. The middle horizontal line represents the median, the rectangle shows the quartiles, the vertical lines represents the 2.5% and 97.5% percentiles, and each circle indicates the number of flies caught in a particular trap

TABLE 1 Generalized linear mixed effect model results for synthetic carcass-baited traps and faeces-baited traps

Term	Estimate	*SE*	Lower CL	Upper CL	*Z*-value	*p*-Value
Synthetic carcass-baited traps						
Intercept—Within group	2.996	1.443	0.114	5.909	[a]	[a]
500 m from group	−1.611	0.411	−2.464	−0.839	−3.920	<0.001
1,000 m from group	−1.880	0.419	−2.732	−1.0852	−4.486	<0.001
ID of capturer: JFG	0.129	0.337	−0.510	0.780	0.382	0.703
Hour trap was set	0.056	0.109	−0.164	0.276	0.510	0.610
Faeces-baited traps						
Intercept—Within group	3.307	1.067	1.117	5.709	[a]	[a]
500 m from group	−2.481	0.308	−3.104	−1.969	−8.056	<0.001
1,000 m from group	−2.337	0.304	−2.951	−1.829	−7.693	<0.001
ID of capturer: JMT	−0.0309	0.261	−0.581	0.471	−0.118	0.906
Hour trap was set	0.0392	0.083	−0.144	0.213	0.470	0.638

[a]Not shown because of not having a meaningful interpretation.

1,000 m from the group were marked. In contrast, 2.05% (11/534) of flies captured in the group on the same days were marked. On August 1, 2013, one marked fly was recaptured with the chimpanzee group that passed through the mangabey group's territory and close to the mangabey group, 628 m from where it was originally marked and 9 days after having been marked in the mangabey group.

3.3 | Fly species

Our analysis suggested that the 96 flies for which good quality *COI* sequences were generated (poor quality sequences were generated for seven flies) belonged to 14 putative species. Most flies were assigned to the family Muscidae (45.8%), Calliphoridae (35.4%) and Sarcophagidae (8.3%), while the remaining 10.4% of flies could not be assigned to a family based on BLAST results (Table 2). BaTS analysis suggested that the type of attractant used to capture a fly was structured on the phylogeny (Table 3). While representatives of nearly all MOTUs were captured using both faeces- and synthetic carcass-baited traps, MOTU ratios measurably differed depending on the bait. For example, 60% of the flies attracted to faeces belonged to MOTU 1 (family Muscidae); only 30% of the flies attracted to synthetic carcass bait belonged to the same MOTU.

3.4 | Mammal DNA

Sooty mangabey DNA was found in 40 of the 53 (tested) flies captured over faeces, while only seven of 45 (tested) flies captured with a synthetic carcass-baited trap contained mangabey DNA. As most of the flies that were captured over faeces belonged to the family Muscidae, mammal positivity was also highest among this group and the BaTS analysis suggested that mammal positivity was structured on the fly phylogeny (Table 3). Duiker DNA was detected in three flies captured over mangabey faeces the day after the mangabey group slept near a duiker carcass.

3.5 | Pathogen screening

3.5.1 | *Bcbva*

Twelve of the 98 flies tested in the mangabey group were positive in duplicate reactions for the *Bcbva* PA qPCR, with copy number estimates ranging from 5.3 to 1,228.0 μl^{-1} (Tables 4 and S5). Of these positive flies, eight tested positive in duplicate reactions for the CapB qPCR, with copy number estimates ranging from 6.5 to 1,175.5 μl^{-1}. Seven of these positive flies were also positive in the *Bcbva* Island IV qPCR with copy numbers ranging from 3.38 to 1,720.0. The highest *Bcbva* copy numbers were observed in flies captured in the mangabey group with faeces on the day after they slept over a dead duiker carcass (Table S6). None of the flies positive for *Bcbva* DNA in all three assays contained mangabey DNA (Figure 5; Table S6) but several contained duiker DNA. Flies containing *Bcbva* DNA were captured with both bait types and all belonged to the family Calliphoridae (Figure 5; Table S6), although the BaTS analysis did not detect a pattern of *Bcbva* positivity on the fly phylogeny. This may be due to the small number of flies positive for *Bcbva* in all three assays. It was possible to culture *Bcbva* from the seven flies positive in the three assays and from the dead mangabey.

In the dead mangabey *Bcbva* isolate 1903, we identified 62 variant sites (Table S7; details on sampling timeline: Figure 1d). The *Bcbva* isolate from fly 3465 differed from the dead mangabey only at a single one of these variant sites; all seven additional variants identified in the *Bcbva* isolate from fly 3465 could not be confidently called in the dead mangabey isolate but were present at lower

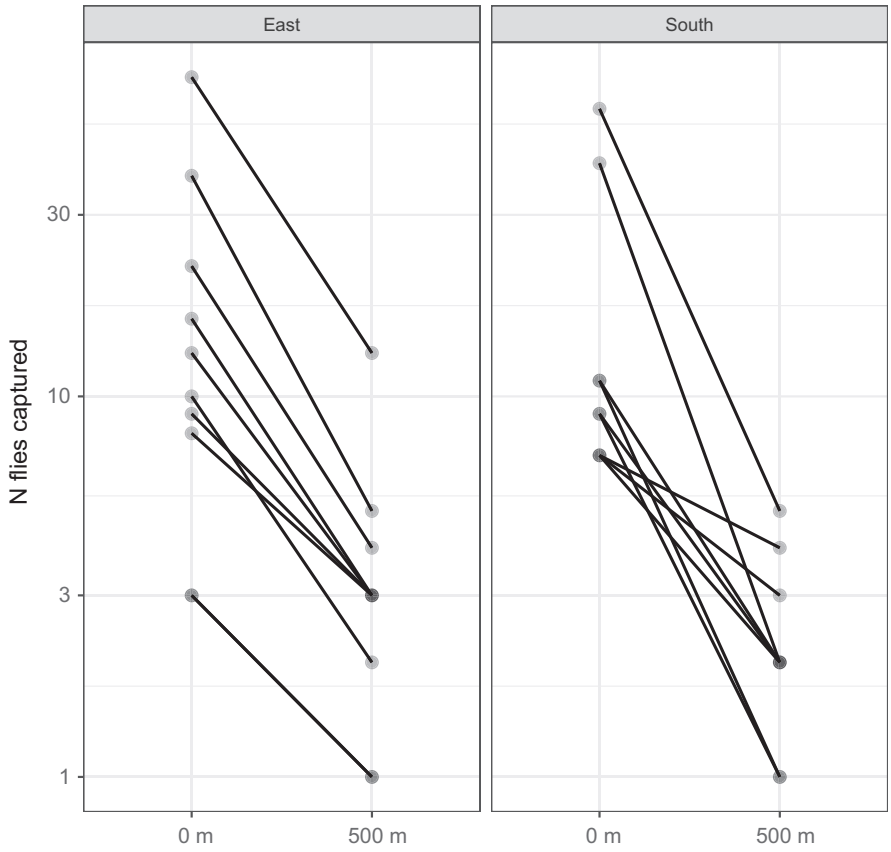

FIGURE 3 The number of flies captured with faeces-baited traps set within and 500 m away from two neighbouring chimpanzee communities. Lines connect paired traps set consecutively on the same day. The *y*-axis is shown using a log scale

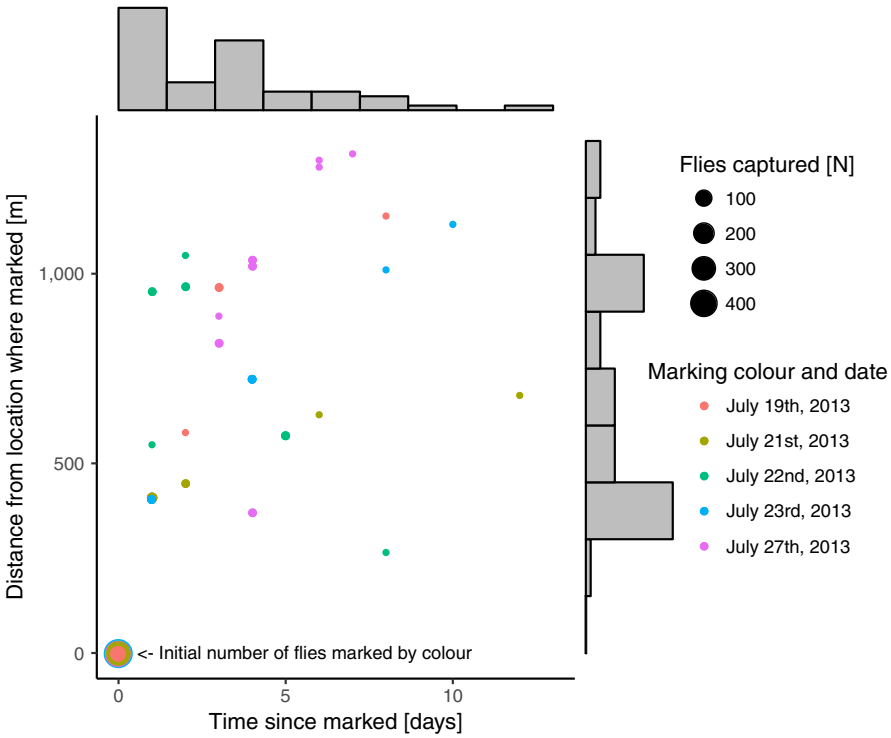

FIGURE 4 Scatter plot of flies recaptured in the sooty mangabey group indicating the distance from the location where they were initially marked and the number of days that elapsed between marking and recapturing. The size of the points is proportional to the number of flies recaptured or marked. The marginal histograms indicate the distribution of the distances from the location where they were marked and the time that elapsed between when they were marked and recaptured, and do not include the initial flies marked. In the lower left corner of the plot, the number of flies originally marked in each colour is indicated with overlapping circles

coverage in this isolate (Table S8). Thus, these isolates were nearly identical, differing by only a single SNP. In contrast, all other flies were more different from the mangabey isolate; the *Bcbva* isolates from flies 3488, 3498, 3487, 3495, 3496 and 3464 respectively differed at eight, 27, 31, 33, 34 and 37 of the mangabey isolate's 62 variant sites. The mangabey isolate differed at five of the additional variants identified in the *Bcbva* isolate from fly 3488, while from flies 3498, 3487, 3495, 3496 and 3464 respectively the mangabey isolate

TABLE 2 Details regarding fly MOTUs

MOTU	Tentative taxonomic assignment based on BLAST	Number of flies	MOTU support
1	Calyptratae: Ostroidea: Muscidae	44	0.84
2	Calyptratae: Ostroidea: Calliphoridae: *Chrysomya*	20	0.89
3	Calyptratae: Ostroidea: Sarcophagidae: *Sarcophaga*	8	0.46
4	Calyptratae: Ostroidea: Calliphoridae: *Chrysomya putoria*	8	0.88
5	Calyptratae	3	0.69
6	Calyptratae: Ostroidea: Calliphoridae	3	0.88
7	Calyptratae	2	0.98
8	Calyptratae	2	0.71
9	Calyptratae: Ostroidea	1	1.00
10	Calyptratae	1	1.00
11	Calyptratae	1	1.00
12	Calyptratae: Ostroidea: Calliphoridae	1	1.00
13	Calyptratae: Ostroidea: Calliphoridae	1	1.00
14	Calyptratae: Ostroidea: Calliphoridae: *Hemigymnochaeta*	1	1.00

1903 differed at nine, five, 13, 13 and five of the additional variant sites, further highlighting the variability of these isolates (Table S8).

3.5.2 | *Treponema pallidum*

Six of 98 (6.12%) flies tested in the mangabey group were positive with the PCR for a short fragment of the *polA* gene (Figure 5; Tables 4 and S6), with sequences showing 100% identity to published *T. pallidum* strains. Only one of those six flies tested positive for a longer fragment of the *cfpA* gene (Table 4). The low sensitivity of the *cfpA* assay may be responsible for the low positivity of this confirmatory test. Of these six flies containing *T. pallidum* DNA, four contained sooty mangabey DNA, including the fly positive in both *T. pallidum* assays (Figure 5; Table S6). *T. pallidum* positive flies were captured using both faeces- and synthetic carcass-baited traps (Figure 5; Table S6) and a number of different fly MOTUs contained *T. pallidum* DNA. The BaTS analysis suggested there was no pattern of *T. pallidum* positivity on the fly phylogeny (Figure 5; Table 3), although this again may be due to the low number of flies containing this pathogen's DNA.

4 | DISCUSSION

Here we document a diverse fly community associated with a mangabey social group containing putative species belonging to fly genera that are reported to be opportunistically necrophagous, coprophagous, haematophagous and/or myiatic. We found that these flies were at a higher density within the mangabey group than at different distances away from the group. We observed a similar pattern in two neighbouring chimpanzee communities, with significantly higher fly densities inside than outside groups. Furthermore, flies marked in the mangabey group moved with the group for up to 12 days and for a straight-line distance of 1.3 km through the rainforest; mangabey groups do not move linearly through this environment, suggesting the actual distance moved by these flies is larger. If the process of marking flies impacted their survival and mobility, our estimates probably represent a further underestimation of the maximum duration and distance travelled by flies in these associations. The finding of mangabey DNA in most of the flies captured in the mangabey group is not surprising given that faeces-baited flies were observed coming into contact with faeces prior to being captured, although many flies captured with the synthetic carcass baits also contained mangabey DNA. This suggests that some of the flies captured with the synthetic bait had come into contact with the mangabeys or their excrement. Flies captured using mangabey faeces were less diverse than those captured with synthetic carcass baits, which supports the idea that some fly species prefer certain foods, although all fly MOTUs that were captured more than three times were captured with both faeces and synthetic carcass baits.

Our results suggest that similar to synanthropic flies that increase human disease risk (Banjo et al., 2005; Förster et al., 2007; Graczyk et al., 2001; Greenberg, 1971), flies associating with NHP social groups have the potential to increase NHP disease risk. Flies captured in the mangabey group contained the DNA of two pathogens that infect wildlife in this ecosystem, *Bcbva* causing anthrax and

TABLE 3 Results of Bayesian tip-association significance testing (BaTS) of bait type, and mammal, *Treponema pallidum*, or *Bcbva* detection on the fly phylogeny (Figure 5)

Parameter	Statistic	Observed mean	Lower 95% CI	Upper 95% CI	Null mean	Lower 95% CI	Upper 95% CI	Significance (*p*-value)
Bait type	AI	3.67	2.92	4.38	5.32	4.62	6.06	**<0.001**
	PS	17.62	16.00	18.00	30.70	28.07	33.22	**<0.001**
	MC (faeces)	7.67	4.00	13.00	3.83	3.16	5.74	**0.020**
	MC (synthetic)	6.17	5.00	7.00	3.48	2.88	4.37	**0.020**
Mammal detection	AI	3.21	2.40	4.01	5.22	4.51	5.90	**<0.001**
	PS	18.10	16.00	19.00	30.47	27.83	32.86	**<0.001**
	MC (yes)	7.00	4.00	12.00	3.91	3.12	5.87	**0.030**
	MC (no)	8.03	8.00	8.00	3.63	2.92	5.87	**0.020**
T. pallidum detection	AI	0.93	0.55	1.27	1.08	0.91	1.25	0.110
	PS	4.98	5.00	5.00	4.94	4.80	5.00	1.000
	MC (yes)	1.02	1.00	1.00	1.06	1.00	1.20	1.000
	MC (no)	19.69	19.00	24.00	19.57	14.16	37.91	0.380
Bcbva detection	AI	0.92	0.43	1.37	1.09	0.80	1.26	0.150
	PS	4.69	4.00	5.00	4.92	4.71	5.00	1.000
	MC (yes)	1.29	1.00	2.00	1.08	1.00	1.27	1.000
	MC (no)	45.89	40.00	58.00	19.90	12.89	40.17	**0.030**

Note: Only flies for which data were available for each of the four parameters were included in this analysis (98 flies). *p*-values less than 0.05 are indicated in bold.
Abbreviations: AI, association index; MC, monophyletic clade statistic indicating the maximum observed exclusive single-state clade size; PS, Fitch parsimony score.

TABLE 4 Summary of pathogen screening for flies captured in the mangabey group

	Treponema pallidum		*Bcbva*			
	% pos. (*N* pos./*N* tested)		% pos. (*N* pos./*N* tested)			
Trap type	*polA* seq.	*cfpA* seq.	PA qPCR	CapB qPCR	Island IV qPCR	Pos. in all three assays
Faeces	5.2 (3/58)	33.3 (1/3)	8.6 (5/58)	50.0 (4/8)	42.9 (3/7)	5.2 (3/58)
Synthetic carcass	8.6 (3/45)	0.0 (0/6)	15.6 (7/45)	33.3 (4/12)	41.7 (5/12)	8.9 (4/45)

probably *TPE* causing yaws, suggesting they may play a role in the ecology and persistence of these pathogens. Caution is warranted in the interpretation of the *Treponema pallidum* result, as the regions amplified by the assays for *TPE* used here are identical in the closely related *Treponema paraluisleporidarum*, as well as other pathogenic *T. pallidum* subspecies. To date, *T. paraluisleporidarum* has exclusively been described from rabbits and hares and no rabbits or hares have been detected in flies in this ecosystem, despite an extensive sampling of flies (unpublished data: C.H., C.S.C., F.H.L.; Hoffmann et al., 2017). Thus, rabbits and hares are unlikely to represent a source of treponemal bacteria found in these flies. The other *T. pallidum* subspecies described to date have not been found in wildlife species, although it is possible that other wildlife species are infected with these or other treponemes that have not yet been described. The mangabey group in which the flies were captured was suffering from a continuous *TPE* outbreak, with ~16% of animals showing visible symptoms, which could represent a source of the treponemal DNA in the flies; further research is needed to definitively link the treponemes detected in flies with infections observed in these mangabeys. To this end, the development of sensitive PCRs allowing for a subspecies distinction of these treponemes will be extremely helpful, as would systems for culturing *TPE* (Edmondson et al., 2018). Laboratory infection studies with model organisms using flies carrying treponemes in the concentrations observed in the wild would ultimately be necessary to determine whether these flies can really lead to infection in another individual when flies come into contact with other animals, for example while feeding on open wounds (Kumm & Turner, 1936).

The fact that we were able to culture *Bcbva* from these flies captured in the mangabey group confirms they contain viable spores and could potentially be spreading *Bcbva* to the mangabeys and their surroundings. In fact, the highest concentration of *Bcbva* DNA (>1,000 copies/µl DNA), some of the highest copy numbers we have observed in flies in this forest (Hoffmann et al., 2017), were found in flies captured on the day after the mangabey group slept near a decaying duiker carcass, suggesting flies may move pathogens from carcasses into monkey groups. Indeed, it was possible to culture *Bcbva* from several of these flies captured on the day the mangabeys slept near a duiker carcass, and these flies contained duiker DNA and their *Bcbva* isolates were nearly identical, suggesting they were exposed to *Bcbva* at a single carcass. A necropsy on a mangabey in the study group that died several days after sleeping near the decaying carcass confirmed that *Bcbva* was the likely cause of death, although comparisons of the *Bcbva* isolate from the dead mangabey with those obtained from flies captured in the week prior to the mangabey's death suggested these cases were not epidemiologically linked. The *Bcbva* isolate from one fly captured a week after the mangabey's death had only a single variant compared to the dead mangabey isolate. It is not possible to know whether this fly picked up this *Bcbva* variant from the mangabey carcass itself, or whether the fly and mangabey both picked up that *Bcbva* variant from some other source.

Flies in nonsylvatic ecosystems have been shown to mechanically spread spores from carcasses through the environment and contaminate plant surfaces, potentially increasing exposure of other animals (Blackburn et al., 2014; Fasanella et al., 2010). This is of particular concern if the flies follow a social group through the forest to feeding sites where they could contaminate food items (e.g., with their regurgitation or faeces). While *Bcbva* was the probable cause of death for 11 of 23 mangabeys for which necropsies have been performed in TNP (Hoffmann et al., 2017), there was not a major mangabey die off while this study was carried out. With so many flies in the mangabey group carrying high concentrations of viable *Bcbva*, it is perhaps surprising that more mangabeys were not dying of anthrax. Laboratory studies using small animal models (e.g., Brézillon et al., 2015) are needed to confirm whether the amount of viable *Bcbva* spores or vegetative bacilli carried by flies are sufficient to cause mortality in a host directly (e.g., through consumption of flies by monkeys or apes or consumption of foods covered in fly vomit spots). Flies following monkeys might explain how arboreal monkey species are exposed to *Bcbva* spores in this ecosystem (Hoffmann et al., 2017). From the perspective of a carrion fly, moving pathogens from carcasses to living animals might increase a fly's fitness by increasing the number of carcasses in the environment for itself or its offspring. This raises the interesting prospect that there could be selection for flies to be able to transmit pathogens efficiently from carcasses to animal groups.

Further research is needed to assess whether these fly associations are found more broadly in different NHP species and in other ecosystems. It will be interesting to understand how these flies are able to track NHP and maintain their associations. Many haematophagous vectors (e.g., mosquitos) are attracted to their hosts via their carbon dioxide production (Gillies, 2009; Kellogg, 1970) and it will be interesting to test whether NHP-associated flies are using similar mechanisms. The finding of two chemosensory receptors that mediate carbon dioxide detection in *Drosophila* suggests the ability

to detect and track carbon dioxide production could be present in other insects as well (Jones, Cayirlioglu, Kadow, & Vosshall, 2007). However these flies are able to maintain such an association, the persistent fly community around an NHP social group could limit fly dispersion and movements between groups and species, limiting pathogen transmission. Our data do not, however, support such a hypothesis; duiker DNA and *Bcbva* DNA was found in flies in this group, a fly marked in the mangabey group shifted to the chimpanzee group, and *Bcbva* DNA in flies captured outside a social group (Hoffmann et al., 2017) had similar *Bcbva* prevalence to that found inside the group in this study, all suggesting movement of flies into and out of association with particular primate groups.

Synanthropic flies have also been suggested to serve as a vector between livestock and humans (Rosef & Kapperud, 1983). Determining whether NHP-associated flies can move out of the forest and into surrounding human populations is an important area of future research. We conducted a small-scale preliminary analysis of the mammalian and *Bcbva* DNA found in 45 flies captured in a village near TNP. Although we did not detect any *Bcbva*, we did detect four wildlife species (*Cephalophus* sp., *Praomys* sp., *Piliocolobus badius* and *Thryonomys* sp.) in these flies (Table S9); the mammal detection rate in the village was noticeably lower in these preliminary results than typical detection rates from flies captured in the forest (9% vs. 20%–40%). While these results may suggest that flies do leave the forest, it is also possible that these flies were exposed to the larger mammal species' DNA through contact with bushmeat in the villages; both duikers and colobines are hunted frequently in the region, and the two rodent species detected are often found in human habitats (with cane rats even being bred for food). Further research, such as a mark–recapture experiment at the forest edge, is needed to conclusively determine whether flies move between human and wildlife populations. Such research will be particularly relevant as expanding human populations come into increasingly close contact with NHP populations and their associated flies. Increasing evidence suggests that human (Köndgen et al., 2008) and domestic animal pathogens (Dobson & Meagher, 1996) can spill-over or spill-back into wildlife populations and cause major population declines. It will thus also be important to understand whether synanthropic flies can move pathogens from human and domestic animal populations into wildlife populations.

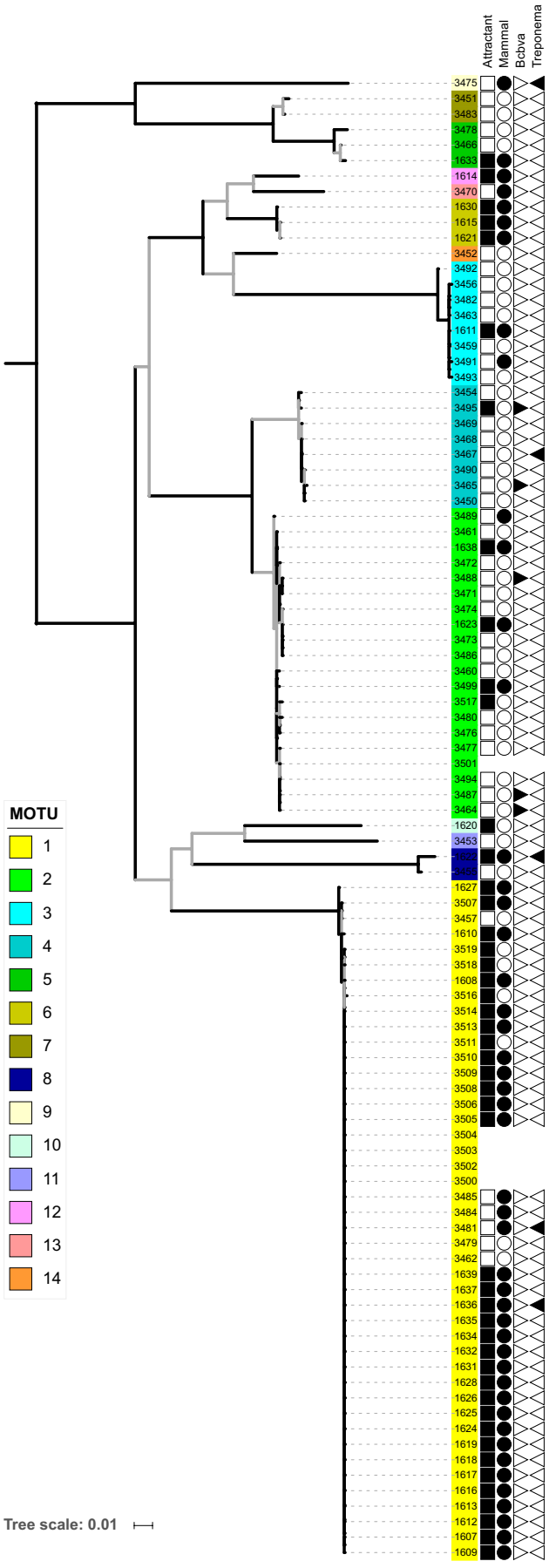

FIGURE 5 A maximum likelihood phylogeny generated using the *COI* sequences from flies captured using either faeces (filled black square) or synthetic carcass (white square) as an attractant. Branches that received less than 0.95 Shimodaira–Hasegawa approximate likelihood ratio test (SH-like aLRT) support are indicated in grey. Numbers at the tips of the branches indicate the unique fly identification number. In total, 14 MOTUs were identified with bPTP and each fly identification number is coloured based on its MOTU. MOTUs are numbered from the most to the least abundant. Flies that contained mammal DNA are shown with a black circle, while those that contained *Bcbva* or *Treponema pallidum* DNA are indicated with a black triangle in the respective column

The DNA found in flies has been shown to be a promising tool for monitoring biodiversity (Calvignac-Spencer et al., 2013; Lee, Sing, & Wilson, 2015; Schubert et al., 2015); where it is usually picked up by flies has not previously been investigated. Because many flies captured in the mangabey group tested positive for mangabey DNA during a time when none of the habituated individuals had died, it is clear that flies were exposed to mangabey DNA through sources other than carcasses. For those flies captured on faeces, the faeces itself represents a likely source of mangabey DNA, but we also found mangabey DNA in flies captured with the synthetic carcass bait. As discussed above, the fly species present appeared to be attracted to both the synthetic carcass baits and faeces, suggesting exposure to faeces might be another source of exposure to mangabey DNA for flies captured using synthetic carcass baits, although landing on a host and direct contact with their fluids is possible as well. Considering that these mangabeys usually defecate several times a day and can live for at least a decade, the amount of faecal biomass generated by a mangabey far outweighs the weight of the carcass it leaves behind—as flies feeding on faeces contain enough mangabey DNA to be detected with our molecular tools, perhaps only a minor proportion of randomly caught flies that contain mammalian DNA acquired it from carcasses. Vertebrate faecal metabolites (urobilinoids) detected in adult blow fly guts represent a potential means to identify when mammalian DNA originated from contact with faeces, which might help future research differentiate the source of mammalian DNA found in flies (Owings, Skaggs, Sheriff, Manicke, & Picard, 2018). Despite the potential rarity of carcass DNA in flies, our results certainly lend further support for such an approach for monitoring bacterial pathogens (Hoffmann et al., 2017; Knauf et al., 2016).

The finding of these two bacterial pathogens in only 103 flies captured in this primate social group suggests that flies represent a cost-effective and comparatively safe tool for pathogen monitoring. Sampling cadavers or anaesthetizing animals to detect pathogens in wildlife requires extensive training and resources and poses a risk to the animals. A number of other highly infectious pathogens circulate in these ecosystems (e.g., monkeypox virus; Radonić et al., 2014, Ebola virus; Le Guenno et al., 1995), making the collection of necropsy samples a dangerous task best left for trained experts; an untrained biologist attempting to collect necropsy samples in TNP was infected with a new strain of Ebola virus (Le Guenno et al., 1995). Thus, sampling flies may prove to be a safe and useful tool for allowing a broader geographical and temporal screening to understand the distribution of these and other pathogens in wildlife populations. We encourage researchers to use caution while trapping flies (e.g., wearing gloves, disinfecting hands after work, disinfecting trapping materials regularly) to minimize the potential risk of pathogen exposure, with the additional by-product that this will help avoid contaminating flies with human DNA.

Collectively, our results suggest that attraction of flies might represent a previously underappreciated cost to forming social groups. Further studies are needed to confirm whether variation in species social organization (solitary vs. group-forming) and behaviour (terrestrial vs. arboreal, group sizes) influences fly density and whether such associations exist with other animals and in other ecosystems. The chimpanzees of Taï forest represent an ideal study system for this purpose: chimpanzee groups undergo fission and fusion, which provides a natural experiment to examine how group sizes influence vector exposure, although our sample sizes were not yet sufficient to explore the impact of this variable on fly densities. In fact, it has been hypothesized that the fission–fusion behaviour of chimpanzees actually evolved to mitigate disease risk (Lehmann, Korstjens, & Dunbar, 2007) and it will be interesting to examine whether smaller parties are indeed less exposed to potential arthropod vectors, including flies. It will also be interesting to assess whether such fly associations also exist in more mobile human hunter-gatherer populations and whether aspects of hunter-gatherer behaviour, such as the repeated moving of camps or the use of latrines, might also serve to reduce exposure to flies and ultimately disease risk. Finally, it will be exciting to explore whether mechanical vectors such as the flies examined here are attracted to social groups more generally and have contributed to shaping the ecology and evolution of social mammals more broadly.

ACKNOWLEDGEMENTS

We thank the Ministère de l'Enseignement Supérieur et de la Recherche Scientifique and the Ministère de Eaux et Fôrests in Côte d'Ivoire, and the Office Ivoirien des Parcs et Réserves for permitting the study. We are grateful to the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire and the staff members of the Taï Chimpanzee Project for their support. We are indebted to the Max Planck Society providing core funding for the Taï Chimpanzee Project since 1997. For assistance in the field we thank Vera Leinert, Bekah Myers, Sylvain Lemoine, and the long-term mangabey field assistants Simon T. Kannieu, Daniel Bouin and Gnimion Florent. For helpful R scripts used for plotting Figure 2 we thank Roger Mundry. For assistance culturing *Bcbva* under biosafety level 3 conditions, we thank Silke Klee, Ursula Thiesen, and Sabine Howaldt. Core-funding for the Taï Chimpanzee Project is provided by the Max Planck Society. This study was supported in part by the DFG Research Group "Sociality and Health in Primates." This article represents work completed in part during the doctoral dissertation of J.F.G. and benefited greatly from the input of his advisor, Jonathan Davies, and his supervision committee, David Marcogliese, Charles Nunn, and Louis Lefebvre.

CONFLICT OF INTERESTS

We have no competing interests.

AUTHOR CONTRIBUTIONS

J.F.G., A.D., J.M.T., S.C.S. and F.H.L. conceived and designed the study with valuable input and discussions with all of the co-authors.

J.F.G., A.D. and J.M.T. collected data in Taï National Park with support from A.M. and R.M.W. who work with the nonhuman primates in this forest. J.F.G., A.D., C.H., B.M. and A.S. conducted experiments in the lab. J.F.G. and S.C.S. conducted data analysis. J.F.G., S.C.S. and F.H.L. drafted the manuscript with subsequent input from all of the co-authors. All authors provided critical revision of the article and gave final approval to the version submitted for publication.

ETHICAL APPROVAL

Fly specimens were collected in accordance with the national laws of the Côte d'Ivoire. The protocols regarding work with the wild primates followed in this study adhered to the American Society of Primatologists principles for the ethical treatment of primates. Appropriate ethical approval and licences were obtained in collaboration with the Ministry of the Environment and Forests, the Ministry of Research, the directorship of the Taï National Park, and the Centre Suisse de Recherches Scientifiques en Côte d'Ivoire in Abidjan.

DATA AVAILABILITY STATEMENT

Next generation sequencing reads for constructing the *Bcbva* genomes are available through the short read archive with the BioProject ID PRJNA545191. All additional DNA sequences generated as part of this analysis have been uploaded to Zenodo (https://zenodo.org/record/3266800) because assignment to a particular organism is always uncertain or redundant with existing sequences. All other raw data are available in the manuscript or in the Appendix S1.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Gogarten JF, Düx A, Mubemba B, et al. Tropical rainforest flies carrying pathogens form stable associations with social nonhuman primates. *Mol Ecol*. 2019;00:1–17. https://doi.org/10.1111/mec.15145