Novel hemotropic mycoplasmas are widespread and genetically diverse in vampire bats

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Summary

Bats (Order: Chiroptera) have been widely studied as reservoir hosts for viruses of concern for human and animal health. However, whether bats are equally competent hosts of non-viral pathogens such as bacteria remains an important open question. Here, we surveyed blood and saliva samples of vampire bats from Peru and Belize for hemotropic *Mycoplasma* spp. (hemoplasmas), bacteria that can cause inapparent infection or anemia in hosts. 16S rRNA gene amplification of blood showed 67% (150/223) of common vampire bats (*Desmodus rotundus*) were infected by hemoplasmas. Sequencing of the 16S rRNA gene amplicons revealed three novel genotypes that were phylogenetically related but not identical to hemoplasmas described from other (non- vampire) bat species, rodents, humans, and non-human primates. Hemoplasma prevalence in vampire bats was highest in non-reproductive and young individuals, did not differ by country, and was relatively stable over time (i.e., endemic). Metagenomics from pooled *Desmodus rotundus* saliva from Peru detected non-hemotropic *Mycoplasma* species and hemoplasma genotypes phylogenetically similar to those identified in blood, providing indirect evidence for potential direct transmission of hemoplasmas through biting or social contacts. This study demonstrates vampire bats host several novel hemoplasmas and sheds light on risk factors for infection and basic transmission routes. Given the high frequency of direct contacts that arise when vampire bats feed on humans, domestic animals, and wildlife, the potential of these bacteria to be transmitted between species should be investigated in future work.

Introduction

Bats (Order: Chiroptera) have been widely studied as reservoir hosts for pathogens of concern for human and animal health [1,2], with particular attention paid to RNA viruses in the *Coronaviridae, Filoviridae, Rhabdoviridae*, and *Paramyxoviridae* families [3–5]. The association of bats with human and animal disease is in part explained by the high diversity of zoonotic viruses that circulate in bats. Per species, bats host more zoonotic viruses than all other mammalian orders and are more likely to share viruses among species, which may be due to aggregations of large colonies, migration, and the multi-species roosts of many bats [6–8]. However, whether bats are equally competent hosts of non-viral pathogens such as bacteria remains an open and understudied question [9,10]. Bacteria such as *Yersinia* spp. and *Leptospira* spp. have been detected in bats [11,12], but the importance of these pathogens for human, wildlife, or domestic animal health remains unknown. For other bacteria such as *Bartonella* spp., phylogenetic analyses have suggested a potential role of bats in the transmission of zoonotic *Bartonella sp.* [13], such as *B. mayotimonensis*, an etiologic agent of human endocarditis [14,15].

Recent studies have also shown hemotropic *Mycoplasma* spp. (hemoplasma) infections in bats [16–19]. Hemoplasmas are facultative intracellular erythrocytic bacteria without a cell wall that were formerly classified as *Haemobartonella* and *Eperythrozoon* spp. based on their 16S rRNA gene sequences and cell morphologic properties [20–23]. These bacteria are thought to be transmitted through direct (blood and saliva) and possibly vector-borne contact [23–26] and can cause acute and chronic anemia in wildlife, humans, and domestic animals [27–30], particularly in immunocompromised hosts [31,32]. Almost all *Mycoplasma* spp., including hemoplasmas, appear to show host specificity that seems to be a result of the host–pathogen interaction during

66 evolution; however, potential zoonotic or inter-species transmission has also been reported
67 [30,31,33–35]. Among bat species studied to date, 16S rRNA gene sequence analyses have
68 shown that hemoplasmas identified in little brown bats (*Myotis lucifugus*) from the United States
69 demonstrated closest homology (~92%) with a hemoplasma detected in a human, *Candidatus*
70 Mycoplasma haemohominis, and with *Mycoplasma haemomuris* detected in a small Japanese
71 field mouse (*Apodemus argenteus*) [16,36]. Recent work on Neotropical bat species from Brazil
72 found velvety free-tailed bats (*Molossus molossus*) were infected with hemoplasmas that shared
73 close identity (93–96%) with a hemoplasma detected in mice, *Mycoplasma coccoides* [19].
74 Surveys of Schreibers' bats (*Miniopterus schreibersii*) and one long-eared bat (*Myotis*
75 *capaccinii*) also detected hemoplasma species with close identity (97%) to *Candidatus*
76 Mycoplasma haemohominis [17]. These phylogenetic relationships between bat hemoplasmas
77 and hemoplasmas from other species suggests possible cross-species transmission in history [37],
78 which may be relevant for zoonotic transmission from bat species with frequent contact with
79 humans.
80 No published data currently exist on evidence for hemoplasma infection in vampire bats
81 or on the prevalence and diversity of these bacteria in hematophagous bats. Yet owing to their
82 direct contact with mammals through blood feeding, vampire bats are an obvious candidate
83 species for which to assess hemoplasma infection and phylogenetic relationships to genotypes
84 previously described in other mammals, including humans and non-human primates. Three
85 species comprise the subfamily *Desmodontinae*: the common vampire bat (*Desmodus rotundus*),
86 the hairy-legged vampire bat (*Diphylla ecaudata*), and the white-winged vampire bat (*Diaemus*
87 *youngi*). Vampire bats occur across diverse habitat types throughout Latin America, ranging
88 from Mexico to northern Argentina [38]. While these species historically feed on wild mammals

and birds, the most abundant species, *D. rotundus*, preferentially feeds on livestock and poultry owing to the greater accessibility and reliability of these novel prey species [39–41]. *D. rotundus* also commonly feeds on humans, making it an important source of human rabies virus outbreaks [3,42–44]. As biting is a possible transmission route for hemoplasmas in other mammals through exposure to infectious blood or saliva [24–26], vampire bat feeding behavior could possibly facilitate transmission to humans, domestic animals, and wildlife. Infection in vampire bats roosting in anthropogenic habitats could also enhance vector-borne transmission cycles [23]. The goals of our study were thus *(i)* to identify hemoplasma species in vampire bats, *(ii)* to assess the position of detected sequences within the broader hemoplasma phylogeny, *(iii)* to identify risk factors for infection, including age and seasonality [45], and *(iv)* to determine hemoplasma presence in vampire bat saliva to assess the possibility for direct transmission of these bacteria.

Materials and methods

Vampire bat sampling

During 2015 and 2016, we sampled 224 vampire bats across 14 sites in Peru (Departments of Amazonas [AM], Apurimac [API], Ayacucho [AYA], Cajamarca [CA], and Loreto [LR]; n=12) and in Orange Walk [OW] District (*n*=2), Belize (Fig. 1). We sampled sites 1–2 times annually (Table S1), in which bats were captured in mist nets or harp traps placed at exits of roosts, along flight paths, or outside livestock corrals from 19:00 to 05:00 [46,47]. Upon capture, bats were held in individual cloth bags and issued a uniquely coded incoloy wing band (3.5 mm, Porzana Inc.). Bats were classified as subadult or adult based on fusion of phalangeal epiphyses [48], and reproductive activity was indicated by the presence of scrotal testes in males and pregnancy or lactation in females. We obtained blood by lancing the propatagial vein with a sterile 23-gauge

needle, followed by sample collection with heparinized capillary tubes. To screen for hemoplasmas by PCR, up to 30 μL blood was stored on Whatman FTA cards to preserve bacterial DNA [49]. Whole blood–impregnated FTA cards were stored in individual pouches at room temperature with desiccant until laboratory analysis. Thin blood smears were prepared on glass slides, stained with buffered Wright–Giemsa (Camco Quik Stain II, Fisher Scientific), and screened by a board-certified veterinary clinical pathologist (MSC) for hemoplasmas using light microscopic examination of a representative area of the blood monolayer at 1000X magnification. For assessment of hemoplasmas presence in vampire bat saliva, we collected oral swabs from Peru; samples were preserved in 2 mL of RNAlater (Invitrogen) at –80°C until laboratory analyses.

All field procedures were approved by the University of Georgia Animal Care and Use Committee (A2014 04-016-Y3-A5) and the University of Glasgow School of Medical Veterinary and Life Sciences Research Ethics Committee (Ref08a/15). Bat capture and sampling were authorized by the Belize Forest Department under permits CD/60/3/15(21) and WL/1/1/16(17) and by the Peruvian Government under permits RD-009-2015-SERFOR-DGGSPFFS, RD-264-2015-SERFOR-DGGSPFFS, and RD-142-2015-SERFOR-DGGSPFFS. Access to genetic resources from Peru was granted under permit RD-054-2016-SERFOR-DGGSPFFS.

DNA extraction, PCR amplification, and sequencing of amplicons

Genomic DNA was extracted from 3–5 2 mm punches of blood preserved on Whatman FTA cards using QIAamp DNA Investigator Kits (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA samples were stored at –80°C until use.

Primary screening for the presence of hemoplasmas was performed with PCR using previously published UNI_16S_mycF and UNI_16S_mycR universal primers for amplification of the partial 16S rRNA hemoplasma genes [50]. Based on our previously published data [50] and recent *in silico* analysis [51] using BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of these universal primers against the different mycoplasma 16S rRNA gene sequences available in GenBank, these primers produce PCR fragments with size of approximately 1000–1035 bp (depending on the target *Mycoplasma* spp.). If samples were considered strongly positive for infection with hemoplasmas in the primary screening PCR, we also amplified the full-length 16S rRNA gene (approximately 1450–1560 nt) using PCR primers designed in this study (16SF – AGAGTTTGATCCTGGCTCAG and 16SR – CTCAAAACTGAAAGYCATCCGC) and then sequenced these amplicons (see GenBank accession numbers KY932674, KY932675, KY932677–KY932680, KY932687–KY932693, KY932695, KY932696, KY932701, KY932703–KY932710, KY932712, KY932716, KY932721, and KY932722). If samples were weakly positive (i.e., a weak band) in the primary screening PCR, we did not amplify the full-length 16S rRNA gene but instead sequenced amplicons of the partial 16S rRNA gene from the primary PCR (see GenBank accession numbers KY932676, KY932681–KY932686, KY932694, KY932697–KY932700, KY932702, KY932711, KY932713–KY932715, KY932717–KY932720, KY932723, KY932724). All PCR in this study was qualitative and thus the load of hemoplasma DNA in individual blood samples was not quantified.

The 16S rRNA amplicons produced were directly sequenced (without cloning into a plasmid vector) by Macrogen. Prior to sequencing, PCR amplicons were purified by electrophoresis through 1.5% agarose gels and extracted with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Amplicons were sequenced with the same primers used for PCR

amplification and then with internal (walking) primers when needed. The amplification mixture for all PCR contained 5 µL of 10X HotStarTaq PCR buffer, 1.5 mM MgCl2, 200mM dNTP mixture, 1 mM of each primer, and 2.5 U HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany) in a final volume of 50 µL, including 3 µL of DNA template. The Vent DNA Polymerase Kit (New England Biolabs), which contains high-fidelity thermophilic Vent DNA polymerase, was also used for the amplification of PCR products for subsequent sequencing. The absence of PCR inhibitors in isolated blood DNA was confirmed by PCR amplification of the *Desmodus rotundus* mitochondrial gene for 16S rRNA as an extraction positive control (with primers 16S_Desmodus_F – AACAGCAAAGCTTACCCCTTGTACC and 16S_Desmodus_R - GTCTGAACTCAGATCACGTAGGAC). Negative (no DNA added) controls were run for each PCR, and *Candidatus* Mycoplasma haemozalophi [50] was used as a positive control.

All PCR reactions were conducted under the following conditions: a polymerase activation step at 95°C for 5 min (or 15 min for HotStarTaq only) followed by 45 cycles of 95°C for 30s, 60°C for 60s, and 72°C for 60s, with a final extension at 72°C for 10 min. PCR products were detected by electrophoresis through 1% TAE-agarose gels containing ethidium bromide concentrations followed by UV visualization.

To avoid the potential presence of chimeric sequences or PCR-derived variants in the data, all hemoplasma 16S rRNA PCR products for phylogenetic analyses were directly amplified from blood DNA samples of vampire bats with two different DNA polymerases (HotStarTaq and Vent) and were directly sequenced without cloning [52,53]. All gene sequences prior to the downstream phylogenetic analysis were subjected to the chimeric sequence analysis using DECIPHER [54] and UCHIME [55]. All sequences available from this study have been deposited in GenBank under the accession numbers KY932674–KY932724.

Phylogenetic analyses

The 16S rRNA sequences detected in this study were compared to those available in GenBank using procedures, algorithms, and methods for phylogenetic tree inference as described elsewhere [50,56,57]. Briefly, the sequences of the 16S rRNA genes were compared to the GenBank nucleotide database. Nucleotide sequences were aligned using the publicly available Clustal X software (http://www.clustal.org). Inter- and intra-species similarity was generated using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Genetic distances were calculated by using the Kimura two-parameter and Tamura–Nei models, and phylogenetic trees were constructed in MEGA 6 software using the minimum evolution algorithm (http://www.megasoftware.net).

Statistical analyses

We first calculated hemoplasma prevalence and 95% confidence intervals using the Wald method in the *prevalence* package of R [58]. We then tested if hemoplasma genotypes detected via sequencing were associated with geography, bat demography, or time using Chi-squared tests with *p* values generated via a Monte Carlo procedure with 1000 simulations [59]. We used the Benjamini and Hochberg correction to adjust *p* values for multiple comparisons [60].

We used generalized mixed effects models (GLMMs) with binomial errors and a logit link to determine risk factors for hemoplasma infection status (positive or negative) [61]. Bat ID was included a random effect to account for multiple sampling of a small number of individuals (n=6); site was not included as a random effect owing to repeatedly failed model convergence. Using a reduced dataset free of missing values (n=220), we compared a set of GLMMs with

country, bat age, sex, reproductive status, year, and season (spring, summer, fall) as fixed effects alongside possible two-way interactions; we limited the number of models to roughly 50% our sample. We compared models with Akaike information criterion corrected for small sample size (AICc) and calculated marginal and conditional R^2 values to assess fit [62,63]. We performed model averaging to compute mean odds ratios (OR) and 95% confidence intervals across the set of GLMMs whose cumulative Akaike weight (w_i) summed to 95%; mean ORs were standardized with partial standard deviation [64]. We used the *MuMIn* and *lme4* packages for model averaging [65,66].

Assessment of hemoplasmas in saliva

To examine the possibility for direct transmission of hemoplasmas through biting or grooming, we used metagenomic data from a parallel study to screen vampire bat saliva samples from the same regions of Peru where blood samples were collected. Although a PCR-based screening of saliva samples would have made for the most comparable set of results, untargeted metagenomic sequencing has been found equally or more sensitive for pathogen detection compared to conventional PCR [67–69]. Five saliva pools were shotgun sequenced, each of which contained nucleic acid extractions from saliva swabs of ten vampire bats from 1–2 colonies within each department (Amazonas, Cajarmarca, Loreto, Ayacucho) or across two neighboring departments (Ayacucho and Apurimac). Pooled samples represent the same regions of Peru, though not necessarily the same colonies or individuals, tested for hemoplasmas in blood through PCR.

Total nucleic acid was extracted from swabs using a modified protocol with the BioSprint 96 One-For-All Vet Kit (Qiagen, Hilden, Germany) and a KingFisher Flex 96 machine (Thermo Fisher Scientific). Swabs were incubated twice consecutively in tubes containing lysis buffer

(Buffer RLT) and Proteinase K for 15 minutes at 56°C; volume from the two tubes was combined prior to the addition of other extraction reagents, and the manufacturer's protocol was subsequently followed. Extractions were quantified using a Qubit RNA HS Assay Kit (Thermo Fisher Scientific) and pooled at approximately 120 ng RNA per sample. Pools were treated for 5 minutes at 35°C with 2U DNase I (Ambion) and cleaned with a 1.8X ratio of Agencourt RNAClean XP beads (Beckman Coulter). Pools were then depleted for host ribosomal RNA using the Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) (Illumina) per the manufacturer's instructions. Prior to library preparation, cDNA synthesis was performed using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and the NEBNext mRNA Second Strand Synthesis Module (New England Biolabs). Samples were library prepared using the KAPA DNA Library Preparation Kit for Illumina (KAPA Biosystems), at which point they were individually barcoded with primers designed based on the NEBNext Multiplex Oligos for Illumina Index Primers (New England Biolabs). The libraries included in this study were combined in equimolar ratios with other metagenomic libraries for sequencing on an Illumina NextSeq500 at the University of Glasgow Centre for Virus Research.

Reads were demultiplexed according to barcode and quality filtered using TrimGalore [70,71] with a quality threshold of 25, minimum read length of 75 bp, and clipping the first 14 bp of the read. Low complexity reads were filtered out using the DUST method and PCR duplicates removed using PRINSEQ [72]. We screened cleaned reads for hemoplasma-like sequences using nucleotide BLAST [73] against a custom database composed of the PCR-generated hemoplasma sequences from this study, retaining only the best alignment for a single query-subject pair. The hemoplasma-like reads were then de-novo assembled using the assembly-only function of

SPAdes [74], and contigs greater than 300 bp were screened for sequences closely matching *Mycoplasma* species using nucleotide BLAST in Genbank.

Results

Hemoplasma genotype detection and phylogenetic analysis

Hemoplasma infection was detected by 16S rRNA PCR in 150/223 (67%; 95% CI=0.61–0.73) of common vampire bats (*Desmodus rotundus*) but was not found in our single sample from a hairy-legged vampire bat (*Diphylla ecuadata*). We did not detect hemoplasmas in any blood samples with light microscopy. Hemoplasma infection prevalence as assessed by PCR ranged from 0–100%, with a mean 67.53% bats per site infected with at least one genotype (Fig. 1).

Figure 2 shows the inferred phylogenetic position of the hemoplasma sequences identified in vampire bats among known hemotropic *Mycoplasma* species using partial 16S rRNA genes (871–890 bp). Vampire bat hemoplasmas represented three main genotypes (Table S2, Fig. 2). One other sample (D141; GenBank accession number KY932724) showed 97% similarity to the 16S ribosomal RNA gene of *Mycoplasma moatsii* strain MK405 (NR_025186), a non-hemotropic *Mycoplasma* spp. isolated from grivit monkeys (*Cercopithecus aethiops*); however, we were not able to amplify the full-length 16S rRNA gene from this sample. Inter-laboratory contamination with *M. moatsii* was excluded as we do not handle this species in our laboratory; the same sequence was also repeatedly amplified from the same blood sample. Vampire bat hemoplasma genotypes 1 and 2 were closely related (97–98% inter-genotype similarity, Table S2) and similar to hemoplasmas detected in common bent-wing bats (*Miniopterus schreibersii*) in Spain (86–87% similarity to GenBank accession numbers KM538691–KM538698), little brown bats (*Myotus lucifugus*) in the United States (88–89%

similarity to KF713538), wild Japanese monkeys (*Macaca fuscata*) (93–94% similarity to AB820288), tufted capuchins (*Sapajus apella*) in the Brazilian Amazon (88–90% similarity to KT314160- KT314164), and to a hemoplasma detected in a human patient with hemolytic anemia and pyrexia in the United States (94% similarity to GU562823). Genotype 3 was most similar to *Mycoplasma coccoides* (93–95% similarity to AY171918), *Candidatus* Mycoplasma turicensis (93–94% similarity to DQ157153), a hemoplasma detected in a capybara (*Hydrochoerus hydrochaeris*) in Brazil (92–93% similarity to FJ667774), and hemoplasmas detected in velvety free-tailed bats (*Molossus molossus*) in Brazil (90-93% similarity to KY356747–KY356751). No chimeras were detected from these 16S rRNA gene sequences.

Within vampire bats, hemoplasma genotype 1 was the most common sequence identified (Fig. 3), infecting 68% of positive bats (100/150). Genotypes 2 and 3 infected 22% (33/150) and 9% (14/150) of positive bats, respectively. The number of genotypes detected per site ranged from one to three, but no coinfection with multiple genotypes was observed. Genotype 1 was detected across all sites, genotype 2 was detected in both Belize sites and 40% of Peru sites (primarily northern and eastern Amazon), genotype 3 was detected in both Belize sites and 40% of Peru sites. The *M. moatsii*–like hemoplasma sequence was detected in only one bat in Belize. The three novel genotypes showed no association with country (X^2=3.09, p=0.31), site (X^2=30.14, p=0.31), department (X^2=13.68, p=0.31), age (X^2=0.28, p=0.88), season (X^2=8.65, p=0.21), or year (X^2=1.48, p=0.59) but were associated with reproduction (X^2=11.32, p=0.03) and were marginally associated with sex (X^2=7.12, p=0.10). Non-reproductive bats showed greater infection with genotype 1 but less infection with genotype 2 than reproductive bats, while males tended to harbor more infection with genotype 1 and 2 than females (Fig. S1).

Risk factors for hemoplasma infection in vampire bats

All hemoplasma genotypes and vampire bat species were pooled for analyses of infection prevalence. The 95% confidence set of GLMMs contained 58/124 of the original models (Table S3), with variable importance as follows: reproductive status (95%), age (75%), season (65%), the interaction between season and reproductive status (48%), sex (44%), country (27%), year (18%), the interaction between season and sex (7%), the interaction between sex and reproductive status (7%), and the interaction between country and year (<1%). These models explained between 9% and 21% of the variation in hemoplasma infection (Table S3). The averaged odds ratios for the effect of being non-reproductive (OR=1.45, 95% CI=1.05–2.02) and being non-reproductive in the spring (OR=0.68, 95% CI=0.50–092) on infection was different than one (Fig. 4A), although the 95% confidence interval for subadults only just overlapped with one (OR=1.50, 95% CI=1.00–2.26; Fig. 4A). A weaker effect was observed for males (OR=1.21, 95% CI=0.85–1.66). Hemoplasma infection prevalence was thus greatest for non-reproductive bats, especially those sampled in the spring, and for subadult bats (Fig. 4B–C). The odds of hemoplasma infection did not vary across the two countries and two years (Fig. 4A). In a small sample of recaptured bats (n=6), we observed two bats move from infected to uninfected status within 367–371 days, while two individuals remained infected across 369–424 days (Fig. S2).

Comparison of Mycoplasma species detected in blood and saliva

Based on the saliva metagenomic data, we identified contigs closely matching to *Mycoplasma* species in all oral swab sample pools from Peru (Table S4). We consistently found long contigs (>1300 bp) with high coverage likely belonging to non-hemoplasma *Mycoplasma* species present in vampire bat saliva. Across all study regions, we also identified shorter contigs (400–500 bp)

317 with high sequence identity (98–100%) to the hemoplasma sequences of genotype 1 and 3 that
318 were detected in blood of *D. rotundus*. This suggests possible presence of similar hemoplasmas
319 in both vampire bat saliva and blood, although the shorter length of these contigs prohibited
320 conclusive identification.
321
322 **Discussion**
323 Our study describes novel and genetically diverse hemoplasmas in common vampire bats.
324 Infection prevalence was relatively high for this species (67%) compared with that from other
325 bats, with all three novel genotypes being geographically widespread. The odds of hemoplasma
326 infection were greatest for non-reproductive bats sampled in the spring and for subadult bats and
327 did not vary between 2015 and 2016 both within and across countries, suggesting individuals
328 important to transmission and endemic infection dynamics. Salivary metagenomics also showed
329 the presence of non-hemotropic *Mycoplasma* species and hemoplasma genotypes
330 phylogenetically similar to those identified in blood, providing indirect data for possible direct
331 transmission of hemoplasmas in vampire bats through biting or grooming.
332 The genus *Mycoplasma* currently comprises twenty hemoplasma species (NIH NCBI
333 Taxonomy). Except for *M. haemocanis*, *M. haemofelis*, and *M. haemomuris*, hemoplasmas have
334 the provisional taxonomic status "*Candidatus*" because they are uncultivated and as a result are
335 incompletely characterized bacterial species [56,75,76]. Here, PCR screening using the
336 previously published UNI_16S_mycF and UNI_16S_mycR universal primers [50] and
337 amplification of the full-length 16S rRNA gene using PCR primers designed in this study
338 illustrate these primers can be used for detection of multiple hemotropic *Mycoplasma* spp.
339 genotypes in vampire bats. This is also the first study in which non-hemotropic *Mycoplasma*

species were detected in vampire bats using metagenomics. *Mycoplasma* species are a part of the normal oral, intestinal, and genital microflora in many animals. We know of no published references on non-hemotropic *Mycoplasma* species in bats, with the exception of a high presence of *Mycoplasma* spp. 16S rRNA gene sequences detected in intestinal biopsy samples of *Cynopterus* spp. bats [18]. Based on our metagenomics data, we cannot infer the significance of finding non-hemotropic *Mycoplasma* in bat saliva on the health of these animals or potential inter-species transmission. More research is needed on the normal *Mycoplasma* microflora in bats.

Phylogenetic studies of the 16S rRNA gene of closely related *Mycoplasma* species (including hemoplasmas) propose to use the arbitrary interspecies sequence similarity value of ≤97% as a minimum level indicating a separate, genetically distant species [76,77]. Data based on the expanded analysis of the 16S rRNA gene sequences of the species within the family *Mycoplasmataceae* generally support this proposition [56]. All three vampire bat hemoplasma genotypes demonstrated low levels (i.e. <97%) of sequence identity to previously described genotypes (or hemoplasma species) detected in other animal species, which suggests that these vampire bat hemoplasmas are novel hemoplasma genotypes or putatively new hemoplasma species not yet described in other animals [56,57].

The vampire bat genotypes are paraphyletic to each other and appear to have common ancestry with hemoplasmas from other bats, rodents, humans, and non-human primates, suggesting that hemoplasmas have a history of host shifts between closely and distantly related species during evolution. Additionally, we observed no geographic clustering for genotypes 2 and 3, suggesting vampire bat hemoplasmas are broadly distributed across Latin America. However, for hemoplasma genotype 1, sequences from Belize and Peru had geography-specific

single nucleotide polymorphism (SNPs) and varied by 2.2% (20 SNPs of 871 nt analyzed sequence); these sequences fell into two country-specific groups (Fig. 2); this might imply more regionally constrained transmission cycles of this hemoplasma genotype.

Hemoplasma infection prevalence observed here in vampire bats (67%) was intermediate compared to that in other bat species. Hemoplasma prevalence in Schreibers' bats (*Miniopterus schreibersii*) and one long-eared bat (*Myotis capaccinii*) in Spain was 97% [17], while only 47% of little brown bats (*Myotis lucifugus*) from the eastern and northeastern United States and only 14% of velvety free-tailed bats (*Molossus molossus*) were infected with hemoplasmas [16,19]. However, the sensitivity of our PCR has not been quantified, so prevalence in vampire bats could conceivably be higher than we detected. Hemoplasmas have not been cultured *in vitro*, and their detection in many species has used PCR with or without analysis of Romanowsky–Giemsa and acridine orange–stained blood smears [20,57]. Prior work on bats has relied on PCR only but either with blood preserved in EDTA or with spleen, liver, or heart tissues [16,17,19]. We instead used blood preserved on Whatman FTA cards to facilitate room-temperature sample storage in remote, tropical field conditions. During primary PCR screening, we found only 30% of positive samples produced a strong band through gel electrophoresis; other positive samples produced average or weak bands and we were mostly unable to amplify the full-length 16S rRNA gene from such samples. Similar problems with amplification of hemoplasma-specific PCR products were recently identified in other bat species in Brazil [19]. Two possibilities for the high number of weak band samples in primary PCR are that hemoplasma concentrations in vampire bat blood are low or that the use of current sample collection or storage methods are inefficient for hemoplasma characterization in bats. We did not detect hemoplasmas in blood using light microscopy, though the sensitivity of this method is relatively low compared to PCR

386 [78]. Further, manual staining in field conditions often results in stain precipitate, which makes
387 definitive detection of hemoplasmas through microscopy difficult.
388 Within vampire bats, we found the odds of hemoplasma infection to be greatest for non-
389 reproductive bats sampled in the spring and for subadult bats. Overall higher prevalence in non-
390 reproductive bats is surprising, given that animals often down-regulate costly immune function
391 during reproductive events and are more susceptible to infection [79,80]. This pattern could
392 possibly reflect seasonal birth pulses and the influx of immunologically naïve bats [81,82],
393 which is corroborated by the OR for non-reproductive bats being greatest in the spring when
394 births peak in vampire bats [83]. Seasonal birth pulses could also explain the trend for prevalence
395 to be greater in subadult bats, though the marginally significant averaged effect of age is likely
396 due to controlling for other factors in the GLMMs. This trend is similar to findings on rabies
397 virus exposure, in which younger vampire bats also showed higher seroprevalence [84].
398 Subadults could also experience greater exposure to hemoplasmas if vectors are more attracted to
399 younger bats [85] or if vampire bat hemoplasmas are transmitted vertically [86]. Unlike with
400 feline and canine hemoplasmas [87,88], the odds of infection did not vary by sex, suggesting
401 sex-biased parasitism may not occur with vampire bat hemoplasmas despite males playing a key
402 role in the spatial dynamics of vampire bat rabies [46]. More extensive sampling of vampire bats
403 over time, alongside infection trials, is necessary to elucidate the transmission routes of these
404 hemoplasmas. We note that even our top GLMMs only explained up to 21% of the variation in
405 infection status (Table S3), which highlights the roles that coinfection with other pathogens [89],
406 differences in host physiology [87,90], or landscape factors such as food availability [57,91]
407 could also play in determining vampire bat susceptibility and exposure to hemoplasmas.

Hemoplasma infection prevalence did not differ between years, across countries, or by season. While more years of data are necessary to corroborate this result, these findings suggest hemoplasmas are endemic within vampire bat populations. Along with relatively high prevalence, this stable temporal trend corroborates other work suggesting bats to be reservoirs of hemoplasmas and potentially other bacterial infections [9,10,17]. The repeated sampling of a small number of recaptured bats in our study sheds further light on the infection dynamics of bat hemoplasmas. We observed two bats move from infected to uninfected within 117–123 days; this could again reflect hemoplasma DNA loads that were too low to be detected by our PCR but could also suggest vampire bats can clear hemoplasma infection. Future longitudinal sampling paired with mathematical models could help infer if vampire bats undergo cycles of latency and reactivation with hemoplasmas or obtain partial immunity from infection [92,93].

To conclude, this study identified novel hemoplasma genotypes in vampire bats that were phylogenetically related to hemoplasmas reported in other mammals, including bats, rodents, humans, and non-human primates. These hemoplasma sequences clustered into three novel genotypes, were most prevalent in young and non-reproductive bats, and were relatively stable in prevalence over time. Future studies should *(i)* explore the host range and specificity of hemoplasmas among bat species and *(ii)* evaluate the pathogenicity of hemoplasmas in vampire bats with hematological and immunological assays. Given the close association between these vampire bat genotypes and those from humans, rodents, and non-human primates, future studies should aim to elucidate the potential for pathogen exchange between vampire bats and sympatric wildlife, humans, and domestic animals. Our metagenomic data identifying *Mycoplasma* species and similar hemoplasma genotypes in vampire bat saliva suggest the possibility for acquisition of hemoplasmas from reservoir hosts or for direct transmission of hemoplasmas through biting

during aggressive encounters with conspecifics [94], blood sharing [95], and feeding on prey [38], but infection trials are needed to confirm this transmission route and its zoonotic potential.

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Conflict of interests

None.

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Figure legends

Figure 1. Location of vampire bat sampling sites in Latin America, with Belize and Peru shown in grey with red outlines (A). Insets show the location of field sites (white) and the prevalence of hemoplasmas per site (B and C) across study years (solid line=2015, dashed line=2016), with red denoting the proportion of infected bats. Points are scaled by sample size.

Figure 2. Dendrogram showing phylogenetic relationships based on nucleotide sequence data for the 16S rRNA gene among the hemoplasma genotypes detected in common vampire bats (*Desmodus rotundus*) with other hemotropic *Mycoplasma* spp. The tree was constructed using the minimum evolution method in MEGA 6. Accession numbers for sequences downloaded from GenBank are shown alongside individual bat ID and country of sampling. The *Desmodus rotundus* samples sequenced in this study are displayed in bold.

Figure 3. Location of vampire bat sampling sites in Latin America, with Belize and Peru shown in grey with red outlines (A). Insets show the location of field sites (white) and the composition of hemoplasma genotypes per site (B and C) across study years (solid line=2015, dashed line=2016). Points are scaled by sample size. Mm denotes the *M. moatsii*-like hemoplasma.

Figure 4. Averaged odds ratios and 95% confidence intervals for all variables within the 95% GLMM set, standardized by partial standard deviation (A). The dashed line shows where the odds ratio equals 1. Raw hemoplasma infection prevalence and 95% confidence intervals for bat reproductive status by sampling season (B) and age class (C).