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Diversity and seasonality of ectoparasite burden on two species of Madagascar fruit bat, *Eidolon dupreanum* and *Rousettus madagascariensis*

Angelo F. Andrianiaina[1*], Santino Andry[2], Gwenddolen Kettenburg[3], Hafaliana Christian Ranaivoson[1,3,4], Vincent Lacoste[4], Philippe Dussart[4], Jean-Michel Heraud[4], Theresa M. Laverty[5], Sarah Guth[6], Katherine I. Young[7], Aristide Andrianarimisa[1] and Cara E. Brook[3]

Abstract

Background Bats are important reservoir hosts for a variety of pathogens, some of which are transmitted by ectoparasite vectors including mites, fleas, lice, ticks, and bat flies (families Nycteribiidae and Streblidae). All these ectoparasite taxa are known to parasitize two endemic fruit bats of Madagascar, *Eidolon dupreanum* and *Rousettus madagascariensis*. We aimed to describe the diversity of ectoparasite infestation for both bat species through morphological observation and DNA barcoding and elucidate ecological and climatic correlates of seasonal nycteribiid parasitism of these hosts.

Methods *Eidolon dupreanum* and *R. madagascariensis* fruit bats were live-captured in northern and central-eastern Madagascar periodically from 2013 to 2020. Ectoparasites on all captured bats were counted and identified in the field and then collected into ethanol. Field identification of a subset of samples was confirmed via microscopy and DNA barcoding of the cytochrome C oxidase subunit 1 (*COI*) and *18S* genes. The seasonal abundance of nycteribiid bat flies on both host bats was analyzed using generalized additive models, and the role of climate in driving this seasonality was assessed via cross-correlation analysis combined with generalized linear models. Phylogenetic trees were generated to compare *COI* and *18S* sequences of Madagascar nycteribiid and streblid bat flies with available reference sequences from GenBank.

Results Ectoparasites corresponding to four broad taxa (mites, ticks, fleas, and bat flies) were recovered from 628 of 873 *E. dupreanum* (71.9%) and 831 of 862 *R. madagascariensis* (96.4%). *Eidolon dupreanum* were most commonly parasitized by *Cyclopodia dubia* nycteribiids and *R. madagascariensis* by *Eucampsipoda madagascariensis* nycteribiids and *Megastrebla wenzeli* streblids. We observed significant seasonality in nycteribiid abundance on both bat hosts, which varied by bat sex and was positively correlated with lagged temperature, precipitation, and humidity variables. Barcoding sequences recovered for all three bat fly species grouped with previously reported sequences, confirming morphological species identification. Our study contributes the first DNA barcodes of any kind reported for *M. wenzeli* and the first *18S* barcodes for *C. dubia*.

*Correspondence:
Angelo F. Andrianiaina
angelo.andrianiaina@ekipafanihy.org
Full list of author information is available at the end of the article

Conclusions This study explores the diversity and abundance of ectoparasite burdens in two Malagasy fruit bat species, highlighting the importance of seasonal ecology and the influence of climate variables on parasitism, which correlates with resource availability.

Keywords Bat fly, Bat ectoparasite, DNA barcoding, *Eidolon dupreanum*, Madagascar, Nycteribiidae, Pteropodidae, *Rousettus madagascariensis*, Streblidae

Background

Bats (order: Chiroptera) are reservoir hosts for highly virulent zoonotic viruses [1], which do not cause clinical pathology in these vertebrate hosts [2]. Bats are also known to host protozoa [3], bacteria [4], fungi [5, 6], and helminths [7], some of which cause significant pathology in bat hosts [5, 6, 8]. In addition, bats are parasitized by a diversity of hematophagous ectoparasites—including mites, fleas, lice, ticks, and bat flies (Order: Diptera; Superfamily: Hippoboscoidea), the most widely recognized bat ectoparasites. Bat flies are obligate pupiparous ectoparasites that comprise two families: the monophyletic and wingless Nycteribiidae, which are occasionally found in the New World but most commonly identified on Old World bats, and the paraphyletic, winged Streblidae, for which disparate New World and Old World clades are recognized, with higher diversity in the New World [9, 10]. Ectoparasites can impact the fitness of obligate bat hosts directly [11, 12] and can also play important indirect roles as pathogen vectors [13, 14]. Bat ectoparasites have been previously implicated in the transmission of *Polychromophilis* spp. [3, 15] and *Trypanosoma* spp. [16–19] protozoa, as well as *Bartonella* spp. [4, 20–28], *Rickettsia* spp. [20, 29, 30], and *Borrelia* spp. bacteria [29, 29–32].

Forty-nine bat species, including 38 endemics, reside in Madagascar, an isolated island nation off the southeastern coast of Africa [33]. Intensive biosurveillance of the island's bats over the past 2 decades has led to the discovery and characterization of numerous bat-borne viruses [34–44], protozoa [3, 45], bacteria [4, 46, 47], and helminths [48]—some of which demonstrate high divergence from taxa reported elsewhere, reflecting the island's longstanding phylogeographic isolation [36, 37, 49, 50], while others show high identity that suggests recent cross-continental genetic exchange [36, 38, 49, 50]. Parasitism of Malagasy bats by mites, fleas, ticks, and bat flies has been previously described [4, 51]. Most prior research on Madagascar's bat flies has focused on elucidating Nycteribiidae diversity [52–55], including through molecular characterization [48, 51]. At least nine species of nycteribiid bat fly infest Malagasy bats, including *Cyclopodia dubia* and *Eucampsipoda madagascariensis,* species-specific ectoparasites of, respectively, the endemic fruit bats, *Eidolon dupreanum* and *Rousettus madagascariensis* [4, 51, 56]. In addition, distinct *Basilia* sp. nycteribiids have been identified as species-specific ectoparasites of the endemic vesper bats, *Scotophilus robustus*, *S. marovaza*, and *Pipistrellus hesperidus*, as well as the pan-African emballonurid bat, *Taphozous mauritianus* [51, 56, 57]. By contrast, at least three nycteribiids (*Penicillidia* sp., *P. leptothrinax*, and *Nycteribia stylidopsis*) are known to parasitize multiple Malagasy bat species, including *Myotis goudoti* and at least eight *Miniopterus* spp. [51, 56]. Only a few early morphological studies describe parasitism of Malagasy bats by Streblidae bat flies: the streblid *Megastrebla wenzeli* has been identified as a species-specific ectoparasite of *R. madagascariensis* [58, 59], but to our knowledge, no molecular data for Malagasy streblids have yet been contributed to the literature.

In addition to systematics and taxonomy, several studies have described potential vector roles for bat flies in Madagascar. In one study, nested *Bartonella* spp. sequences were identified in *C. dubia* and their obligate *E. dupreanum* fruit bat hosts, suggesting a possible vectorial function for the bat flies [4]. Another study identified *Bartonella* spp. bacteria in *Basilia* sp., *P. leptothrinax*, and *N. stylidiopsis* nycteribiids without investigating the bat host [60]. Nested sequences of *Polychromophilus melanipherus* protozoa were also detected in both *P. leptothrinax* and *N. stylidiopsis* nycteribiids and their obligate bat hosts, *Miniopterus aelleni, M. manavi,* and *M. gleni*, again suggesting a vector role [3]. To our knowledge, experimental confirmation of true vector-pathogen relationships has not yet been carried out for any Malagasy bat fly.

More recent work has provided deeper insights into the ecology of parasite-host relationships for Malagasy bat flies parasitizing *R. madagascariensis* bats. One study in Ankarana National Park, northern Madagascar, showed higher rates of *E. madagascariensis* parasitism of *R. madagascariensis* male vs. female bats and a higher prevalence of parasitism during the Malagasy dry (September) vs. wet season (January) [61]; the sex ratios of *E. madagascariensis* also skewed towards males [61]. Within each season, the authors identified a significant positive correlation between bat body condition index (a proxy for bat health) and *E. madagascariensis* abundance, which they hypothesized might result from larger available surface

area on bigger bats to facilitate nycteribiid fixation [62]. Field studies in both northern [62] and central Madagascar [63] have shown that fruit bat body conditions improve during Madagascar's resource-abundant wet season (~December–April) compared to the resource-poor dry season. Other work has also documented *R. madagascariensis* consumption of both nycteribiid and streblid bat flies, a habit which provides a likely important protein source to these frugivorous bats in the dry season [64–66]. No prior work has identified any differences in parasitism intensity across host sex, age, or time of sampling for the less prevalent *M. wenzeli* streblid parasite of *R. madagascariensis* [61].

Here, we aimed to characterize ecological patterns of ectoparasite-host association for two species of cave-dwelling, endemic Malagasy fruit bats, *E. dupreanum* and *R. madagascariensis.* Using data from longitudinally monitored roost sites in northern and central Madagascar, we quantified seasonal variation in bat fly parasitism for these two bat host species and elucidated a possible role for climate in explaining this variability. Finally, we expanded prior molecular studies of Madagascar bat flies to include *M. wenzeli* streblid parasites of *R. madagascariensis*.

Methods

Bat sampling and ectoparasite collection

Endemic Malagasy fruit bats were captured at 6-week intervals at longitudinally monitored roost sites in central-eastern Madagascar (*Eidolon dupreanum*: Angavokely, Angavobe, Lakato caves; *Rousettus madagascariensis*: Maromizaha cave) and in Ankarana National Park in northern Madagascar between November 2013–March 2020 in part with ongoing studies characterizing seasonal viral dynamics in these bat populations (Additional file 2: Table S1) [34, 36–38, 63]. All field research was carried out in accordance with research permits obtained from the Madagascar Ministry of Forest and the Environment (permit nos. 251/13, 166/14, 75/15, 92/16, 259/16, 019/18, 170/18, and 007/19, 14/20) and under guidelines of the American Veterinary Medical Association. All field protocols employed were pre-approved by the Animal Care and Use Committees of Princeton University (2013–2016; IACUC Protocol #1926) or UC Berkeley (2017–2020; IACUC Protocol # AUP-2017-10-10393), and every effort was made to minimize discomfort to animals.

Bats were captured using mist nets hung at cave entrances at dusk and dawn. Upon capture, all bats were removed from nets and placed individually in clean, cloth bags for processing (all bags were washed prior to reuse on a new individual). During processing, bats were weighed (in g) using a Pesola scale, and forearm measurements (in mm) were collected with a caliper. All bats were visually examined for ectoparasites, and any observed ectoparasites were removed with forceps and counted in the field into broad taxonomic categories (ticks, mites, fleas, and bat flies in family Nycteribiidae or Streblidae). After counting, all ectoparasites collected from a single bat were stored collectively in a tube filled with 70% ethanol and labeled corresponding to the sample number of the host bat.

Morphological identification of ectoparasites

Following field studies, all ectoparasite samples collected from *E. dupreanum* and *R. madagascariensis* bats captured between February 2018 and November 2019 were examined under a standard light microscope (OMAX M8311) and subject to additional morphological assessment (hereafter, the 'morphological data subset'). Under the microscope, different ectoparasite species were sorted morphologically and recounted into broad taxonomic categories (ticks, mites, fleas, and Nycteribiidae or Streblidae bat flies) following previously published taxonomic guides. These included general guides for bat ectoparasites broadly [67], specific references for bat mites [68, 69], and specialized guides for Malagasy bat flies in both Nycteribiidae [53–55] and Streblidae [58, 59] families. Where possible, ectoparasites were further categorized by genus and species, and bat flies were grouped by male and female sex. Photographs were taken of all observed genera of any ectoparasite taxa.

Molecular identification of bat flies

Ectoparasite specimens from samples collected between February 2018 and November 2019 were exported to the University of Chicago for molecular identification. A random subset of bat flies corresponding to all three species observed during morphological study were selected for DNA extraction and barcoding (Additional file 2: Table S2). For all selected specimens, DNA was extracted using the Zymo Quick-DNA 96 Plus Kit, following the manufacturer's instructions and including the step for Proteinase K digestion. Following extraction, DNA quality was verified on a nanodrop, and 10 ng/µl of high-quality DNA samples (260/230 ratio = 2.0–2.2; 260/280 ratio = 1.7–2.0) was barcoded via amplification of the well-conserved cytochrome C oxidase subunit 1 gene (*COI*) (650 bps), using LCO1490 and HCO2198 primers that have been previously published [70] and previously applied in molecular studies of Malagasy bat flies [51, 56]. All polymerase chain reactions (PCR) were conducted in 25-µl reaction mixtures containing 12.5 µl GoTaq colorless master mix (Promega, Madison, WI), 8 µl deionized water, 0.25 µl of each primer, and 3 µl extracted DNA. The amplification profile was 95 °C for 2 min, followed by

35 cycles of 30 s at 95 °C, 30 s at 49 °C, and 30 s at 72 °C. A final extension step of 7 min at 72 °C was realized. Positive (*Drosophila melanogaster*) and negative (water) controls were included in all PCR runs. PCR products were separated by electrophoresis on 1% agarose gel, stained with SYBR Safe DNA gel stain (Invitrogen: S33102), and visualized under UV light. PCR products were purified and sequenced at the University of Chicago genomics core using both forward and reverse primers.

Following sequencing and phylogenetic analysis, we subsequently elected to additionally amplify the *18S* gene of a subset of representative samples of each of the three bat fly species to compare against available reference sequences. Here, we used previously published 1.2F and 7R PCR primers to target a 1600 bp region of the *18S* gene of extracted DNA samples [51, 71, 72] in a conventional (one-step) PCR protocol. All *18S* PCRs were conducted in 25-µl reaction mixtures following the same proportions as used for *COI* amplification, with the following amplification profile: 95 °C for 4 min, followed by 40 cycles of 40 s at 95 °C, 30 s at 57 °C, and 60 s at 72 °C. A final extension step of 10 min at 72 °C was realized. As before, positive (*Drosophila melanogaster*) and negative (water) controls were included in all PCR runs. PCR products were separated by electrophoresis, purified, and then sequenced at the University of Chicago genomics core following the same protocol used for *COI* barcoding.

Following barcoding, recovered sequences were manually curated in Geneious Prime 2022.1.1 (www.geneious.com): the 5′ and 3′ ends of all forward and reverse sequences were trimmed, low-support nucleotide calls were eliminated, and a consensus sequence was generated from paired forward and reverse sequences for all samples. Following cleaning, sequences were submitted to the Barcode of Life Database (BOLD) [73] and then subsequently uploaded to NCBI GenBank.

Climate data

Meteorological data used in these analyses were downloaded from NASA Earthdata Program using the Giovanni tool (https://giovanni.gsfc.nasa.gov/giovanni/) in raster format. We downloaded monthly temperature (°C), precipitation (mm), and diurnal humidity (% relative humidity, RH) data for all of Madagascar from January 2013 to December 2019 (Additional file 2: Table S1). Monthly averages per year were calculated in R (v4.4.1) [74] for a 30-km buffer surrounding the Angavokely and Maromizaha roost sites (respectively for *E. dupreanum* and *R. madagascariensis*), for which we evaluated seasonal patterns in parasitism. The 30-km buffer was chosen as an appropriate spatial resolution to account for variation in the resolution of the different meteorological datasets as well as to encompass the average distance a bat may travel in a typical foraging night.

Data analysis

All analysis was conducted in R. All data and code can be accessed freely through our open-access Github repository: https://github.com/brooklabteam/Mada-Ectoparasites.

Host-ectoparasite associations and correlations with field studies

Using the 'bipartite' package in R [75], we first constructed an alluvial plot to group host-ectoparasite relationships from the morphological data subset by bat species and associated ectoparasites categorized into class, order, superorder, family, and genus.

Next, to evaluate the accuracy of our parasitological classifications in the field and evaluate whether field estimates of ectoparasite counts by taxonomic group across our entire 2013–2020 time series could be used to test ecological hypotheses, we compared morphological counts under the microscope of nycteribiid bat flies (*C. dubia* for *E. dupreanum* hosts and *E. madagascariensis* for *R. madagascariensis* hosts) against raw field counts of the same species. To this end, we used a simple linear regression to test the strength of association between nycteribiid count via microscopy in the laboratory and nycteribiid count in the field using the morphological data subset which reported both metrics. Because we only began reliably recognizing and recording counts of *M. wenzeli* several years into our time series, we did not attempt to compare field and laboratory counts of streblid parasites of *R. madagascariensis* but instead limited ecological analyses to nycteribiid bat flies only.

Correlates of seasonal nycteribiid abundance using Generalized Additive Models (GAMs)

Because our comparison of field- and laboratory-derived nycteribiid counts suggested that we accurately estimated ectoparasite burden in the field (see "Results"), we carried out all subsequent analysis of seasonal patterns in parasitism using the field-derived dataset, which spanned from August 2013–March 2020. Seasonal analyses reported in the main text were restricted to the subset of our data collected in central-eastern Madagascar (*E. dupreanum* roosts: Angavobe and Angavokely; *R. madagascariensis* roost: Maromizaha), where *E. dupreanum* were sampled in 11/12 months of the year (missing May only) and *R. madagascariensis* were sampled in all months of the year. We also report seasonal analyses for our northern Madagascar site (Ankarana National Park) in the Supplementary Materials, with the caveat that the temporality of these data are more limited: *E. dupreanum* were sampled

in March–April and August–November in this site and *R. madagascariensis* in March and August–November only. For both study regions, we further restricted seasonal analyses only to adult bats to allow for comparison of the impact of sex and body condition on nycteribiid bat fly abundance within each bat host species.

We used the 'mgcv' package in R [76] to construct generalized additive models (GAMs) aimed at identifying ecological correlates of the response variable of the abundance of nycteribiid ectoparasites infesting our two bat host species separately for our two study regions. We first fit a series of Poisson GAMs to our data, evaluating the correlation of a suite of diverse predictor variables against the response variable of nycteribiid count separately for *E. dupreanum* and *R. madagascariensis*. We tested the hypothesis that ectoparasite abundance varied seasonally by allowing for a smoothing spline predictor of 'day of year' and a random effect of 'sampling year' in each GAM. We tested hypotheses that allowed for host sex-specific differences in the seasonality of parasitism (incorporating 'bat sex' in the smoothing spline 'by' term) vs. a composite seasonality across the two sexes. We also compared models which additionally incorporated random effect smoothing splines for the categorical variable of host bat sex and thinplate smoothing splines for mass: forearm residual (MFR), a measure of host body condition that we have previously shown to vary seasonally in these populations, tracking resource availability [63]. We calculated MFR as the residual of the regression of $\log_{10}$ mass (in g) per $\log_{10}$ forearm length (in mm) for each separate sex (male vs. female) and species (*E. dupreanum* vs. *R. madagascariensis*) subset of our data. For all central-eastern analyses, we fixed the seasonal smoothing knots ('k') at 7 as recommended by the package author [76]; for northern Madagascar data, we limited smoothing knots to 4 due to more limited seasonality in the data. In all cases, we modeled 'day of year' as a cyclic cubic spline to force continuity from the end of 1 year to the beginning of the next. We compared all GAM formulations by Akaike Information Criteria (AIC) to determine the best fit to the data.

For the central-eastern study region, we additionally reran our GAM analysis on the morphological data subset, this time including the additional categorical predictor of bat fly sex, which we recorded during microscopy. As with the complete field datasets, we compared model fits by AIC and plotted significant predictor variables for both bat species and sex combinations. Too few individuals were morphologically evaluated from our northern Madagascar field site to allow for similar analysis for this region. Additionally, using the morphological data subset we carried out a two-sided Student's t-test comparing the mean abundance of male vs. female nycteribiids observed on *E. dupreanum* and *R. madagascariensis* bats for the two localities (central-eastern and northern) surveyed.

Cross-correlation analysis of nycteribiid association with climate

Because our GAMs indicated significant seasonality in nycteribiid abundance across our time series (see "Results"), we next evaluated the role of climate in driving this seasonal variation. To this end, we carried out cross-correlation analysis in the R package 'sour' [77] to calculate the optimal lag between the mean nycteribiid bat fly (*C. dubia* for *E. dupreanum* and *E. madagascariensis* for *R. madagascariensis*) count per bat per month from 2013–2019 and the monthly average of our three climate variables (mean monthly diurnal humidity, mean monthly precipitation rate, and mean monthly temperature) for the corresponding locality (Angavokely cave for *E. dupreanum* and Maromizaha cave *R. madagascariensis*) across the same timespan. We considered monthly time lags up to 1 year by which climate variables preceded ectoparasite burden. Because our GAM analyses indicated significant seasonal deviations by host bat sex in ectoparasite burden (see "Results"), we calculated optimal lags to disparate ectoparasite burden time series for male and female host bats for the two species. Additionally, for visualization purposes, we summarized monthly averages across the entire study period for all three climate variables and for bat fly abundance for both bat hosts.

Climate correlates of nycteribiid abundance using Generalized Linear Models (GLMs)

Following cross-correlation analysis, we next constructed a composite dataset that included the three optimally lagged climate variables alongside the corresponding ectoparasite burden for each bat species and sex. Then, we compared a series of Poisson family generalized linear models (GLMs) to evaluate linear predictors of nycteribiid bat fly count separately for *E. dupreanum* and *R. madagascariensis*. In addition to the three climate variables, models included predictor variables of bat sex and MFR. We compared model fits by AIC and reported the incidence rate ratio of all significant correlates in the top-performing model for each species.

Phylogenetic analysis

Finally, using data generated from DNA barcoding, we constructed one *COI* and one *18S* maximum likelihood (ML) phylogenetic tree comparing Madagascar bat fly (nycteribiid and streblid) DNA sequences with available reference sequences downloaded from NCBI and reported in previous studies [9, 51, 78, 79]. We rooted both phylogenies with *Drosophila melanogaster*; see

Additional file 2: Table S3 for NCBI accession numbers for all sequences (both new and reference) included in our phylogenetic analyses. For both *COI* and *18S* phylogenies, we aligned sequences using the default parameters in MAFFT v7 [80, 81] and checked alignments manually for quality control in Geneious Prime. We carried out all subsequent phylogenetic analyses on both a trimmed alignment of the conserved region of each gene (*COI*: 336 bp; *18S*: 391 bp) and an untrimmed version. As results were comparable across the two methodologies, we reported results of only the untrimmed alignments here. All sequence subsets and alignment files (including trimmed versions) are available for public access in our GitHub repository: https://github.com/brooklabteam/Mada-Ectoparasites.

Following quality control, alignments were sent to Modeltest-NG [82] to assess the best fit nucleotide substitution model appropriate for our data. Both alignments (*COI* and *18S*) were subsequently sent to RAxML-NG to construct the corresponding phylogenetic trees [83] using the best-fit nucleotide substitution model as estimated by Modeltest-NG [82]. Following best practices outlined in the RAxML-NG manual, 20 ML inferences were made on each original alignment, and bootstrap replicate trees were inferred using Felsenstein's method [84], with the MRE-based bootstopping test applied after every 50 replicates [85]. Bootstrapping was terminated once diagnostic statistics dropped below the threshold value, and support values were drawn on the best-scoring tree. We plotted the resulting phylogenetic trees using the 'ggtree' package in R [86].

Results

Bat fly detection and host-parasite associations

From 2013–2020, we captured 873 *E. dupreanum* bats (408 male, 465 female) and 862 *R. madagascariensis* bats (457 male, 405 female), which we surveyed for ectoparasites (nycterbiid and streblid bat flies, fleas, mites, ticks) (Additional file 2: Table S1). Among those captured bats, we successfully counted, identified, and collected ectoparasites from 628 *E. dupreanum* (290 male, 338 female) and 831 *R. madagascariensis* (438 male, 393 female). Ectoparasites from bats captured between February 2018 and November 2019 were subject to additional microscopy (363 *E. dupreanum* and 477 *R.* madagascariensis, which comprised the 'morphological data subset'), from which we identified 264 (72.7%) *E. dupreanum* and 462 (97.2%) *R. madagascariensis* that hosted bat flies (family: Nycteribiidae or Streblidae); 114 (31.4%) *E. dupreanum* and 2 (< 0.5%) *R. madagascariensis* that hosted fleas, 279 (78.9%) *E. dupreanum* and 366 (76.7%) *R. madagascariensis* that hosted mites, and 83 (22.9%) *E. dupreanum* and 35 (7.3%) *R. madagascariensis* that hosted ticks (Fig. 1; Additional file 1: Fig. S1). Simultaneous parasitism by multiple ectoparasite taxa was common on any individual bat: *E. dupreanum* were simultaneously parasitized by a mean 1.44 [95% confidence interval (CI) 1.36–1.51] different ectoparasite taxa (nycterbiids, streblids, fleas, mites, or ticks), while *R. madagascariensis* were parasitized by a mean 2.11 [95% CI 2.05–2.17] ectoparasite taxa.

One species of nycteribiid bat fly, *C. dubia*, was identified on *E. dupreanum* bats. Both the nycteribiid *E.*

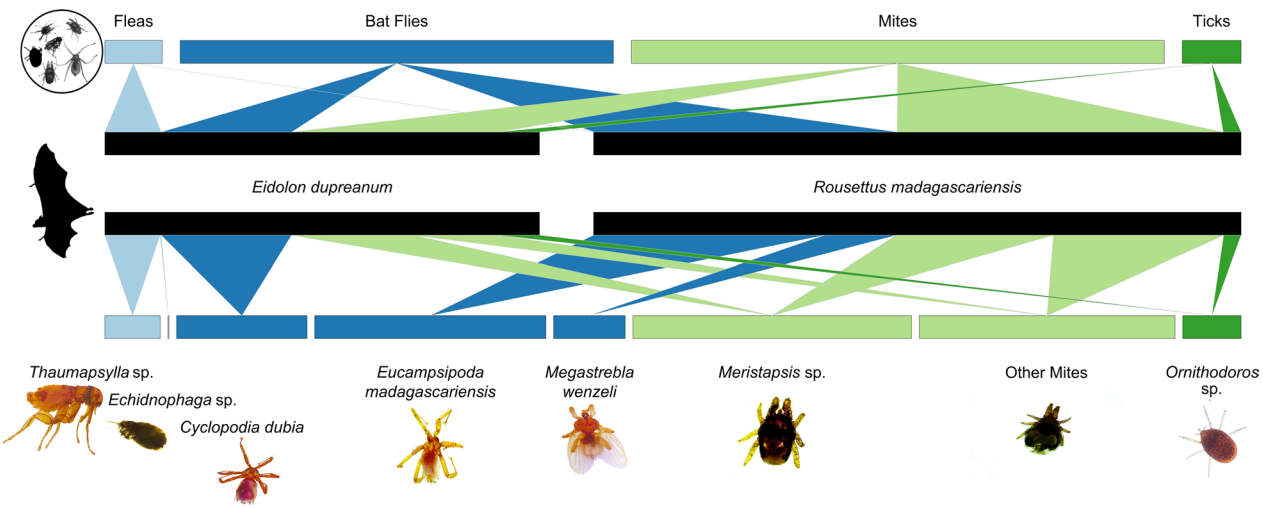

Fig. 1 Alluvial plot showing bat host species (center) associations with broad ectoparasite clades (top) and genus-species classifications (bottom). Fleas and bat flies in order Diptera are colored blue, while mites and ticks in class Arachnida (respectively, superorder Acariformes and Parasitiformes) are colored green. Images taken under the microscope at 40 × magnification are shown below the names of corresponding species

madagascariensis and the streblid *M. wenzeli* were identified on *R. madagascariensis* bats. Parasitism of *R. madagascariensis* by *E. madagascariensis* was more frequent and occurred at higher abundance than did parasitism by *M. wenzeli* (Fig. 1). When observed, *M. wenzeli* parasitism almost always co-occurred with *E. madagascariensis* parasitism: 96.9% (123/127) of the *R. madagascariensis* observed to be parasitized by *M. wenzeli* were also parasitized by *E. madagascariensis*. Most bat fleas infesting *E. dupreanum* were *Thaumapsylla* sp. previously reported on this host [4], while a few *Echidnophaga* sp. were also observed. Two fleas that keyed to family *Ischnopsyllidae* were also observed on two *R. madagascariensis* bats. Most mites parasitizing either bat host species belonged to the genus *Meristapsis*, though non-*Meristapsis* mites were also observed [68, 69]. Ticks observed on both bat species keyed to the genus *Ornithorodos* in the soft-bodied tick family Argasidae (Fig. 1) [67]. Downstream molecular assay is needed to confirm genus- and species-level identifications of flea, mite, and tick ectoparasites.

A linear regression comparing the laboratory recount of *C. dubia* on *E. dupreanum* and *E. madagascariensis* on *R. madagascariensis* against the raw field count observations demonstrated a highly significant positive correlation in both cases (Additional file 1: Fig. S2; *C. dubia:* $r=0.92$, $P<0.001$; *E. madagascariensis:* $r=0.91$; $P<0.001$), indicating that our field counts could be used representatively to explore broad seasonal patterns in our dataset.

Correlates of seasonal nycteribiid abundance from GAMs

For both *E. dupreanum* and *R. madagascariensis* bat hosts in central-eastern Madagascar, the top-performing GAM to recover seasonal nycteribiid abundance included a cyclic smoothing spline predictor by 'day of year' with a 'by' term of 'bat sex' allowing for disparate seasonal trends of parasitism for male vs. female bat hosts (Fig. 2; Additional file 2: Table S4). For both host species, the top-performing model included a predictor of MFR, though this variable was only significant in *R. madagascariensis* models (Fig. 2B, D). The abundance of *C. dubia* on *E. dupreanum* peaked in ~late May/early June for female bats (preceding the onset of the gestation period) and late June for male bats (at the onset of the nutritionally scarce dry season). The abundance of *E. madagascariensis* on *R. madagascariensis* peaked in late February/early March for females (~5 months preceding gestation) and March for males during the resource-abundant wet season. Despite improving overall model fit, MFR showed no significant variation with bat fly abundance for *E. dupreanum* (Fig. 2B). For *R madagascariensis*, extremely low MFR values were associated with lower bat fly burden, and high MFR values were associated with slightly elevated bat fly burden (Fig. 2D).

GAMs demonstrated similar results when refit to the February 2018–November 2019 morphological data subset (Additional file 1: Fig. S3; Additional file 2: Table S4). Inclusion of additional categorical predictors of bat host sex and bat fly sex improved model performance against the morphological data subset, but partial effects for these predictors were not significant. For GAMs fit to the morphological data subset, MFR had a significant effect on bat fly count for both *E. dupreanum* and *R. madagascariensis,* recapitulating trends from the full field dataset (Fig. 2B, D). For *E. dupreanum* hosts, low MFR was associated with high bat fly burden and high MFR with lower bat fly burden (Additional file 1: Fig. S3B); patterns were reversed for *R. madagascariensis* hosts, where low MFR was again associated with low bat fly burden, and high MFR was associated with higher bat fly burden (Additional file 1: Fig. S3D).

Student's t-test demonstrated no significant difference in the average count of male vs. female *C. dubia* recovered on *E. dupreanum* hosts ($P=0.015$) or male vs. female *E. madagascariensis* recovered on *R. madagascariensis* hosts ($P=0.07$) in the central-eastern data, where sampling was representative across the entire calendar year (Additional file 1: Fig. S4). We did observe a significantly higher mean count of male vs. female nycteribiids for both *E. dupreanum* and *R. madagascariensis* hosts in the morphological data subset from northern Madagascar ($P<0.001$ in both cases), where sampling was restricted to just the dry season months of the year (Additional file 1: Fig. S4).

For the northern Madagascar field-derived dataset, the best-fit model for both *E. dupreanum* and *R. madagascariensis* hosts included predictor variables of host sex-specific seasonal smoothing splines, in addition to MFR and a random effect of host bat sex (Additional file 1: Fig. S5; Additional file 2: Table S5). Though the seasonal duration of these data was more limited, we estimated a slightly later peak in nycteribiid burden compared with central-eastern study sites, with highest abundance in ~late August/early September for female *E. dupreanum* and September for males and in ~late September/early October for female *R. madagascariensis* and October for males (Additional file 1: Fig. S5). The limited sampling window of our data does not preclude the possibility of a second peak in bat fly abundance early in the calendar year. Models fit to data from northern Madagascar recapitulated patterns observed in central-eastern Madagascar for ectoparasite relation to MFR: no significant correlations were observed for *E. dupreanum,* though patterns trended to higher bat fly load in low MFR individuals (Additional file 1: Fig. S5B). Significant trends

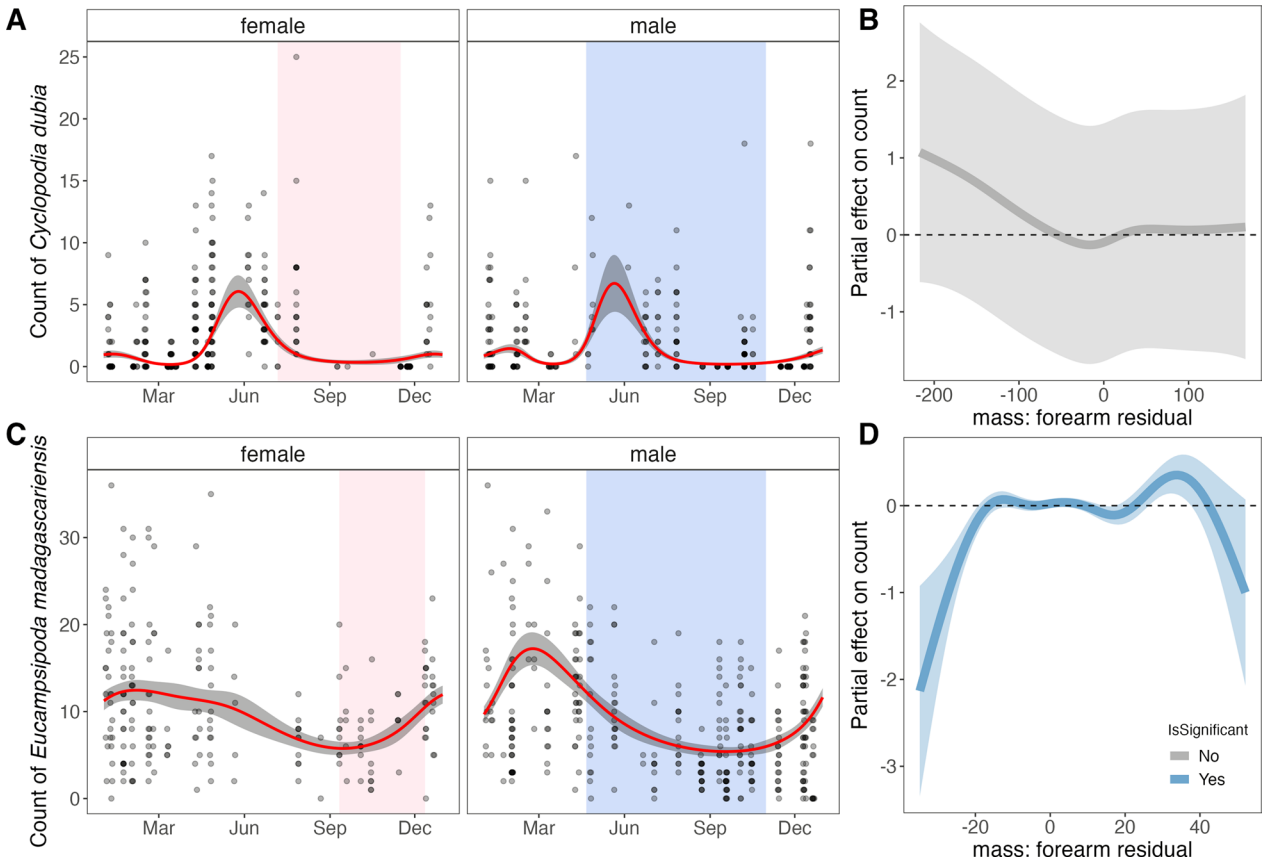

Fig. 2 Seasonal variation in the abundance of Nycteribiidae bat flies counted on **A**, **B** *Eidolon dupreanum* and **C**, **D** *Rousettus madagascariensis* bats captured at roost sites in central-eastern Madagascar (respectively, Angavobe/Angavokely and Maromizaha caves). **A** and **C** Seasonal ectoparasite count predictions (red line) from best-fit GAMs for male and female bats, with 95% CI by standard error shaded in gray. Background points in black show raw data from 2013–2020. Background shading in pink indicates gestation period for each species from [63], and shading in blue indicates the nutritionally deficient dry season for the region. **B** and **D** Partial effect (y-axis) of bat host mass: forearm residual (x-axis) on bat fly count, respectively, for *E. dupreanum* and *R. madagascariensis*. Solid lines (gray = non-significant; blue = significant effects) show mean effects, with 95% CIs by standard error in translucent shading

were observed for *R. madagascariensis,* again showing the opposite pattern, with lower bat fly burden in bats with the lowest MFR (Additional file 1: Fig. S5E). Though inclusion of bat sex improved model fits to this northern Madagascar data subset, no significant partial effects by sex were observed (Additional file 1: Fig. S5C, F).

Cross-correlation analysis of nycteribiid association with climate

Because GAM analyses indicated significant host sex-specific seasonality in bat fly burden for both bat species, we next investigated the correlation between site-specific climate variables and seasonal variation in nycteribiid bat fly count for *E. dupreanum* and *R. madagascariensis.* We first plotted the monthly average of three key climate variables (daily humidity, precipitation rate, and temperature) for each roost site (Angavokely and Maromizaha caves) compared to the monthly average bat fly count per bat for the two species (Fig. 3). We observed a substantial lag between monthly peaks in precipitation and temperature climate variables and the corresponding peak in ectoparasite abundance for both localities studied. We next quantified these lags using cross-correlation analysis of the monthly average for each climate variable per year from 2013–2019 compared to the time series of bat fly abundance on male and female bats of both species (Additional file 1: Fig. S6; Additional file 2: Table S5A). For *E. dupreanum,* the cross correlation between climate variable and bat fly abundance was maximized at no lag for humidity and abundance on male bats and a 5-month lag for females; at a 4-month lag for precipitation and abundance on male bats and a 5-month lag for females; and at a 3-month lag for temperature and abundance on male bats and a 6-month lag for females (Additional file 1: Fig. S6; Additional file 2: Table S5). For *R. madagascariensis*, the cross correlation between climate and bat

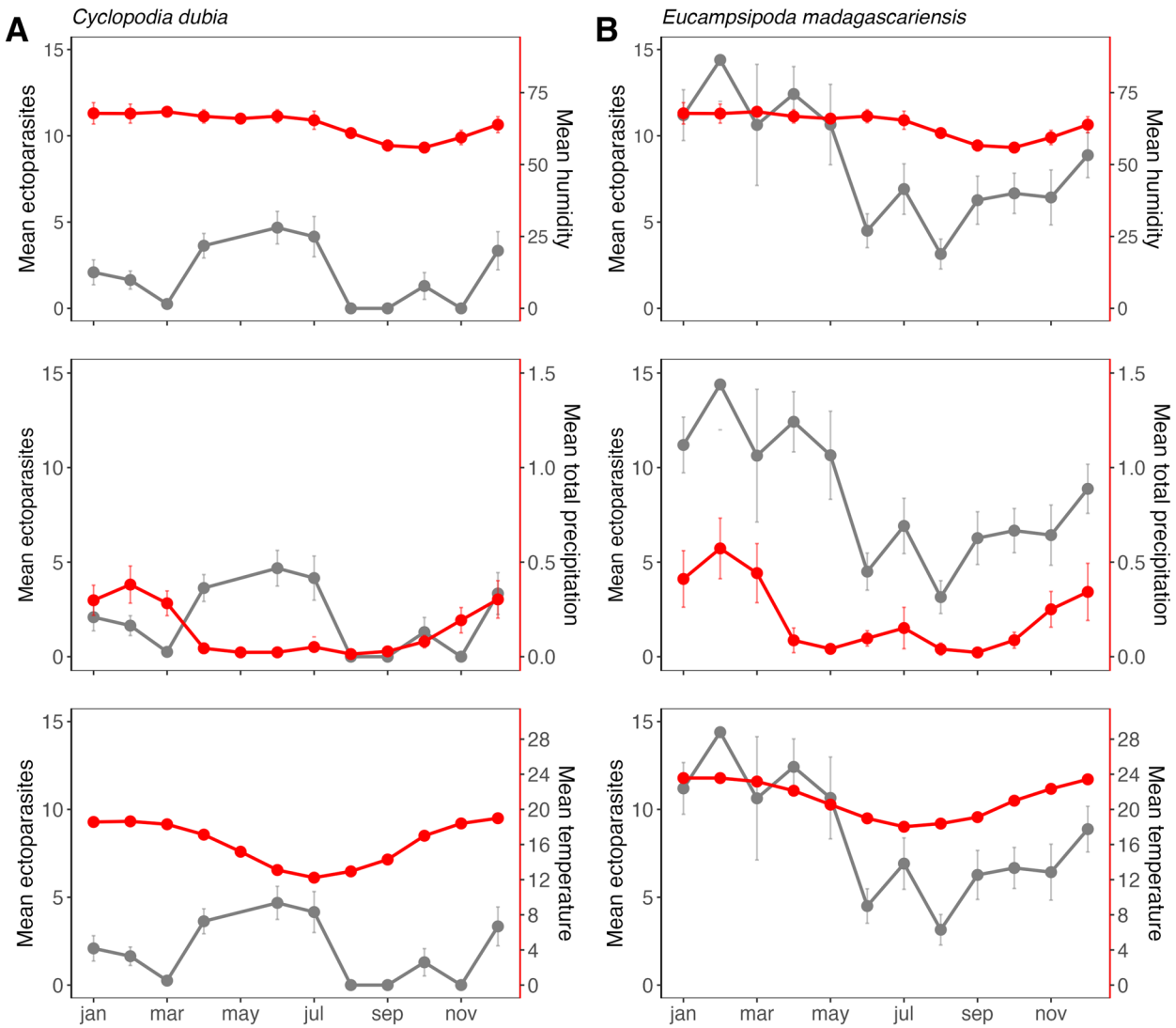

Fig. 3 Mean monthly nycteribiid count per bat for **A** *Cyclopodia dubia* parasitizing *Eidolon dupreanum*, from 2013–2020 for Angavokely roost (gray lines and points; left y-axis), compared with monthly averages for different climate variables in the region (horizontal panels; red lines and points; right y-axis): humidity (% relative humidity), total precipitation (mm), and temperature (°C). **B** *Eucampsipoda madagascariensis* parasitizing *Rousettus madagascariensis*, mirroring structure from **A**, for Maromizaha roost; 95% CIs by standard error are shown for both ectoparasite and climate data

fly time series for both male and female bats was maximized at no lag for humidity and at 1 month for both precipitation and temperature time series (Additional file 1: Fig. S6B).

Climate correlates of nycteribiid abundance from GLMs

Using the optimally lagged climate time series, we next identified linear predictors of nycteribiid bat fly burden across our field-derived dataset for both bat hosts (Fig. 4). For *C. dubia* abundance on *E. dupreanum*, the best-fit GLM included all predictor variables tested: all three climate variables (optimally lagged by bat host sex), in addition to bat sex and MFR (Fig. 4A). Lagged precipitation and temperature were the most influential variables contributing to overall model performance (Fig. 4A). All three climate variables were positively correlated with bat fly burden, while male bat sex was negatively correlated with bat fly abundance, and the effect of MFR was not significant (Fig. 4B). For *E. madagascariensis* abundance on *R. madagascariensis*, the best-fit GLM included all predictor variables tested except for MFR (Fig. 4C). Here, lagged temperature was the most influential variable contributing to overall model performance (Fig. 4C). As with the *E. dupreanum* model, all climate variables in

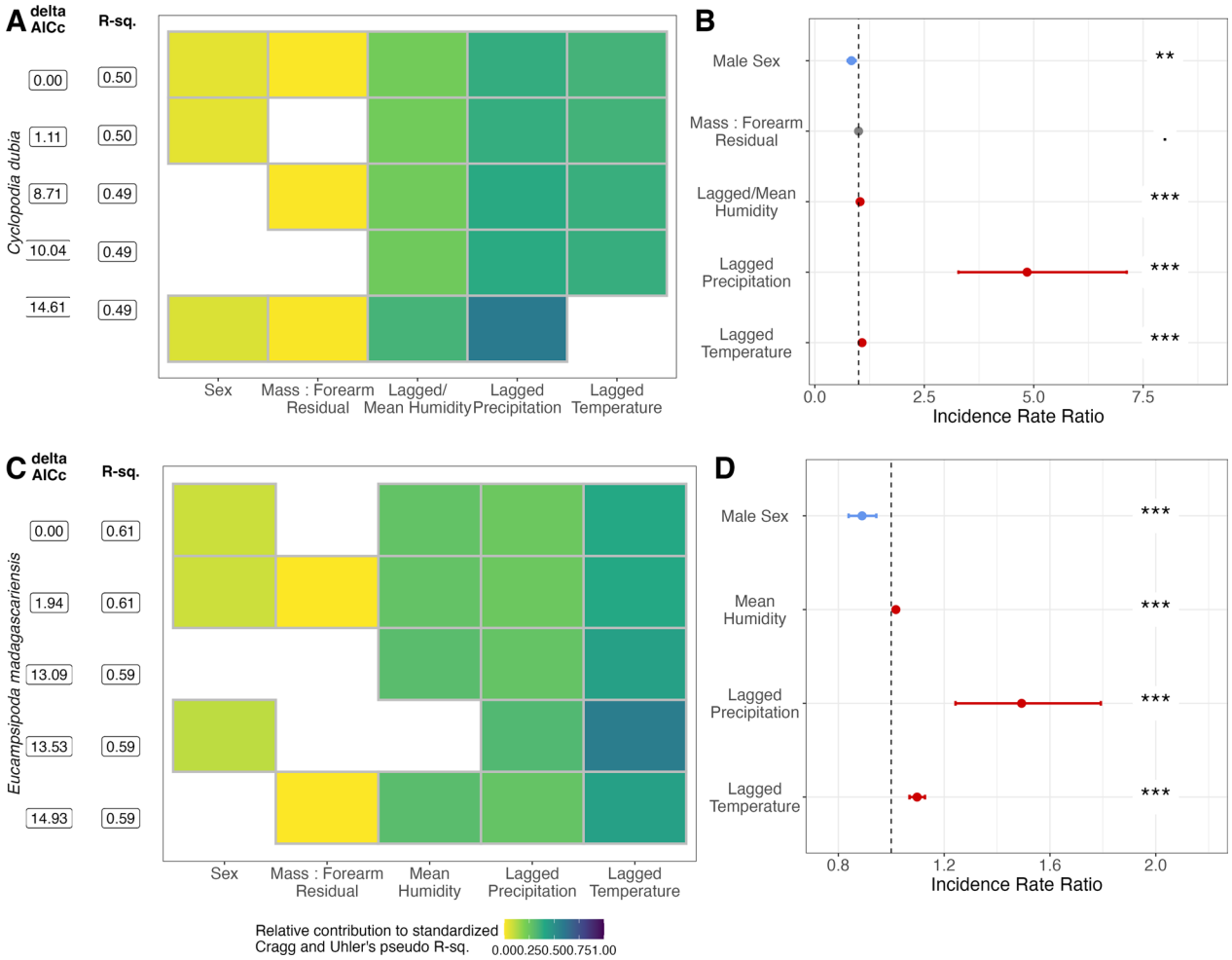

Fig. 4 Association of climate and demography with abundance of nyteribiid bat flies for **A**, **B** *Cyclopodia dubia* on *Eidolon dupreanum* and **C**, **D** *Eucampsipoda madagascariensis* on *Rousettus madagascariensis*. **A**, **C** Top five GLMs using optimally lagged climate variables to predict bat fly abundance, ranked by δAICc. Rows represent individual models and columns represent predictor variables. **B**, **D** Incidence rate ratios of linear predictors from top-fit models indicated in **A**, **C**. Significant positive correlates are colored red, significant negative correlates blue, and insignificant correlates gray; 95% CIs by standard error are shown as horizontal error bars

the *R. madagascariensis* model were positively correlated with bat fly burden, and male bat sex was negatively associated with bat fly count (Fig. 4D).

Phylogenetic inference

Nycteribiid and streblid sequences from bat flies of Malagasy *E. dupreanum* and *R. madagascariensis* recovered from *COI* and *18S* DNA barcoding were deposited to GenBank under accession numbers listed in Additional file 2: Table S2 (43 *COI* and 12 *18S* sequences) and then aligned with available reference sequences for phylogenetic analysis (Additional file 2: Table S3). Modeltest-NG [82] identified the best-fit nucleotide substitution model as GTR+I+G4 for the *COI* phylogeny and TIM2+I+G4 for the *18S* phylogeny. Correspondingly, RAxML-NG [83] recovered similar topologies for both ML phylogenies (Fig. 5; Additional file 1: Figs. S7, S8). *COI* sequences recovered from *C. dubia* parasitizing *E. dupreanum* clustered with previously-published sequences from this same species in a monophyletic clade with other *Cyclopodia* spp. identified from other Pteropodidae fruit bat hosts (Fig. 5; Additional file 1: Fig. S7); our *18S C. dubia* sequences represent the first *Cyclopodia* spp. contributions for this gene to GenBank (Additional file 1: Fig. S7). Likewise, both *COI* and *18S* sequences recovered from *E. madagascariensis* parasitizing *R. madagascariensis* clustered with previously published sequences from this species. The *Eucampsipoda* spp., including *E. madagascariensis*, formed a different monophyletic clade within the Nycteribiidae family, with each disparate parasite

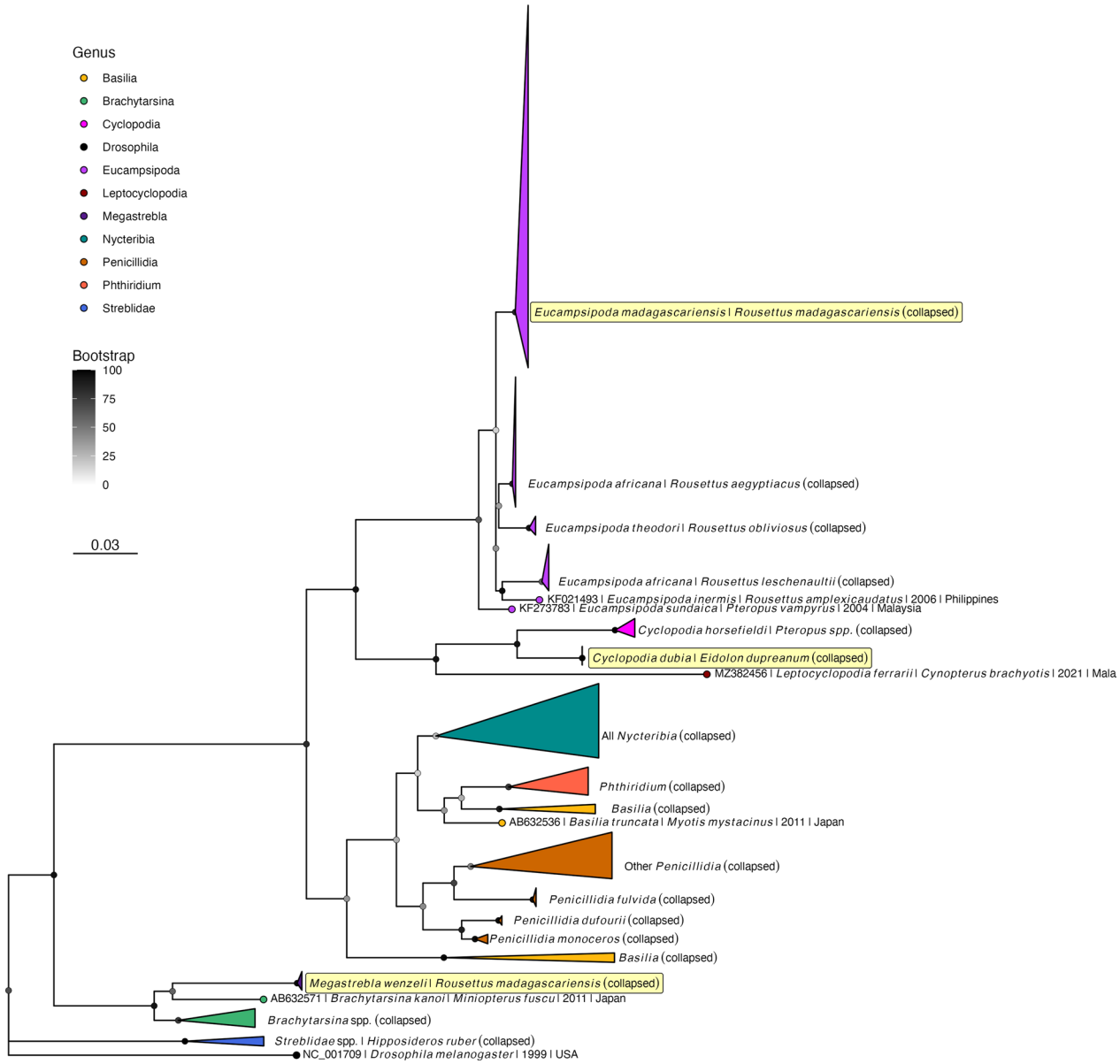

Fig. 5 Maximum likelihood phylogeny of *COI* ectoparasite sequences from untrimmed alignment (RAxML-NG, GTR + I + G4) [83]. Bootstrap support values computed using Felsenstein's method [84] are shown as shaded circles on each node, corresponding to legend. Sequences are collapsed into single species or genus clades for visualization; see Additional file 1: Fig. S7 for full phylogeny with individual sequences labeled. Tip shapes are colored by genera. Tip labels for the three Madagascar clades (*Eucampsipoda madagascariensis, Cyclopodia dubia, Megastrebla wenzeli*) are highlighted in yellow. Tree is rooted in *Drosophila melanogaster,* accession number NC_001709. Branch lengths are scaled by nucleotide substitutions per site, corresponding to scalebar

species resolving into disparate subclades associated with a unique pteropodid fruit bat host species (Fig. 5; Additional file 1: Figs. S7, S8).

Both *COI* and *18S* sequences recovered from *M. wenzeli* parasitizing *R. madagascariensis* represent the first molecular record of this streblid ectoparasite available on GenBank (Fig. 5; Additional file 1: Figs. S7, S8). While to our knowledge no *COI* reference sequences are currently available for the *Megastrebla* genus, our *18S* sequences from *M. wenzeli* clustered within a monophyletic clade of previously reported *Megastrebla* spp. sequences recovered from parasites of other pteropodid fruit bats (Additional file 1: Fig. S8) [9].

Discussion

We report diversity and seasonality in ectoparasite infestation of two species of endemic Malagasy fruit bat, *E. dupreanum* and *R. madagascariensis*. Both bat species were frequently infested with several ectoparasites, most commonly Nycteribiidae family bat flies: *C. dubia* for *E. dupreanum* and *E. madagascariensis* for *R. madagascariensis*, consistent with previously published work [51, 53–56]. In addition, we report the first molecular records documenting parasitism of *E. dupreanum* bats by the streblid ectoparasite, *M. wenzeli*; sequences recovered from our study place this parasite in a monophyletic clade of Old World streblids including previously reported sequences for *M. nigriceps* and *M. parvior*, streblid bat flies collected from *Eonycteris spelaea* fruit bats in Malaysia [9]. Our morphological observations of *M. wenzeli* are consistent with prior records describing this ectoparasite in Madagascar [58, 59, 61]. In addition to bat flies, we reconfirmed previous reports of *E. dupreanum* parasitism by *Thaumapsylla* sp. fleas [4] as well as reports of both *E. dupreanum* and *R. madagascariensis* parasitism by mites and ticks [4, 51]. Additional molecular studies are needed to confirm species-level identity of fruit bat ectoparasites beyond bat flies in the superfamily Hippoboscoidea.

The bulk of our analyses centered on understanding seasonal variation in nycteribiid parasitism of the two fruit bat hosts. Previous work corresponding to this theme has been published for *E. madagascariensis* parasitism of *R. madagascariensis* in northern Madagascar (Ankarana National Park) [61, 62]. To our knowledge, our study is the first to document seasonal patterns of parasitism for *C. dubia* on *E. dupreanum* as well as the first to document these patterns for either nycteribiid in central-eastern Madagascar (Districts of Manjakandriana and Moramanga), which has a cooler climate profile than the north. In general, our seasonal analyses in northern Madagascar mirrored those previously reported for *E. madagascariensis* parasitism of *R. madagascariensis* [61, 62]: we observed highest abundance of ectoparasite load per bat during the regional dry season (~ September), though our observations were too limited during the wet season (December–April) to rule out the possibility of a second annual peak. Some recent evidence suggests that *R. madagascariensis* may undergo two annual breeding seasons in northern Madagascar [87], a pattern previously reported for sister species *Rousettus aegyptiacus* in more tropical localities on the African continent [88, 89]. As hormonal changes associated with reproduction are known to impact the seasonality of ectoparasite burden in other host-parasite systems [90–92], including bat systems [93–95], these reproductive changes may influence ectoparasite seasonality in Madagascar as well. Parasitism of *E. dupreanum* by *C. dubia* in our northern Madagascar locality also peaked in September, though limited data in wet season months again precluded inference earlier in the year.

At the well-sampled central-eastern Moramanga site, we observed only one peak in nycteribiid burden for *R. madagascariensis* towards the end of the wet season (March) for this locality; in related studies, we only observed a single annual gestation period between September and December for *R. madagascariensis* in the same site [63, 96]. Also in the central-eastern study region, we observed a single peak in ectoparasite burden for *C. dubia* parasitism of *E. dupreanum*, here preceding the onset of the female gestation period for this bat species, at the start of the dry season (June) in this region. We note that, while we grouped both the Angavokely roost for *E. dupreanum* and Maromizaha roost for *R. madagascariensis* within the central-eastern region of Madagascar, these sites are located over 60 km apart (Additional file 2: Table S1), and mean monthly temperatures were on average just under 5 °C cooler in Angavokely vs. Maromizaha across our study period (Fig. 3). This suggests different climatic influences on both the reproductive calendar for the bat hosts and the seasonality of ectoparasite burden in the two localities. Our GLM analyses highlight an important role for climate, particularly precipitation and temperature, in driving seasonality in ectoparasite burden, whether directly through impacts on ectoparasite physiology or indirectly through modulation of bat host physiology or both. Clearly, seasonal patterns in ectoparasite burden are more comparable between the two bat species when sampled in the exact same northern Madagascar locality (Ankarana National Park) than when sampled in climatically different sites in central-eastern Madagascar. Nonetheless, despite the clear influence of climate, one key finding from our study is the repeated support we recovered across both study sites and both bat host species for sex-specific differences in the seasonality of ectoparasite burden, with ectoparasite burden on female bats always preceding that for males of the same species in the same locality. These patterns suggest that, independent of climate, seasonal differences in bat physiology, likely related to reproduction, are important drivers of ectoparasite burden.

In addition to seasonality, our analyses of the impact of bat host body condition (MFR) on ectoparasite load mirrored previous reports for *E. madagascariensis* on *R. madagascariensis* [62]: we found higher parasite loads in individuals with better body condition (higher MFR), which, as previously hypothesized, could be related to larger surface area available for nycteribiid infestation in these relatively small (~ 60 g) fruit bats. We consistently observed the inverse trend, with low host MFR

associated with higher nycteribiid burden for larger (~250 g) *E. dupreanum* bats, perhaps because of immunocompromising effects of host nutrition. It is possible that *R. madagascariensis* fall below a certain size threshold below which available surface area scales positively with ectoparasite burden, while above this threshold, the effects of host physiology and immunology prevail. Further research at the minimum and maximum size distributions for these different species is needed to parse these divergent trends.

In contrast to a previous study in northern Madagascar which identified higher nycteribiid parasitism intensity on adult male vs. female *R. madagascariensis* [61], we found that, after controlling for climate predictors, nycteribiid abundance was lower on male vs. female *R. madagascariensis* and *E. dupreanum* bats in our central-eastern Madagascar sites (Fig. 4). No significant effects of host bat sex were observed for either species in the more limited northern Madagascar dataset. Our findings in central-eastern Madagascar are consistent with previous reports of ectoparasite preference for female bat hosts in other systems [97, 98] and suggest that previous reports in northern Madagascar may reflect seasonal biases in data collection, as hypothesized by the study authors. Prior work in northern Madagascar additionally identified a significant male sex bias in the *E. madagascariensis* ectoparasites themselves [61]. We observed a similar bias in our northern Madagascar site, for which morphological observations were only conducted during the dry season (Additional file 1: Fig. S4). As our more complete seasonal time series in central-eastern Madagascar showed no significant differences in sex distribution for either *C. dubia* or *E. madagascariensis* bat flies, we hypothesize that this previous observation may also reflect seasonal bias in the sampling. Indeed, previous reports in the literature suggest that seasonal variation in the sex ratio of nycteribiid bat fly populations may be common [99–101]. Further field study is needed to more clearly delineate these patterns for *C. dubia* and *E. madagascariensis.*

Our study has several limitations, most obviously our reliance on publicly available coarse-scale climate data in lieu of direct climate records collected from the interior of bat cave roosts. Previous studies have demonstrated critical impacts of microclimate differences in bat roosts on seasonal dynamics in ectoparasite communities [102, 103]. As we observed clear differences in the seasonality of ectoparasite burden across study sites within the same broad geographic region, future work will greatly benefit from more careful study of local climate. In addition, our more limited seasonal sampling of northern Madagascar localities (due to access challenges in the rainy season) precludes some comparisons between the two study regions; equally intensive seasonal study of northern Madagascar sites could offer additional insight in the future. We focused the bulk of our ecological analyses on seasonal variation in the abundance of nycteribiid bat flies; future work should attempt to carry out similar investigations into the ecology of *M. wenzeli* parasitism, in addition to the several other ectoparasite taxa observed during our field and laboratory studies. Finally, confirmation of species-level identity of non-bat fly ectoparasites of *E. dupreanum* and *R. madagascariensis* using molecular techniques is a major research priority.

Conclusions

Ectoparasites of bats, including nycteribiid and streblid bat flies, fleas, mites, and ticks, can play important roles in pathogen transmission. Here, we describe the diversity of ectoparasite burden for two Malagasy fruit bats, *E. dupreanum* and *R. madagascariensis*, and expand the molecular record to include streblid bat fly ectoparasites of *R. madagascariensis*. We highlight seasonal variation in nycterbiid burden for these two bat hosts, which mirrors seasonal variation in nutritional resource availability and the reproductive calendar across northern and central-eastern Madagascar. As bats are important reservoirs for several highly virulent zoonotic pathogens, understanding ecological patterns of bat parasitism is of critical public health importance. Because ectoparasites can cause negative fitness impacts on their hosts, our work is additionally informative for conservation efforts for these two Pteropodidae fruit bats, both ranked as 'Vulnerable' on the IUCN Red List of Threatened Species [104].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-025-06805-z.

Additional file 1.

Additional file 2.

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Author contributions

AFA, SA, HCR, SG, and CEB collected the field data in part with a large project overseen by CEB, JMH, PD, and VL. AFA and SA carried out microscopy on field-collected ectoparasite samples, with support from HCR and SG. AFA, SA, and GK conducted DNA barcoding of field-collected ectoparasite samples, with support from KIY and CEB. AFA analyzed the resulting data in R with

support from TML, KIY, AA, and CEB. AFA and CEB wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are attached as supplementary tables to this article and also available in our open-access GitHub repository: https://github.com/brooklabteam/Mada-Ectoparasites.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Zoology and Animal Biodiversity, University of Antananarivo, Antananarivo, Madagascar. [2]Department of Entomology, University of Antananarivo, Antananarivo, Madagascar. [3]Department of Ecology and Evolution, University of Chicago, Chicago, IL, USA. [4]Virology Unit, Institut Pasteur de Madagascar, Antananarivo, Madagascar. [5]Department of Fish, Wildlife and Conservation Ecology, New Mexico State University, Las Cruces, NM, USA. [6]Department of Biology, Skyline College, San Bruno, CA, USA. [7]Department of Biological Sciences, El Paso, TX, USA.

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