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Incidence and taxonomic richness of mosquitoes in the diets of little brown and big brown bats

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Bats have been portrayed as important consumers of mosquitoes, but evidence supporting this claim is surprisingly scant. We collected the fecal material of 2 common North American bats at 22 sites in Wisconsin, United States and screened samples for mosquitoes using a recently improved molecular method for detecting arthropod DNA. Overall, we detected 17 discrete operational taxonomic units assigned to the mosquito family (Diptera: Culicidae), 15 of which were assigned at the species level. We detected mosquitoes in 71.9% of samples and at all sampling sites for little brown bats (*Myotis lucifugus*). By comparison, we detected mosquitoes in 33.3% of samples and one-half of the sampling sites for big brown bats (*Eptesicus fuscus*). Our results suggest that the incidence and taxonomic richness of mosquito prey consumed by bats is considerably higher than has been previously shown. In light of globally declining bat populations, we propose that future studies reassess the importance of trophic interactions between bats and mosquitoes.

Key words: bat diet, citizen science, Culicidae, DNA barcoding, *Eptesicus fuscus*, *Myotis lucifugus*, next-generation sequencing

Predators can regulate populations of their prey, and the loss of predators can thus result in significant changes to biological communities and ecosystem services (Ritchie and Johnson 2009; Estes et al. 2011). Bats are globally widespread consumers of arthropods and have been cited as important regulators of pests, including agriculturally relevant insects as well as mosquitoes of human health concern (Kunz et al. 2011). While agricultural pest suppression services provided by bats have strong empirical support (Cleveland et al. 2006; Kalka et al. 2008; Boyles et al. 2011; Maine and Boyles 2015), claims regarding mosquito suppression, or even the degree of consumption, are less well substantiated. Commonly referenced studies have lead to suggestions that bats consume as many as 1,000 mosquitoes per hour (Griffin et al. 1960) and significantly reduce mosquito oviposition rates (Reiskind and Wund 2009). These studies, however, consisted of enclosure experiments that may not represent natural conditions, and the question of whether bats impact mosquito populations in the wild remains largely unanswered. Consequently, the potential effect of declining bat populations (O'Shea et al. 2016) on the future abundance of mosquitoes is indeterminate.

Diet studies represent a first, but important, step in understanding the potential impacts of a predator on populations of their respective prey (Jedlicka et al. 2016; Krauel et al. 2018). While characterizing bat diets based on the morphological remains of arthropods within stomach contents or guano has provided information on the taxonomy of prey, resolution beyond the ordinal or family level is often limited, especially among soft-bodied prey items such as mosquitoes (Whitaker et al. 2009). The use of DNA barcoding methods has greatly improved the assignment of taxonomic identity to the genetic material present in fecal samples, but molecular studies have generally found that mosquitoes represent a small portion of the diet of wild bats and that the taxonomic richness of mosquitoes consumed by bats is low (Belwood and Fenton 1976; Dickman and Huang 1988; Clare et al. 2009; Gonsalves et al.

2013; Clare et al. 2014a, 2014b). However, mosquitoes could be underrepresented in molecular-based investigations of bat diets due to issues such as primer biases, polymerase errors, and other biases that commonly challenge such studies (Acinas et al. 2005; Clarke et al. 2014; Deagle et al. 2014; Brandon-Mong et al. 2015). Thus, uncertainty still exists regarding the extent to which bats actually consume mosquitoes, a paucity of evidence that is perhaps surprising considering the importance of mosquitoes as global vectors of disease and local backyard irritants (Juliano and Lounibos 2005; Dickinson and Paskewitz 2012).

We screened 2 common North American bat species (little brown bat, *Myotis lucifugus*; big brown bat, *Eptesicus fuscus*) for evidence of mosquito consumption using a recently refined high-throughput amplicon sequencing technique for dietary analyses of insectivores that builds on previous molecular approaches (Jusino et al. 2017). This method includes an improved primer set, the use of an extensive mock prey community, and a customized bioinformatics pipeline that collectively result in lower false negative rates than other commonly used approaches. As such, it provides opportunities to characterize bat diets with greater taxonomic resolution and accuracy and constitutes a key next step for understanding trophic interactions between bats and their prey. Our results suggest that little brown and big brown bats consume a greater variety of mosquito taxa, and do so more frequently, than has been shown previously.

Materials and Methods

We collected bat guano samples below 12 little brown and 10 big brown bat maternity roosts across Wisconsin, United States during the summer of 2014. These sites, which included roosts in houses, barns, outbuildings, and bat houses, were monitored as part of an existing network of citizen scientists coordinated by the Wisconsin Department of Natural Resources (Fig. 1). Landscape conditions at these study sites included a range of habitat types, which were assessed using the USDA National Agricultural Statistics Service Cropland Data Layer (https://nassgeodata.gmu.edu/CropScape/). Within a 3-km buffer around roosts, the average composition was 25% forest (ranging from 4% to 61%), 22% grass or pasture (ranging from 0% to 52%), 21% agricultural (ranging from 0% to 59%), and 10% wetland (ranging from 0% to 36%). Less-common landcover types included open water, developed areas, and other miscellaneous vegetation types. Bat guano was collected during 3 separate sampling periods: 1) late May to early June ("early"); 2) late June to early July ("mid"); and 3) late July ("late"), which roughly correspond to lactation, pup pre-volancy, and pup post-volancy reproductive stages, to characterize potential seasonal shifts in mosquito consumption. During the time period from the earliest collection (May 17) to the latest (July 29), the average temperatures recorded at the centrally located Dane County Regional Airport (Madison, Wisconsin) weather station was 27°C with a high of 34°C and a low of 19°C. A total of 31 cm of rainfall occurred during this time.

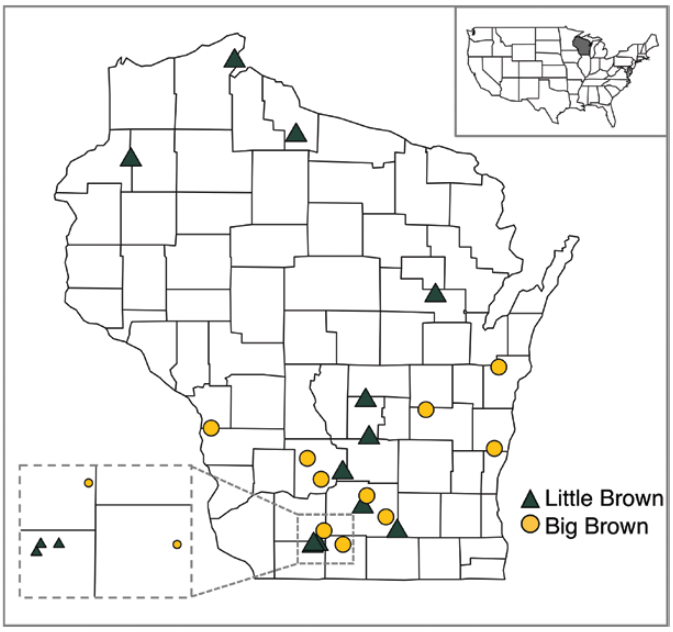

Fig. 1.—Map of little brown (*Myotis lucifugus*, $n = 12$) and big brown (*Eptesicus fuscus*, $n = 10$) bat guano sampling sites in Wisconsin, United States.

Bat species identity was determined visually by directly observing bats and was verified based on guano pellet size at the time of collection. Clean plastic sheets were placed under each roost for 1 week during each of the 3 sampling periods and, at the end of 1 week, a 50 ml storage tube was filled with guano. Thus, each sample likely contained fecal material from multiple individuals. Samples were initially stored at −20°C, and then transferred to −80°C for long-term storage. A subsample of 100 mg (~10 pellets) was selected from each sample for genetic analyses. Some sites did not have samples collected at all 3 time periods as a result of bat movements or field constraints, resulting in a total sample size of 53 guano collections. All sample collection methods were carried out in accordance with guidelines of the Wisconsin Department of Natural Resources and the American Society of Mammalogists (Sikes et al. 2016). Experimental protocols were approved by the Wisconsin Natural History Inventory (NHI) Program and the University of Wisconsin-Madison College of Agricultural and Life Sciences (CALS) Animal Care and Use Committee (ACUC).

DNA was extracted from each guano subsample using a Qiagen DNA Stool mini kit (Qiagen, Inc., Germantown, Maryland), and a 180 bp cytochrome c oxidase subunit 1 (*COI*) amplicon was then amplified using ANML primers according to Jusino et al. (2017). These primers amplify a 180 bp segment of *COI* and were originally designed to detect a broad range of arthropod taxa. However, in this study, we focused on the use of these methods for the specific purpose of screening mosquito DNA present in bat fecal material. Amplification of the extracted DNA used the following reagent volumes per 15 µl reaction: 7.88 µl molecular grade water, 3 µl Green GoTaq 5× buffer (Promega), 0.12 µl of 20 mg/ml bovine serum albumin (NEBiolabs), 0.3 µl of each 10 µM primer, 0.3 µl dNTPs, 0.1 µl of 5 U/µl GoTaq

polymerase (Promega Corp., Madison, Wisconsin), and 3 µl of extracted arthropod template DNA. The thermocycler parameters followed Hebert et al. (2003) except with the final extension at 72°C increased from 5 to 7 min. As a positive control for downstream analyses, a single-copy mock community of 46 known arthropod constituents (Jusino et al. 2017) was also separately amplified under the same PCR conditions. Libraries were then sequenced on an Ion Torrent Personal Genome Machine platform (PGM; ThermoFisher Scientific, Inc., Sante Fe, New Mexico) according to manufacturer's recommendations using the following kits: Ion PGM Hi-Q View OT2 400 bp Kit, Ion PGM Hi-Q View Sequencing Kit, and Ion PGM 318v2 chip.

Ion Torrent PGM sequencing data were processed using AMPtk v0.8.5 (Palmer et al. 2017). Briefly, the raw sequence data were de-multiplexed using the unique barcode index sequence, forward–reverse primers were stripped, and full-length reads (reads where both forward and reverse primers were found) were retained. The resulting sequence data were then run through the DADA2 module of AMPtk, which quality-filters data using expected error trimming (Callahan et al. 2016). Data were then "de-noised" in the DADA2 algorithm, followed by 97% clustering into operational taxonomic units (OTUs), and finally an OTU table was assembled using the de-multiplexed sequencing reads. Taxonomy was assigned to the OTUs using the built-in *COI* database in AMPtk (Jusino et al. 2017). All OTUs identified as family Culicidae were manually vetted using 3 criteria: 1) each OTU shared at least 99% identity and 99% query cover with known sequences from a single taxon represented in GenBank, or 2) each OTU had at least a 99% match with known sequences from a single taxon represented in the BOLD Full Length Record Barcode Database (Ratnasingham and Hebert 2007), and 3) identities assigned to OTUs were from taxa that have been previously found in the state of Wisconsin (Roberts et al. 1956; DeFoliart et al. 1967; Grimstad and DeFoliart 1975). Any remaining OTUs that were not classified at least to order (e.g., identified only to class Insecta, Arthropoda, or Animalia) were manually searched against both GenBank and the BOLD databases. We recognize that species assignments based on OTUs and reference databases represent approximations based on currently available taxonomic delineations (see Supplementary Data SD1). For the purposes of this study, taxon assignments reflect specimen identification guidelines following Collins and Cruickshank (2013).

Molecular methods such as those used in this study do not yield reliable estimates of the number of individuals consumed for several reasons, including that the number of sequence reads does not necessarily correspond to the amount of genetic material present in a sample. Thus, in order to assess mosquito consumption by both bat species, we estimated and compared both incidence (defined here as the number of occurrences of mosquito detection in guano from a roost sample from a specific site during a given time period) and taxonomic richness (the number of unique OTUs). Taxonomic richness was compared between species and sampling periods using Welch's *t*-tests and 1-way analysis of variance, and incidence was compared using chi-squared tests.

Results

Processing of the Ion Torrent PGM data (including de-multiplexing, de-noising–clustering, and OTU table filtering using the mock community) resulted in a total of 5,913 processed reads assigned to the family Culicidae. These reads corresponded to 17 supported Culicidae OTUs, with a range of 6–3,005 ($\bar{\chi}$ = 348) reads per OTU. Overall, 15 of the 17 discrete mosquito OTUs (88.2 %) met the aforementioned criteria for assigning species identity. Two OTUs did not meet these criteria at the taxonomic levels of species or genus and were therefore assigned taxon identities as separate unclassified OTUs belonging to the subfamily Culicinae (Diptera: Culicidae). These unclassified OTUs were included in richness and incidence analyses and classified as "unknown Culicinae A" and "unknown Culicinae B."

Mosquito taxonomic richness was greater in the diet of little brown bats, as this species consumed all 17 OTUs compared to only 7 OTUs consumed by big brown bat. When samples were pooled by sites, little brown bats had a significantly higher mean number of mosquito OTUs ($\bar{\chi}$ = 5.25 ± 2.22, n = 12) in their diets than big brown bats ($\bar{\chi}$ = 1.1 ± 0.92, n = 10; $t_{14.4}$ = 3.81, P = 0.002). At the individual sample level, little brown bats had, on average, more mosquito OTUs in their diets ($\bar{\chi}$ = 1.97 ± 0.62, n = 32) than big brown bats ($\bar{\chi}$ = 0.57 ± 0.44, n = 21; $t_{50.113}$ = 3.74, P < 0.001). The mean number of mosquito OTUs did not differ significantly between the 3 sampling periods for either little brown bats ($F_{2,29}$ = 0.30, P = 0.745) or big brown bats ($F_{2,18}$ = 0.81, P = 0.459; Fig. 2).

We detected mosquitoes in 30 of 53 (56.6%) bat guano samples and at 17 of 22 maternity roosts (77.3%) when data were pooled between the 2 bat species. Mosquito incidence was significantly greater in the diet of little brown bats (χ^2 = 5.18, *d.f.* = 1, P = 0.023), and we detected at least 1 mosquito OTU at 100% of little brown bat sites (n = 12) compared to 60% of big brown bat sites (n = 10). When considered at the level of individual samples, little brown bats had a significantly higher incidence of mosquitoes in their diets (71.9%; n = 32) than big brown bats (33.3%, n = 21; χ^2 = 6.18, *d.f.* = 1, P = 0.013). Incidence of mosquitoes in little brown bat samples was numerically greatest in the early sampling period (at least 1 OTU present in 81.2% of samples) and declined during the mid- and late sampling period (66.7% for both the mid- and late sampling periods); this difference, however, was not significant (χ^2 = 0.81, *d.f.* = 2, P = 0.459). Incidence of mosquitoes in big brown bat samples remained constant across the sampling periods (33.3%). For individual mosquito OTUs, incidences ranged from 3.1% to 34.4% ($\bar{\chi}$ = 11.6%) in little brown bat samples and from 4.8% to 19.0% ($\bar{\chi}$ = 7.5%) in big brown bat samples. Seasonal differences in the incidence of mosquito taxa consumed were observed, but were not significantly related to time or site differences (Fig. 3).

Discussion

We detected a high incidence and taxonomic richness of mosquitoes in bat diets, particularly for little brown bats. Both the incidence and taxonomic richness of mosquitoes in little brown bat

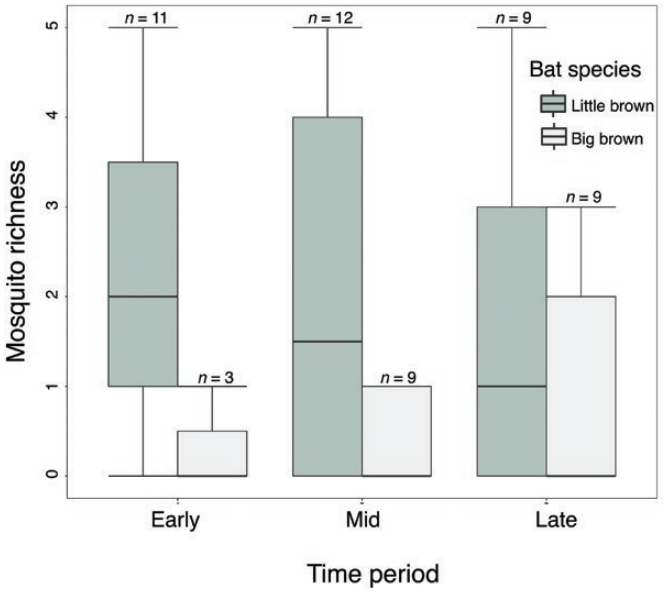

Fig. 2.—Total mosquito taxonomic richness in little brown bat (*Myotis lucifugus*) and big brown bat (*Eptesicus fuscus*) guano samples ($n = 53$) across sites ($n = 22$), separated by sampling time period. "Early" sampling period took place from late May to early June, "Mid" sampling period took place from late June to early July, and "Late" sampling period took place in late July. Horizontal lines indicate the median, box shows the interquartile range (IQR), and whiskers are 1.5×IQR. Points indicate the number of bat guano samples with each respective richness value.

diets were greater than detected by any previous molecular-based study of bat diets (see Supplementary Data SD2). While some diet studies have found high incidence or taxonomic richness, no study, either molecular or morphological, has detected both a high incidence and richness of mosquitoes in bat diets. A single morphological study detected a higher incidence of mosquitoes than this study, but prey items were not classified below the family level (Diptera: Culicidae), limiting the ability to identify which specific taxa were consumed (Anthony and Kunz 1977). Certainly, differences between our results and those from previous studies may, in part, be attributed to geographic, temporal, or bat species differences among studies. Moreover, difficulties in comparing bat diets across studies can arise from differences in methodological approaches such as the use of morphological versus molecular methods, sample pooling (e.g., sampling individuals versus roosts), and the metrics reported (e.g., volume, percent, or incidence). However, our sampling occurred across the state of Wisconsin, in both agricultural and forested landscapes, and during multiple time periods (May through July), suggesting that bats consumed mosquitoes under a broad range of ecological conditions. Perhaps more importantly, the laboratory and bioinformatic methods we used can reduce false negative rates for arthropod DNA present in fecal samples (Jusino et al. 2017). As part of previously published experimental trials, the approach we used detected 8 of 8 distinct Dipteran taxa (including 2 of 2 mosquito taxa), yielding greater confidence in estimates of both mosquito incidence and taxonomic richness (Jusino et al. 2017). In contrast, Zeale et al. (2011), using the ZBJ primer pair, detected only 4 of 8 of the same Dipteran taxa (including 1 of 2 mosquito taxa). Other common primer pairs used for DNA barcoding for insectivore diets, including LEP (Hebert et al. 2004; Smith et al. 2006) and CO1 L/H (Folmer et al. 1994), also demonstrated taxonomic biases against certain groups, although these 2 primers were considerably better at detecting Dipteran taxa, with 8 of 8 and 7 of 8 Dipterans, respectively, and 2 of 2 mosquitoes detected by Jusino et al. (2017). By implementing a method that has demonstrated improved amplification across arthropod taxa, the results from our study suggest that mosquitoes (and potentially other arthropod taxa of interest) may have been underrepresented in prior studies of bat diets, in part due to methodological constraints.

Both the incidence and taxonomic richness of mosquitoes consumed by little brown bats were more than double those of big brown bats, consistent with previous studies suggesting that smaller bats more commonly consume mosquitoes and other small, soft-bodied Dipteran prey (Barclay and Brigham 1991; Gonsalves et al. 2013). Our study found a significantly higher incidence of individual mosquito OTUs across little brown bat sites ($\bar{\chi} = 11.6 \pm 4.9\%$) in comparison with a previous study that reported mosquito incidence among the same species ($\bar{\chi} = 3.7 \pm 2.7\%$; $t_{23.9} = 3.01$, $P = 0.006$—Clare et al. 2014a). Constraints related to body size may affect the maneuverability of bats foraging on small prey like mosquitoes, and such prey may also provide inadequate caloric content to meet the energy demands of larger bats. Nevertheless, despite their larger body size and presumed preference for larger arthropod taxa (e.g., beetles—Moosman et al. 2012; Clare et al. 2014b), we found that big brown bats still consumed a range of mosquito taxa. Thus, our results demonstrate that even medium-sized arthropodivorous bats can consume several different mosquito species and do so more commonly than reported in prior studies.

The detection of different mosquito taxa in bat guano did not display any obvious temporal patterns. Our sampling periods roughly corresponded to different bat reproductive stages, with the "early" sampling period occurring during pregnancy and lactation, the "mid" sampling period occurring during pup pre-volancy, and the "late" sampling period occurring during pup post-volancy. These different reproductive stages not only affect foraging patterns and nutritional demands in pregnant and lactating female bats, but also correspond with changing population densities and different foraging patterns between adults and juveniles (Anthony et al. 1981; Adams 1997; Wilkinson and Barclay 1997). Notably, 2 of the most commonly detected mosquito species, *Culex restuans* and *Aedes vexans*, display multivoltine life cycle types with multiple adult emergences per year (Crans 2004), suggesting that patterns of mosquito consumption by bats also may be related to the natural history and relative abundance of different mosquito species.

Evidence from our study supports previous claims that bats, by virtue of the incidence and taxonomic richness of mosquitoes in their diets, have the potential to provide pest suppression services by consuming mosquitoes (Kunz et al. 2011). Indeed, we found that little brown bats consumed 9 mosquito species known to harbor West Nile virus (*Aedes cinereus*, *Ae. vexans*, *Coquillettidia perturbans*, *Culex territans*, *Cx. restuans*, *Culiseta morsitans*, *Ochlerotatus canadensis*, *Oc. sticticus*, and *Oc.*

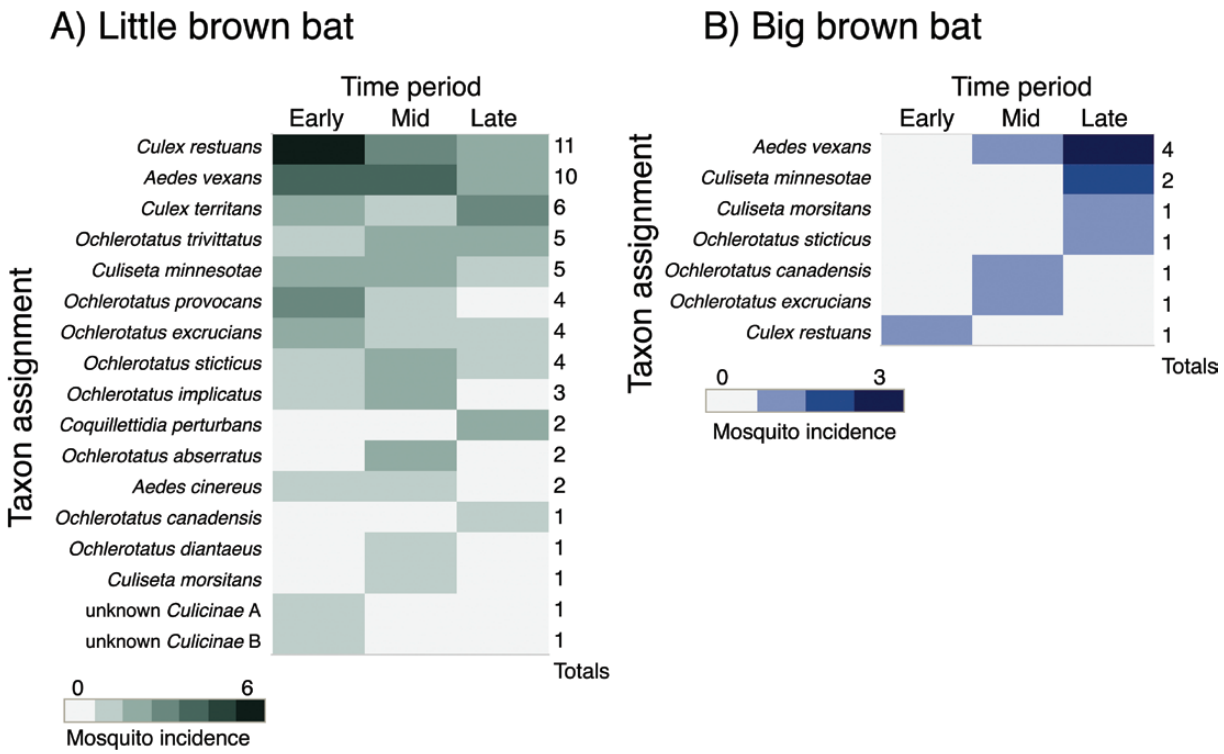

Fig. 3.—A) Incidence of individual mosquito taxa detected in little brown bat (*Myotis lucifugus*) guano samples (n = 32) across sites (n = 12), separated by time period. B) Incidence of mosquito taxa present in big brown bat (*Eptesicus fuscus)* guano samples (n = 21) across sites (n = 10), separated by time period.

trivittatus), a disease that poses risks for humans as well as many bird species (Kramer et al. 2008; Centers for Disease Control and Prevention 2017). Moreover, *Ae. vexans*, one of the most abundant mosquitoes in the region (Meece et al. 2003), was the most commonly detected species in diets of both little brown and big brown bats. Thus, our results suggest that questions regarding the extent to which bat species actually consume mosquitoes in the wild (and how consumption varies spatially, temporally, and taxonomically) should be revisited. Molecular-based diet studies such as ours, however, only provide information on the presence and identity of prey in individual guano samples and cannot reliably provide measurements of prey abundance; the proportion of sequences does not necessarily correspond to dietary mass (Deagle et al. 2013). Additionally, mosquitoes constitute only part of a larger diet including many other components.

Testing for mosquito suppression would require experimental manipulations of bat abundances in the wild, quantitative PCR assays with specificity across the family Culicidae that could provide estimates of the actual quantity of prey consumed by bats, or more ecological studies of bat–mosquito interactions, including the potential for preferential consumption of particular individuals (e.g., gravid females—Reiskind and Wund 2009). The influence of environmental factors, including rain, temperature, and human activities such as pesticide application, may also prove more important than top-down predator control of mosquito populations. Nonetheless, future studies could shed light on the trophic interactions between bats and mosquitoes, particularly if replicated for different bat species across different regions. Our study represents a 1st step in revisiting important questions regarding bat consumption of mosquitoes, and thus lays the groundwork for future investigations of potential suppressive effects. As bats continue to decline globally due to habitat loss and wind turbines, and in North America due to white-nose syndrome (O'Shea et al. 2016), we propose that their potential role as mosquito control agents be reevaluated.

Sequence Data Availability

The corresponding sequence data for this paper can be found at SRA (SRP108780).

Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—AMPtk, BOLD, and NCBI BLAST identity matches for Culicidae OTU taxon assignment.

Supplementary Data SD2.—Summary of prior detection of Culicidae in key bat diet studies. Where possible, volume, incidence, or frequency values were standardized for comparability across studies.

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