VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 22, Number 11, 2022

DOI: 10.1089/vbz.2022.0025

Straw-Colored Fruit Bats (*Eidolon helvum*) and Their Bat Flies (*Cyclopodia greefi*) in Nigeria Host Viruses with Multifarious Modes of Transmission

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Abstract

Background: Bat flies (Diptera: Hippoboscoidea: *Nycteribiidae* and *Streblidae*) are increasingly appreciated as hosts of ''bat-associated'' viruses. We studied straw-colored fruit bats (*Eidolon helvum*) and their nycteribiid bat flies (*Cyclopodia greefi*) in Nigeria to investigate the role of bat flies in vectoring or maintaining viruses.
Methods: We captured bats and bat flies across northern Nigeria. We used metagenomics to identify viruses in 40 paired samples (20 flies from 20 bats). We characterized viruses using genomic and phylogenetic methods, and we compared infection frequencies in bats and their bat flies.
Results: In 20 bats, we detected two individuals (10%) infected with eidolon helvum parvovirus 1 (BtPAR4) (*Parvoviridae*; *Tetraparvovirus*), previously described in Ghana, and 10 bats (50%) with a novel parvovirus in the genus *Amdoparvovirus* (*Parvoviridae*). The amdoparvoviruses include Aleutian disease virus of mink and viruses of other carnivores but have not previously been identified in bats or in Africa. In 20 paired bat flies (each fly from 1 bat) all (100%) were infected with a novel virus in the genus *Sigmavirus* (*Rhabdoviridae*). The sigmaviruses include vertically transmitted viruses of dipterans. We did not detect BtPAR4 in any bat flies, and we did not detect the novel sigmavirus in any bats. However, we did detect the novel amdoparvovirus in 3 out of 20 bat flies sampled (15%), including in 2 bat flies from bats in which we did not detect this virus.
Discussion: Our results show that bats and their bat flies harbor some viruses that are specific to mammals and insects, respectively, and other viruses that may transmit between bats and arthropods. Our results also greatly expand the geographic and host range of the amdoparvoviruses and suggest that some could be transmitted by arthropods. Bat flies may serve as biological vectors, mechanical vectors, or maintenance hosts for ''bat-associated'' viruses.

Keywords: Chiroptera, *Nycteribiidae*, *Rhabdoviridae*, *Parvoviridae*, *Amdoparvovirus*, *Tetraparvovirus*

Introduction

The order Chiroptera (bats) currently contains >1400 recognized species and >20 recognized families, making it the second most diverse mammalian order, after Rodentia (Burgin et al. 2018). Bats play important beneficial roles in maintaining ecosystems worldwide (Kunz et al. 2011), but they also host an abundance of potentially zoonotic viruses (Olival et al. 2017, Letko et al. 2020, Van Brussel and Holmes 2022). Bats also host diverse arthropod ectoparasites that are highly adapted to life on bats (Dick and Patterson 2006, Whitaker et al. 2009, Szentivanyi et al. 2019).

Perhaps the most specialized of these are the bat flies (Diptera: Nycteribiidae and Streblidae), which are obligate hematophagous parasites that spend nearly their entire lives on bats (Dick and Patterson 2006, Szentivanyi et al. 2019). The family Nycteribiidae currently contains ~300 recognized species and 20 genera, whereas the family Streblidae

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contains ~200 recognized species and 30 genera, with taxonomic diversity of nycteribiids higher in the Eastern Hemisphere and taxonomic diversity of streblids higher in the Western Hemisphere (Dick and Patterson 2006, Szentivanyi et al. 2019).

Several viruses of agricultural, medical, and veterinary importance have been reported in bat flies (Feng et al. 2017, Goldberg et al. 2017, Bennett et al. 2020, Ramírez-Martínez et al. 2021, van Vuren et al. 2017, Xu et al. 2019, 2022). Research suggests that some of these viruses may not be vectored by bat flies in the traditional sense, nor are they strictly vertically transmitted; rather, some appear to be transmitted both vertically (fly to fly) and horizontally (fly to bat and bat to fly), with varying efficiencies (Goldberg et al. 2017, Bennett et al. 2020, Ramírez-Martínez et al. 2021).

Opportunistic feeding by bat flies on humans also occasionally occurs, leading to zoonotic infections (Wenzel and Tipton 1966, Dick and Patterson 2006). The role of bat flies as sometimes-vector, sometimes-host, and sometimes-reservoir may explain the enigmatic nature of many ''bat-associated'' viruses, which are ecologically associated with bats but rarely or never found in bats themselves (Ramírez-Martínez et al. 2021).

The straw-colored fruit bat (*Eidolon helvum*) is the most common megabat in Africa, the most hunted, migrates long distances, and often roosts in large colonies in or near human population centers (Peel et al. 2017, Oyewo et al. 2021). These characteristics have made *E. helvum* a suspected reservoir of zoonotic viruses, including ebolaviruses (*Filoviridae*: *Ebolavirus*) (Mbu'u et al. 2019, Markotter et al. 2020). Relatives of known zoonotic viruses have been identified in large, urban *E. helvum* colonies, but the actual zoonotic risk of these agents remains unclear (Fagre and Kading 2019, Mbu'u et al. 2019, Baker et al. 2020, Coertse et al. 2021). In Nigeria, for example, *E. helvum* has been extensively sampled for molecular and serologic evidence of infection with Lagos bat virus, a relative of rabies virus discovered in 1956 (Markotter et al. 2020, Coertse et al. 2021).

In this study, we sampled *E. helvum* bats and their bat flies across northern Nigeria. We analyzed matched samples of bats and bat flies, to identify viruses maintained in this highly coevolved host–parasite system and their potential mode(s) of transmission. Our results confirm that bat flies and the bats they parasitize harbor novel viruses with multifarious modes of transmission, and that some of these viruses are potentially important for human and animal health.

Methods

Ethical statement

Before handling bats, we obtained approval from the Animal Use and Care Committee (AUCC) of the National Veterinary Research Institute (NVRI), Vom, Nigeria (protocols AEC/02/59/18 and AEC/03/65/19). These protocols adhere to internationally accepted guidelines for the humane handling of wild mammals in research (Sikes et al. 2011). Samples were imported into the United States under CDC permit 20190411-2529A.

Study sites

We sampled bats from January to March 2019. We sampled roosts within or next to human habitations in four locations in three states in northern Nigeria: near a hostel in Nasarawa Toto (8.3892° N 7.0781° E), Nasarawa State; in a zoological garden in Jos (9.8965° N 8.8583° E), Plateau State; and in two villages, Toro (10.0596° N 9.07069° E) and Sabon Gari Narabi (10.3912° N 9.1686° E), both in Bauchi State (Fig. 1).

Bat capture and sampling

We captured bats at their roosting sites using nets constructed from local materials. We placed bats individually in clean nylon bags to await processing. We removed bat flies from bats or their nylon bags using forceps and placed them in coded vials containing RNAlater nucleic acid preservation buffer (Thermo Fisher Scientific, Waltham, MA, USA). We then humanely sacrificed bats and harvested tissues, for this and other collaborative research studies. For this study, we placed spleens from each bat in labeled vials containing RNAlater and kept them on ice in the field and during transportation to the NVRI in Vom, Plateau State, Nigeria. We stored samples at −20°C and shipped them to the University of Wisconsin-Madison, USA, at ambient temperature (4 days in transit), at which point we stored them at −20°C until processing.

Bat fly characterization

We visually examined all bat flies collected for this study in the field and identified them morphologically under a stereomicroscope based on the presences of distinctive chaetotaxy (bristles) on the abdomens of females and claspers on males (Theodor 1967). We imaged flies with a digital camera affixed to an illuminated dissecting microscope as previously described (Ramírez-Martínez et al. 2021). We then conducted DNA barcoding of the mitochondrial cytochrome oxidase subunit 1 (cox1) gene for all bat flies, also as previously described (Ramírez-Martínez et al. 2021).

Metagenomics and phylogenetics

We processed bat flies and bat spleens for virus characterization using previously described protocols applied to these sample types (Goldberg et al. 2017, Bennett et al. 2020, Ramírez-Martínez et al. 2021). In brief, we enriched for virus particles in spleen homogenates using density centrifugation and used nuclease digestion to reduce nonencapsidated nucleic acids, which in combination are as sensitive as quantitative real-time polymerase chain reaction for detecting viruses (Toohey-Kurth et al. 2017). We then extracted nucleic acids from sample homogenates using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), converted RNA to cDNA using the Superscript IV system (Thermo Fisher), and prepared DNA libraries using the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA).

We sequenced libraries on an Illumina MiSeq instrument (V2 chemistry, 300 cycle kit; Illumina). We trimmed resulting sequences (reads) at Phred quality scores of ≥Q30 and at lengths ≥50 using CLC Genomics Workbench 22 (Qiagen), then we assembled trimmed reads into contiguous sequences (contigs) using metaSPAdes (Nurk et al. 2017). We queried raw reads and assembled contigs (nucleotide and deduced amino acid sequences) against representative eukaryotic viral protein sequences available in the National Center for Biotechnology Information (NCBI) databases as of January 9, 2022, using the blastx homology detection

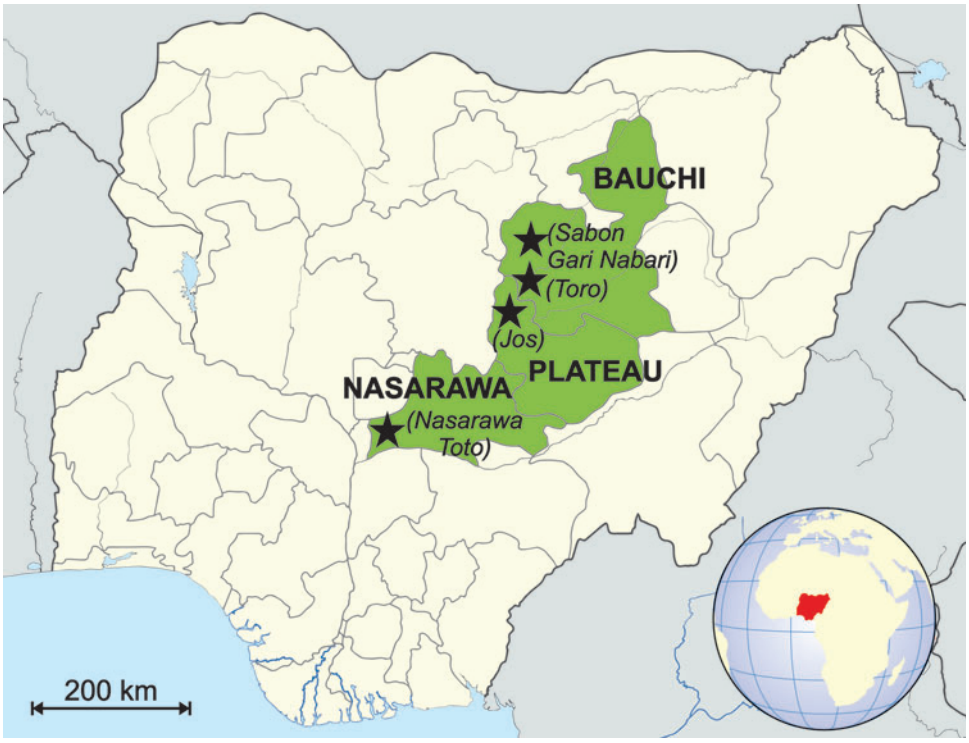

FIG. 1. Map of Nigeria showing study sites.

algorithm (Altschul et al. 1997) within CLC Genomics Workbench, and we used Cenote-Taker 2 (Tisza et al. 2021) to verify results and to create draft genome annotations, which we hand edited as needed.

We aligned resulting virus sequences with related sequences in NCBI (including outgroups) using a codon-guided version of the T-Coffee algorithm (Notredame et al. 2000) with Gblocks (Talavera and Castresana 2007) applied to remove poorly aligned regions, implemented in TranslatorX (Abascal et al. 2010). We then inferred phylogenies using PhyML 3.0 (Guindon et al. 2010) with smart model selection (Lefort et al. 2017) and 1000 bootstrap replicates to assess statistical support of clades. We displayed resulting trees using Dendroscope 3.8.2 (Huson and Scornavacca 2012). We estimated the abundance of each virus in each sample as log transformed viral reads per million total reads per kilobase of target sequence ($\log_{10}$(vRPM/kb)), which is a metagenomic proxy for viral load that correlates with quantitative real-time polymerase chain reaction data (Toohey-Kurth et al. 2017, Negrey et al. 2020).

Results

Bat fly identification

All bat flies were morphologically consistent with *Cyclopodia greefi*, which is the typical bat fly found on *E. helvum* throughout its range (Kamani et al. 2014) (Fig. 2). Sequences of the *coxI* gene (636 bp) from the 20 bat flies (GenBank acc. nos. ON324520.1–ON324539.1) were identical to each other, except for one sequence that differed from the others at a single nucleotide position. The sequences were between 95.60% and 95.75% similar to sequences of *Cyclopodia dubia* (GenBank acc. no. MF462038.1) and between 92.92% and 93.71% similar to sequences of *Cyclopodia horsfieldi* (GenBank acc. no. KF273782.1). No *C. greefi* sequences were available in NCBI databases for direct comparison at the time of this writing.

Virus characterization

From the 20 bat spleen samples processed, we generated a total of 35,748,949 sequence reads (average of 1,787,447 reads per sample ±132,610 standard error of the mean [SEM]) after quality trimming, with an average length of 108 nucleotides. From these data, we identified the previously described eidolon helvum parvovirus 1 (*Parvoviridae*: *Tetraparvovirus*; BtPAR4; (Canuti et al. 2011), represented by a 1008-base contig (NCBI acc. no. ON324117.1) containing partial gpp1 and gpp2 genes and 99.2% similar to the published BtPAR4 sequence (NCBI acc. no. NC_016744.1).

We also obtained a coding-complete genome sequence of a novel parvovirus in the genus *Amdoparvovirus*, which we named sabeidhel virus 1 (SBEHV-1; derived from Sabon Gari Narabi *Eidolon helvum*; NCBI acc. no. ON324118.1). SBEHV-1 is most similar to gray fox amdoparvovirus (GFAV), sharing; 58.5% and 57.8% amino acid sequence similarity in the putative NS1 and VP proteins, respectively. SBEHV-1 is sister taxon to GFAV on a phylogenetic tree of the amdoparvoviruses (Fig. 3). SBEHV-1 contains two long open reading frames (ORFs) and a short middle ORF, as is typical of the amdoparvoviruses (Canuti et al. 2015).

From the 20 bat fly samples processed, we generated a total of 51,740,672 sequence reads (average of 2,587,034 ± 92,576 SEM) after quality trimming, with an average length of 120 nucleotides. From these data, we obtained a coding-complete genome sequence of a novel member of the genus *Sigmavirus* (*Rhabdoviridae*). This virus, which we named jopcycgri virus 1 (JPCGV-1; derived from Jos Plateau *C. greefi*; NCBI acc. no. ON324119.1) forms a distinct lineage within the sigmaviruses and is most closely related to a clade of sigmaviruses from fruit flies (Drosophilidae and Tephritidae) and louse flies (Hippoboscidae) (Fig. 3). JPCGV-1 contains the canonical N, P, G, M, L gene arrangement of the rhabdoviruses (Walker et al. 2015). However, JPCGV-1 does not contain an

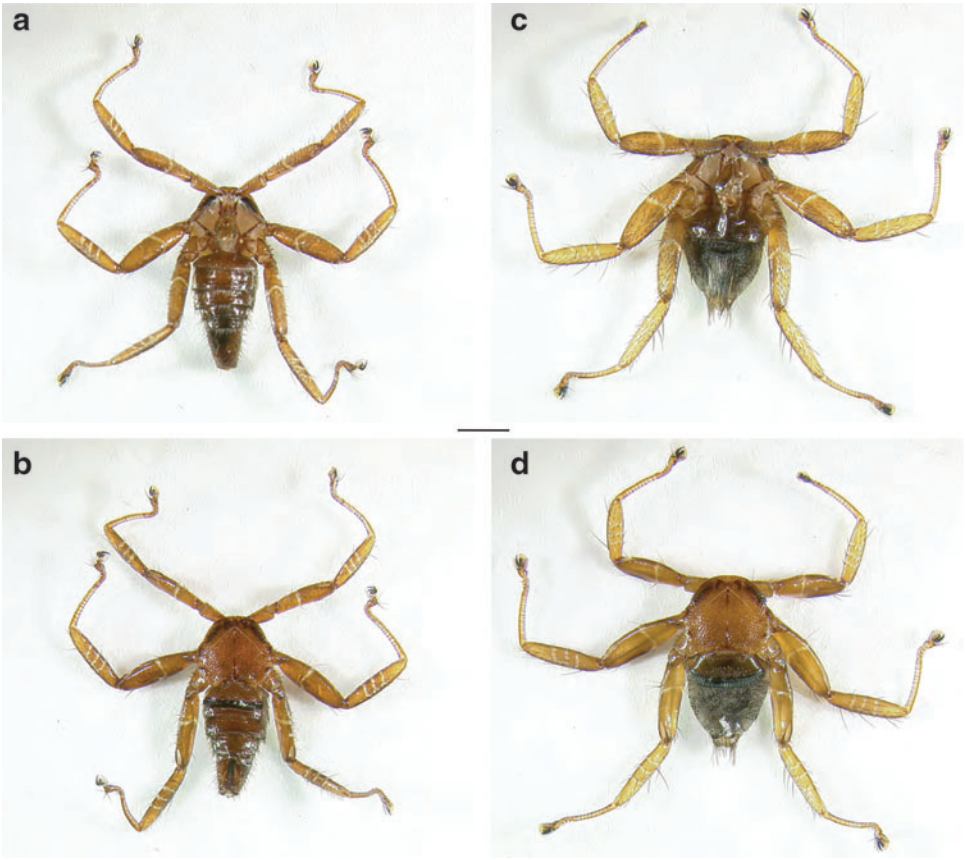

FIG. 2. Images of *Cyclopodia greefi* bat flies from straw-colored fruit bats in Nigeria. A male specimen (**a**: dorsal, **b**: ventral) and a female specimen (**c**: dorsal, **d**, ventral) are shown. Scale bar = 1 mm. DNA barcoding at the cox1 gene revealed these flies to be genetically intermediate between *Cyclopodia dubia* and *Cyclopodia horsfieldi*, consistent with established relationships within the genus.

ORF X (U1) gene between P and M, making its genome architecture more similar to sigmaviruses of other hippoboscid flies than to sigmaviruses of fruit flies (Walker et al. 2020).

We detected BtPAR4 in 2/20 (10%) of bats but in no bat flies (0%) (Fig. 4). Normalized BtPAR4 read depths in positive bats ranged from 12.7 to 30.7 per million. We detected JPCGV-1 in all 20 bat flies (100%) but in no bats (0%) (Fig. 4). Normalized JPCGV-1 read depths in infected bat flies ranged from 25.7 to 22278.1 per million (Fig. 4). Interestingly, we detected SBEHV-1 in both bats (10/20; 50%) and in bat flies (3/20; 15%) (Fig. 4). Normalized SBEHV-1 read depths ranged from 1.5 to 1981.7 per million in bats and from 9.8 to 33.6 per million in bat flies.

One bat fly in which we detected SBEHV-1 came from a bat also positive for SBEHV-1 (bat/fly pair 12 in Fig. 4). However, the two other bat flies in which we detected SBEHV-1 came from bats in which we did not detect this virus (bat/fly pairs 18 and 19 in Fig. 4). To investigate whether ingestion of SBEHV-1 by bat flies during a recent bloodmeal might explain this pattern, we mapped reads from each bat fly to the *E. helvum* mitochondrial cytochrome b (cytb) DNA sequence (NCBI acc. no. NC_046903) as described earlier. We found sequence reads matching *E. helvum* cytb in eight bat flies (40%; bat fly numbers 5, 7, 8, 9, 11, 15, 19, and 20 in Fig. 4). Thus, one bat fly in which we detected SBEHV-1 (number 18 in Fig. 4) came from a bat in which we did not detect SBEHV-1. We note that the abdomen of bat fly 18 was not engorged (as would be the case had it ingested a recent bloodmeal).

Discussion

Our results show that bats and their bat flies can host viruses with markedly different modes of transmission. We identified BtPARV4 only in bats at a prevalence of 10%, which is consistent with the 7% prevalence of BtPARV4 reported in *E. helvum* in Ghana (Canuti et al. 2011). Our detection of the virus in spleen tissue also concords with previous data showing that spleen and kidney are the most likely replication sites for this virus in *E. helvum* (Canuti et al. 2011). These findings support the idea that BtPARV4 replicates in bats and is transmitted horizontally among bats, perhaps through the fecal–oral route (Canuti et al. 2011). By contrast, we identified JPCGV-1 only in bat flies. We detected JPCGV-1 in 100% of sampled bat flies, which concords with previous reports of sigmaviruses in dipteran insects (Fleuriet 1988, Longdon et al. 2011). We suspect that future studies will reveal an ovarian tropism for this virus. If so, these findings would support the idea that JPCGV-1 replicates in bat flies and is transmitted vertically from female bat flies to their offspring.

To our knowledge, no amdoparvoviruses have been described previously in noncarnivore hosts or in Africa (Canuti et al. 2015, Markarian and Abrahamyan 2021). Our finding of SBEHV-1, therefore, substantially extends the host and geographic range of the amdoparvoviruses. Amdoparvoviruses infect animals within various carnivore families, including the Ailuridae, Canidae, Mephitidae, Mustelidae, and probably others (Shao et al. 2014, Canuti et al. 2015, Alex et al. 2018). It is, therefore, intriguing that

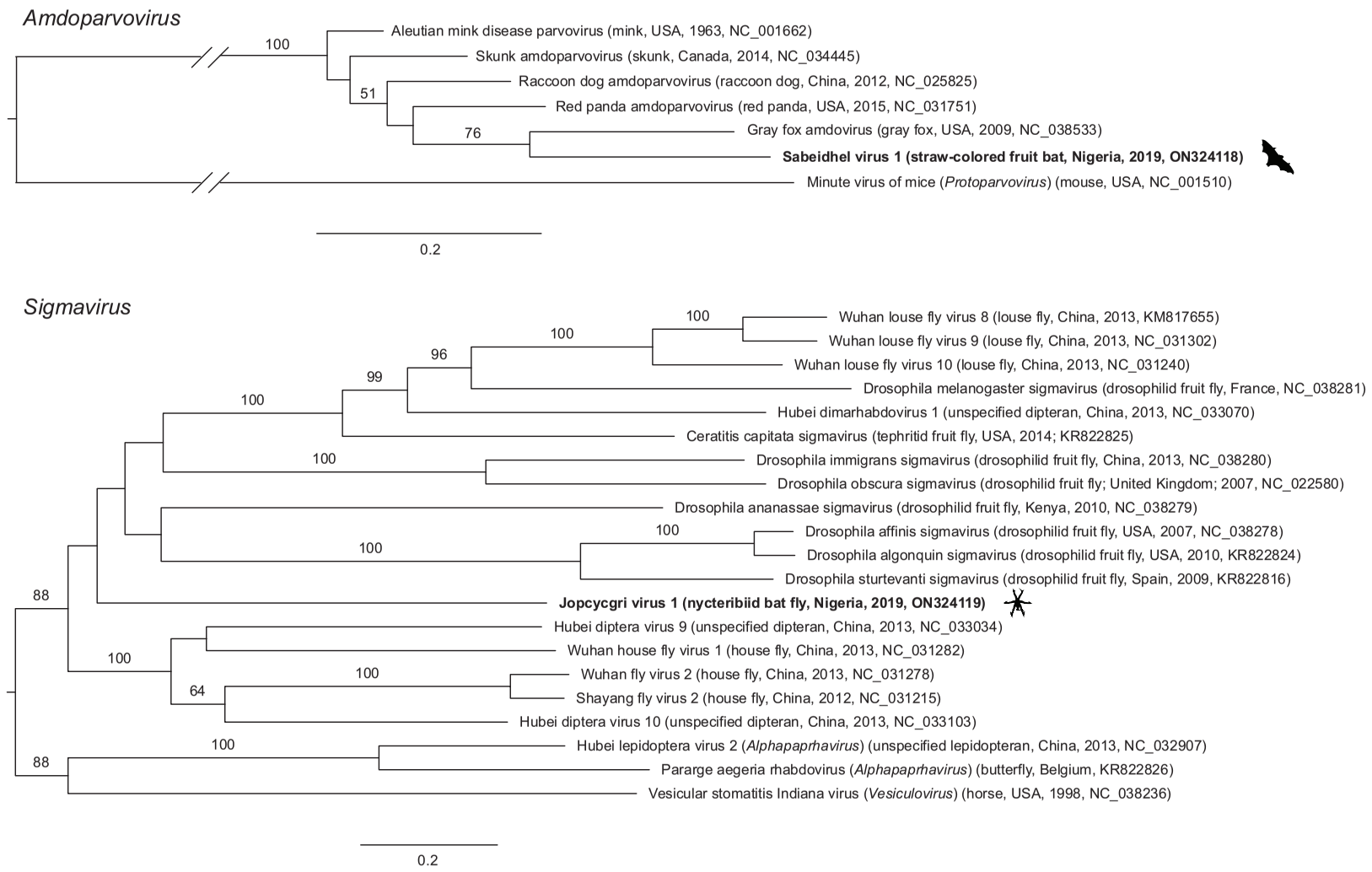

FIG. 3. Maximum likelihood phylogenetic trees of amdoparvoviruses and sigmaviruses. Virus names are followed (in *parentheses*) by host, country, year of collection (where available), and accession number. Viruses identified in this study are shown in *bold type* with silhouettes. Outgroup viruses are followed in *parentheses* by names of the genera to which they belong. Numbers beside branches are bootstrap values based on 1000 replicates; only values ≥50% are shown. Scale bar = amino acid substitutions per site.

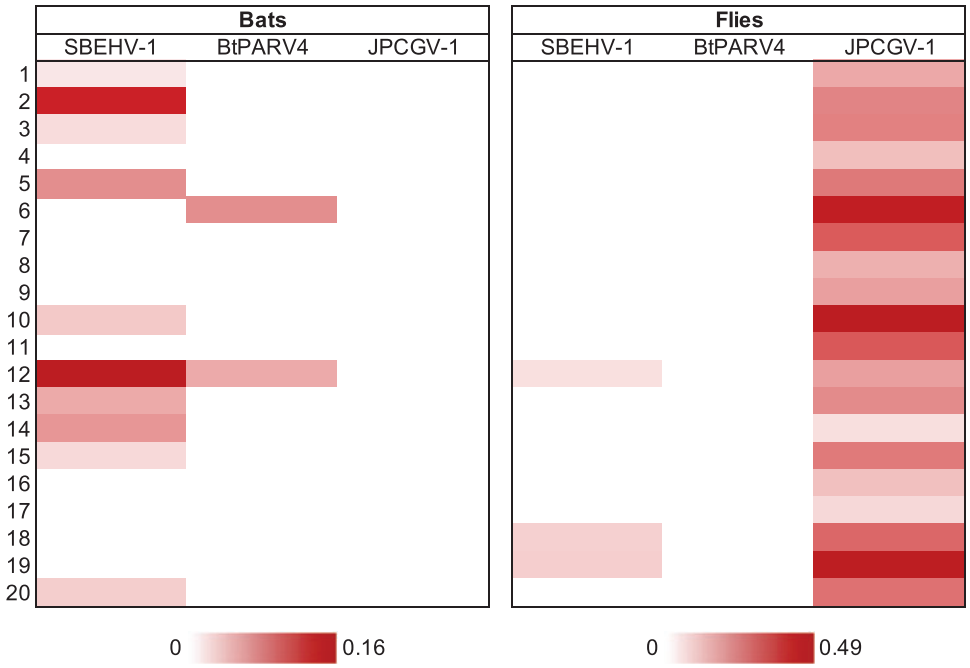

FIG. 4. Heatmap of normalized metagenomic read depths of viruses in *Eidolon helvum* bats and their *Cyclopodia greefi* bat flies. Viruses are SBEHV-1, BtPARV4, and JPCGV-1. Each row represents a single bat and a single bat fly parasitizing that bat. Cells are shaded in proportion to $\log_{10}$ viral reads per 10^6 total reads per kilobase of target. BtPARV4, eidolon helvum parvovirus 1; JPCGV-1, jopcycgri virus 1; SBEHV-1, sabeidhel virus 1.

SBEHV-1 is not divergent from the other amdoparvoviruses but rather is sister taxon to GFAV within the amdoparvovirus clade (Fig. 3).

This finding is contrary to what might be expected given the large evolutionary distance between bats and carnivores and the large geographic distance between Nigeria and the Holarctic ranges of known carnivore amdoparvovirus hosts (Albery et al. 2020). We speculate that an unknown diversity of amdoparvoviruses exists in other noncarnivore hosts, and that, eventually, the amdoparvoviruses might show a pattern of evolutionary host switching similar to that observed for other genera within the subfamily *Parvovirinae* (Cotmore et al. 2019).

Our finding of SBEHV-1 in bat flies is also intriguing. We initially hypothesized that SBEHV-1-positive bat flies recently fed upon bats infected with SBEHV-1, merely carrying the virus in their gastrointestinal tracts. However, of the three bat flies in which we found SBAV-1, two were collected from bats in which we did not detect SBEHV-1. Moreover, in one of these two SBEHV-1-positive bat flies from SBEHV-1-negative bats, we did not detect *E. helvum* DNA, which weakens the argument that this particular bat fly recently ingested a bloodmeal. It is, therefore, possible that bat flies could play a role in the maintenance or transmission of SBEHV-1, either as mechanical or biological vectors.

Members of the *Parvovirinae* are not thought to be vector-borne, although canine parvovirus (genus *Protoparvovirus*) can remain infectious for 28 days in experimentally infected *Haemaphysalis longicornis* ticks (Mori et al. 2015) and was found in a wild *Rhipicephalus sanguineus* tick in Palestine (Ravi et al. 2019). We also cannot exclude the possibility that these two bat flies had recently switched *E. helvum* bats, as bat flies do (ter Hofstede et al. 2004, Dick and Patterson 2006). Other lines of evidence, such as visualization of SBEHV-1 in the tissues of bat flies (*e.g.*, localization to the salivary glands) or experimental infection studies, would be needed to resolve whether SBEHV-1 is transmissible between bats and bat flies.

On rare occasions Aleutian disease virus of mink (ADV) can infect humans. Mink farmers have tested positive for ADV and ADV-directed antibodies and have experienced symptoms and pathologies (eventually fatal) evocative of Aleutian disease (Jepsen et al. 2009). Occult infection with ADV might even explain elevated rates of malignant lymphoma in mink farmers relative to other agricultural workers (Wiklund et al. 1988, Jepsen et al. 2009). Furthermore, bat flies occasionally bite people (Osborne et al. 2003, Dick and Patterson 2006, Reeves and Lloyd 2019). In this light, and given the wide geographic range, large population size, and peridomestic roosting habits of *E. helvum* (Peel et al. 2017, Oyewo et al. 2021), the zoonotic potential of SBEHV-1 should not be dismissed. Should SBEHV-1 prove to be vectored by bat flies or other hematophagous arthropods of bats, this risk would be amplified, even if zoonotic transmission is inefficient.

Our finding of JPCGV-1 in *C. greefi* is comparatively unsurprising. Bat flies host diverse rhabdoviruses (Goldberg et al. 2017, Bennett et al. 2020, Ramírez-Martínez et al. 2021), and sigmaviruses are ubiquitous and vertically transmitted among dipterans (Fleuriet 1988, Longdon et al. 2011, 2015, Shi et al. 2016). It is nevertheless interesting that JPCGV-1 is most closely related to viruses of fruit flies and not to viruses of other hippoboscid flies, based on phylogenetic analysis of the L gene (Fig. 3).

JPCGV-1 lacks a U1 gene between P and M, which is a unifying feature of fruit fly sigmaviruses, whereas absence of this gene is a unifying feature of hippoboscid fly sigmaviruses (Walker et al. 2020). The reasons for this discrepancy between phylogenetic position and genome architecture are unclear, especially because negative sense RNA viruses such as sigmaviruses do not recombine. It may reflect incomplete/biased geographic and host taxonomic sampling of sigmaviruses or convergent evolution (*i.e.*, parallel loss of U1 upon switching from a drosophilid to a hippoboscid host).

Overall, our results reaffirm that bats and their bats flies host viruses that have evolved to exploit the multifarous

transmission opportunities that this highly coevolved host–parasite system affords. Our results also confirm that, although bats in general (*i.e.*, the order Chiroptera) host a great diversity of viruses (Tian et al. 2022, Van Brussel and Holmes 2022), metagenomic studies of individual bat populations, tissue types, and time points may yield few viruses, if any (Bergner et al. 2020, Paskey et al. 2020, Simic et al. 2020).

Viruses in bats and their bat flies may be transmitted with varying efficiencies between host types, sometimes maintained neither in bats nor in bat flies alone but rather collectively in both, reminiscent of the ''holobiont'' concept as applied to viruses (Grasis 2017, Roossinck and Bazan 2017). By incidentally biting humans, bat flies may cause zoonotic infections that appear to have resulted from direct exposure to bats but are, in fact, only indirectly associated with bats through their arthropod parasites (Ramírez-Martínez et al. 2021). Bat flies and other specialized bat ectoparasites will likely continue to shed light on the intriguing ecology of ''bat-associated'' viruses.

Acknowledgments

We thank I. Abubakar and E. V. Kamani for assistance with field work. We thank C. Dunn for assistance with sequencing and D. Young and the University of Wisconsin-Madison Department of Entomology for assistance with bat fly imaging.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This project was funded by the University of Wisconsin-Madison John D. MacArthur Professorship Chair (to T.L.G.).

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