DNA Metabarcoding-based Evaluation of the Diet of Big Brown Bats (*Eptesicus fuscus*) in the Mid-Atlantic Region

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Abstract - High-throughput DNA sequencing can generate large genetic datasets in a cost-effective manner. Although the diet of *Eptesicus fuscus* (Big Brown Bat) has been studied widely in natural and rural systems using visual identification of prey items in feces, our aim was to more completely assess diet using a metabarcoding approach across a wide urban–natural landscape gradient in the mid-Atlantic region. Concordant with our expectations and previous Big Brown Bat diet studies from visual identification, we observed a high abundance of Coleoptera (beetles) relative to other insect orders. Although a possible improvement over visual techniques for studying food habits, we suggest caution in interpreting metabarcoding results in diet studies. We noted observations of environmental or contaminant taxa within these data, and designed a stringent filtering method that we used to eliminate these taxa, but that also removed previously documented prey taxa from our dataset.

Introduction

Insectivorous *Eptesicus fuscus* (Palisot de Beauvois) (Big Brown Bat) occur across a diverse range of New World habitats from near-boreal to tropical, as well as in natural, semi-natural rural, and urban ecosystems (Agosta 2002, Kurta and Baker 1990). Variation in Big Brown Bat diet reflects their broad distribution and habitat associations, as they consume insect taxa from at least 13 orders and 50 families (Agosta and Morton 2003; Brack and Whitaker 2004; Hamilton 1933; Long et al. 2013; Phillips 1966; Whitaker 1972, 1995). Additionally, Big Brown Bat diet varies on spatial and temporal scales (Agosta 2002; Agosta and Morton 2003; Whitaker 1995, 2004). Although their diet is flexible, they generally are known as Coleoptera specialists, with a jaw and dental morphology capable of more easily consuming hard-bodied insects than many other North American bats (Brigham and Saunders 1990; Griffith and Gates 1985; Hamilton 1933; Hamilton and Barclay 1998; Whitaker 1972, 1995).

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Sex and reproductive condition of the bat also may influence diet because of contrasting energy requirements and foraging techniques (Hamilton and Barclay 1998, Menzel et al. 2001, Wilkinson and Barclay 1997). Pregnant and lactating female bats often forage for longer periods and/or have more-frequent foraging bouts within areas near the roost, because unlike males, they are limited to maintaining proximity to their maternity colonies (Altringham 2011, Barclay 1989, Clark et al. 1993, Rintoul and Brigham 2014, Rydell 1993).

Most studies of bat diets, including those for Big Brown Bats, have been based on visual identification of insects or insect parts in dissected fecal pellets or stomach contents (Agosta 2002). Visual methods bias the results toward difficult-to-digest taxa and require high identification proficiency (Razgour et al. 2011). Additionally, classification of insects to genus or species is not always possible if the prey items, such as soft-bodied insects, have been digested; therefore, many Big Brown Bat studies simply describe diet at the order or family levels (Griffith and Gates 1985, Hamilton 1933, Whitaker 1995) and accordingly may overestimate Coleopteran representation in the diet. Newer genetics-based approaches, however, have identified prey to lower taxonomic levels and include soft-bodied prey that would not be identified with visual techniques (Clare et al. 2014, Long et al. 2013, Zeale et al. 2011).

High-throughput sequencing comprises a collection of DNA sequencing technologies, with applications including metabarcoding, that can produce large taxonomic data sets in a relatively rapid, cost-effective manner by sequencing many DNA fragments simultaneously (Ekblom and Galindo 2011). Metabarcoding techniques have been used to analyze diet habits for many taxa, including insectivorous bats (Clare et al. 2014, Deagle et al. 2010, O'Rourke et al. 2022, Razgour et al. 2011). However, metabarcoding results require critical evaluation to assess the degree to which these methods can address relevant questions before scaling-up to addressing research questions relating bat diets to broader ecological questions (Alberdi et al. 2019, Ekblom and Galindo 2011, Schattanek et al. 2021). Amplification of degraded DNA, such as from dead or partially digested animals, can lead to taxonomic misidentifications (Valentini et al. 2009). Further, metabarcoding cannot distinguish between extraneous environmental DNA consumption (such as that ingested from grooming), and amplification can unevenly replicate DNA sequence reads (Pompanon et al. 2012). Finally, a lack of standardization in OTU inclusion criteria introduces additional variation between studies; researchers often identify and explore these differences within their studies (Clare et al. 2014, Gordon et al. 2019, Razgour et al. 2011).

The purposes of our analysis were to document the diet of Big Brown Bats within a mid-Atlantic landscape and to assess the potential and limitations of DNA metabarcoding methods to describe food habits and as a tool to serve as an "early warning" system for invasive insects. We expected that we might identify new prey items or prey distributions than were not observed in earlier visual studies, thereby providing novel insights into Big Brown Bat diets because of the coarser resolution of previously used techniques and general lack of sampling in urban environments.

We expected that the diet of Big Brown Bats would vary based on sample area and day of year, as well as on bat sex and reproductive condition.

Field-site Description

We collected fecal samples of Big Brown Bats from a large region in the western Chesapeake Bay watershed in Maryland, Virginia, West Virginia, and the District of Columbia (DC). The study areas consisted of: Marine Corps Base Quantico and the National Park Service (NPS) units at Antietam National Battlefield (AN), Catoctin Mountain Park (CA), Chesapeake and Ohio Canal National Historical Park (CH), Gettysburg National Battlefield (GE), George Washington Memorial Parkway (GW), Harpers Ferry National Historic Park, Manassas National Battlefield Park (MA), Monocacy National Battlefield (MO), National Capital Parks-East (NA), Prince William Forest Park, Rock Creek Park (RO), and Wolf Trap National Park for the Performing Arts (WO) (Fig. 1). We added Harpers Ferry National Historic

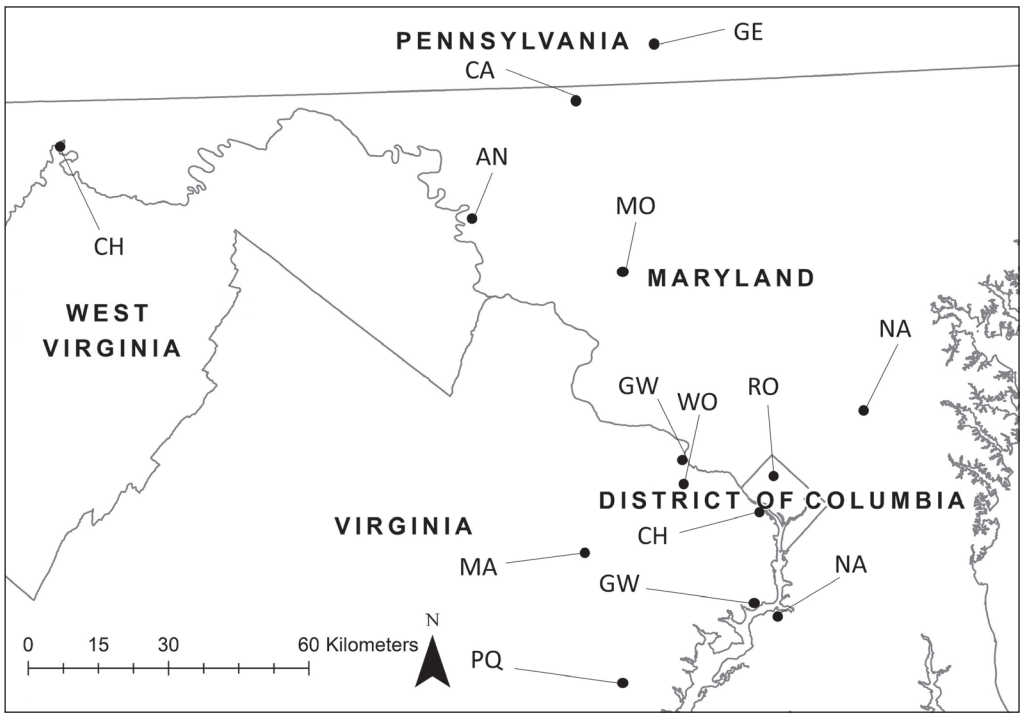

Figure 1. Bat survey study areas where Big Brown Bat (*Eptesicus fuscus)* fecal samples were collected between May and August 2017–2018. Area abbreviations are: Antietam National Battlefield (AN), Catoctin Mountain Park (CA), Chesapeake and Ohio Canal National Historical Park and Harpers Ferry National Park (CH), Gettysburg National Battlefield (GE), George Washington Memorial Parkway (GW), Manassas National Battlefield Park (MA), Monocacy National Battlefield (MO), National Capital Parks-East (NA), Prince William Forest Park and Marine Corps Base Quantico (PQ), Rock Creek Park (RO), and Wolf Trap National Park for the Performing Arts (WO). CH and GW follow the Potomac River between the 2 labeled points. NA points indicate the southern-most and northern-most parks within those units.

Park samples to CH, as the units are adjacent. We also combined Marine Corps Base Quantico and Prince William Forest Park and designated them as site PQ. They are also adjacent and share similar habitat types, although Quantico contains forests that are more actively managed (i.e., using harvest and prescribed burning) as well as open fields and developed area.

Of the study areas, CH is the most diverse, as it is a narrow, linear unit following the Potomac River from downtown DC through suburban, agricultural, and private and state forested areas west to Cumberland, MD. CA is a large contiguous forest, surrounded by agricultural and forested lands, and has lower levels of urbanization. Several NPS units consist of highly fragmented forest patches surrounded by agricultural fields (AN, MA, MO) and others have high levels of urbanization (GW, WO, NA). However, GW also contains larger contiguous forest patches in its northern portions. RO is a mostly contiguous, large forest patch in the center of the highly urbanized DC area.

From east to west, the survey areas are distributed across the Coastal Plain (CH, GW, NA, PQ, WA), Piedmont (CH, GE, GW, MA, MO, PQ, RO, WO), Blue Ridge Mountains (CA, CH), and Ridge and Valley (AN, CH, HA) physiographic regions. The western portions of the study area are associated with higher elevations and less urbanization than the eastern areas. Temperatures during sampling varied from 2.1 to 35.6 ºC, with western areas generally having lower temperatures (PRISM Climate Group 2019). Precipitation was highly variable, but the region accumulated 350–723 mm in 2017 and 562–1127 mm in 2018 (PRISM Climate Group 2019).

Methods

We collected fecal samples for genetic analyses from captured Big Brown Bats from 17 May to 10 August 2017 and 7 May to 12 August 2018. All capture and handling procedures were in accordance with Virginia Tech Institutional Animal Care and Use Protocol #16-240. We used mist nets to capture bats, then placed each bat in a new, unused paper bag for ~10 minutes before identification to species, measurement of mass, and determination of age, sex, and reproductive condition. We identified all bats caught to species using dichotomous keys (Menzel et al. 2002) and determined their age category by observing epiphyseal ossification and the reproductive condition of females through palpation of the abdomen and condition of mammary glands (Anthony 1988, Racey 1988). After releasing the bat, we inspected the bag for feces and used a BrandTech 780500 (Essex, CT) polypropylene 1.5-mL microcentrifuge tube to scoop and store the feces in 100% ethanol.

We extracted DNA from bat feces using the QIAamp Fast DNA Stool Mini Kit (Qiagen # 19593; Hilden, Germany). We amplified the mitochondrial cytochrome oxidase c subunit 1 (CO1) gene using forward (LCO1490) and reverse (CO1-CFM-Ra) primers developed by Folmer et al. (1994) and Jusino et al. (2019), respectively: 5’-GGTCAACAAATCATAAAGATATTGG-3’ (forward) and 5’-GGWACTAATCAATTTCCAAATCC-3’ (reverse), i.e., the ANML primer pair combination and thermocycling conditions (94 °C for 2 minutes; 40 cycles of: 94 °C for 60 seconds, 50 °C for 90 seconds, 72 °C for 60 seconds; 72 °C for 10 minutes) demonstrated

by Jusino et al. (2019). GENEWIZ, Inc. (South Plainfield, NJ) prepared the DNA library using the NEB Next Ultra DNA Library Prep kit following the manufacturer's recommendations (Illumina, San Diego, CA). These libraries contained PCR amplicons as inserts, barcodes for individual identification during multiplexing, and Illumina universal adapter sequences that allowed the library to bind to features on the Illumina flow-cell. We ligated end-repaired adapters after adenylation of the 3' ends and enrichment by limited-cycle PCR, validated the DNA libraries, and loaded pooled DNA libraries onto the Illumina DNA-sequencing instrument according to manufacturer's instructions using a 250 paired-end configuration, followed by image analysis and base calling with Illumina Control Software on the Illumina MiSeq 2 x 250 bp instrument.

We then processed the DNA sequence data through the AMPtk software pipeline (Palmer et al. 2018) to associate DNA sequences with prey taxa (Jusino et al. 2019). We used UPARSE clustering and the default "hybrid" taxonomy assignment algorithms within the AMPtk pipeline. We used the R package 'phyloseq' (McMurdie 2013, R Core Team 2019) to exclude low-quality samples (with <2000 reads) and to subsequently filter samples by operational taxonomic units (OTUs). Noting that inclusion thresholds for bat diet vary greatly by study (Clare et al. 2014, Gordon et al. 2019, O'Rourke et. al. 2022), we filtered out OTUs with <97% confidence based on AMPtk confidence scores and percentage of mapping identity of 250 base pairs (Palmer et al. 2018) and also those present in only 1 sample. Taxa from Class Chordata that remained were unlikely to have been prey items for Big Brown Bats, and we interpreted them as host DNA (Chiroptera), environmental DNA (orders likely consumed from drinking water, or the consequence of uptake of tissue such as hair residue from roosts shared with other mammals), or sample contamination. To prioritize minimizing false positives (Alberdi et al. 2018), we eliminated all taxa with number of reads less than that for the most highly represented such taxon (*Sciurus carolinensis* Gemelin [Gray Squirrel; Order Rodentia] = 21,701) from the dataset. Rodentia reads were present in 6.8% of ≥97% confidence samples (AN = 1, CH = 9, GE = 1, PQ = 1, RO = 6), with 5 samples from 1 sampling night. We eliminated 2 samples with no remaining OTUs. We refer to these methods to as our "stringent filtering criteria". We noted that even OTUs with 100% confidence included members of Rodentia.

We determined the orders, families, genera, and species present in each sample. We compared our prey-diversity results with those of previous diet studies. To evaluate whether our study included enough sampling effort and distribution to ensure replication (Pompanon et al. 2012) and better understand required sample sizes for future work, we also calculated collector's curves for different taxonomic levels, an analog to species-accumulation curves, using the R package 'vegan' (Oksanen et al. 2015, R Core Team 2019). We calculated collector's curves for the 3 areas with larger sample efforts within a relatively small spatial scale: GE, PQ, and RO. We identified the temporal distribution of samples with the presence of each prey order, and the richness of the respective families within the most-common order (Coleoptera) by sex and reproductive status.

Results

We collected and sequenced DNA isolated from 286 fecal samples. Overall, we observed 7,515,556 DNA sequences. Per-sample reads varied from 204 to 622,016, and we identified 7720 OTUs.

We identified 82 taxa from 3037 OTUs of ≥97% confidence, which were collected from 264 samples (Table 1). After implementing all of our stringent filtering

Table 1. Numbers of samples with observations of each order among fecal samples from 264 *Eptesicus fuscus* (Big Brown Bat) collected within the District of Columbia, Maryland, Pennsylvania, and Virginia, 2017–2018. Criteria for taxa inclusion are 97% (operational taxonomic unit identification confidence ≥97%), 100% (operational taxonomic unit identification confidence = 100%) and our stringent filtering criteria (operational taxonomic unit identification confidence ≥97%, > 21,701 reads, and presence within at least 2 samples). [Table continued on following page.]

	≥97%		100%		Stringent filtering criteria	
Order	Samples	%	Samples	%	Samples	%
Amblypygi	1	0.4	1	0.4	-	-
Amphipoda	18	6.8	16	6.1	-	-
Anguilliformes	4	1.5	4	1.5	-	-
Anura	29	11	24	9.1	-	-
Araneae	163	61.7	98	37.3	-	-
Argentiniformes	14	5.3	-	-	-	-
Blattodea	58	22	52	19.8	48	18.3
Blenniiformes	3	1.1	1	0.4	-	-
Calanoida	4	1.5	3	1.1	-	-
Callionymiformes	4	1.5	4	1.5	-	-
Chiroptera	129	48.9	54	20.5	51	19.5
Cichliformes	4	1.5	4	1.5	-	-
Clupeiformes	1	0.4	1	0.4	-	-
Coleoptera	260	98.5	255	97	238	90.8
Cumacea	3	1.1	3	1.1	-	-
Cyclopoida	34	12.9	8	3	-	-
Cypriniformes	4	1.5	-	-	-	-
Decapoda	49	18.6	38	14.4	-	-
Dermaptera	14	5.3	3	1.1	-	-
Diplostraca	2	0.8	1	0.4	-	-
Diptera	248	93.9	203	77.2	-	-
Embioptera	20	7.6	20	7.6	-	-
Entomobryomorpha	43	16.3	32	12.2	-	-
Ephemeroptera	48	18.2	37	14.1	-	-
Gadiformes	1	0.4	1	0.4	-	-
Geophilomorpha	1	0.4	-	-	-	-
Gobiiformes	2	0.8	1	0.4	-	-
Harpacticoida	2	0.8	1	0.4	-	-
Hemiptera	209	79.2	186	70.7	74	28.2
Hymenoptera	204	77.3	194	73.8	19	7.3
Isopoda	12	4.5	9	3.4	-	-
Isoptera	4	1.5	2	0.8	-	-
Ixodida	30	11.4	23	8.7	-	-
Labriformes	2	0.8	-	-	-	-
Lepidoptera	201	76.1	163	62	18	6.9

criteria, the dataset contained 4,253,929 reads across 59 OTUs (Insecta = 58, Mammalia = 1) and 262 samples (Table 2). Big Brown Bat OTUs were present in 51 samples and Insecta OTUs included 7 orders, 18 families, 33 genera, and 34 species (Table 3). All OTUs documented in 2017 were also documented in 2018.

Our stringent filtering criteria eliminated many ≥97% confidence OTUs with few reads, including orders consumed by many individuals (e.g., Diptera) and taxa detected as environmental DNA present in samples (e.g., Anura) (Table 1).

Table 1, continued.

	≥97%		100%		Stringent filtering criteria	
Order	Samples	%	Samples	%	Samples	%
Lithobiomorpha	2	0.8	2	0.8	-	-
Lophiiformes	1	0.4	1	0.4	-	-
Mantodea	9	3.4	8	3	-	-
Mecoptera	2	0.8	-	-	-	-
Megaloptera	6	2.3	4	1.5	4	1.5
Mesostigmata	22	8.3	17	6.5	-	-
Myctophiformes	2	0.8	2	0.8	-	-
Myliobatiformes	2	0.8	-	-	-	-
Neuroptera	23	8.7	16	6.1	-	-
Odonata	11	4.2	4	1.5	-	-
Orthoptera	35	13.3	5	1.9	-	-
Ovalentaria	1	0.4	1	0.4	-	-
Pantopoda	4	1.5	4	1.5	-	-
Passeriformes	3	1.1	3	1.1	-	-
Perciformes	4	1.5	3	1.1	-	-
Plecoptera	8	3	7	2.7	-	-
Pleuronectiformes	3	1.1	2	0.8	-	-
Poduromorpha	25	9.5	23	8.7	-	-
Poecilostomatoida	2	0.8	-	-	-	-
Polydesmida	4	1.5	2	0.8	-	-
Primates	4	1.5	4	1.5	-	-
Pseudoscorpiones	5	1.9	4	1.5	-	-
Psocodea	27	10.2	22	8.4	-	-
Raphidioptera	2	0.8	2	0.8	-	-
Rodentia	18	6.8	17	6.5	-	-
Sarcoptiformes	25	9.5	20	7.6	-	-
Scorpaeniformes	1	0.4	1	0.4	-	-
Scorpiones	24	9.1	13	4.9	-	-
Siluriformes	5	1.9	5	1.9	-	-
Siphonostomatoida	4	1.5	4	1.5	-	-
Soricomorpha	2	0.8	1	0.4	-	-
Spariformes	1	0.4	-	-	-	-
Squamata	10	3.8	9	3.4	-	-
Stomiiformes	1	0.4	1	0.4	-	-
Symphypleona	5	1.9	4	1.5	-	-
Tetraodontiformes	1	0.4	1	0.4	-	-
Thysanoptera	21	8	15	5.7	-	-
Trichoptera	39	14.8	36	13.7	10	3.8
Trombidiformes	24	9.1	19	7.2	-	-

The number of samples with dipteran OTUs indicated that almost all bats are consuming prey items from this order, but the number of reads from the 2 most common dipteran OTUs (present in 80 and 38 samples, respectively) encompassed less than 0.001% of the total Insecta reads. The dipteran OTU with the most reads (Family Ceratopogonidae [midges], n = 142,764) came from 1 sample. Eliminating OTUs present in only 1 fecal sample excluded 3 OTUs (1 each of Diptera, Coleoptera, and Ichneumonidae). Hereafter, we only present results from our stringent filtering criteria.

As expected, Coleoptera were present in most samples (90.8%), followed by Hemiptera, Blattodea (cockroaches and termites), Hymenoptera (sawflies, wasps, bees, and ants), Lepidoptera (butterflies and moths), Trichoptera (caddisflies), and Megaloptera (alderflies, dobsonflies and fishflies; Tables 2, 3; Fig. 2). Every NPS unit included a sample with Coleoptera, and Hemiptera were present in prey items for every unit except WO. Elateridae (Order Coleoptera; click beetles) was the family we most commonly observed, and *Melanotus* (Family Elateridae; click beetles) was the most common genus (Table 2). Families Carabidae (ground beetles), Elateridae, Pentatomidae (stink bugs or terrestrial turtle bugs), and Scarabaeidae (scarabs) were present in the highest proportions of samples. Proportions of samples for each family varied greatly among sample areas and time (see Supplemental File 1, available online at https://www.eaglehill.us/NENAonline/suppl-files/n29-4-N1935-Deeley-s1, and for BioOne subscribers, at https://www.doi.org/10.1656/

Table 2. *Eptesicus fuscus* (Big Brown Bat) demographic information for fecal samples collected 2017–2018 with mist netting in: Antietam National Battlefield (AN), Catoctin Mountain Park (CA), Chesapeake and Ohio Canal National Historical Park and Harpers Ferry National Historic Park (CH), Gettysburg National Battlefield (GE), George Washington Memorial Parkway (GW), Manassas National Battlefield (MO), National Capital Parks-East (NA), Prince William Forest Park and Marine Corps Base Quantico (PQ), Rock Creek Park (RO), and Wolf Trap National Park for the Performing Arts (WO). Reproductive status of the bats: D = descended testes, NR= non-reproductive male; P = pregnant, L = lactating, PL = post-lactating, U = unknown reproductive status or non-reproductive female, and Missing = no data was recorded for that bat.

		Female						Male				
				Reproductive condition						Reproductive condition		
Area	Total samples	Adult	Juvenile	P	PL	L	U	Adult	Juvenile	D	NR	Missing
AN	2	0	0	0	0	0	0	0	2	0	2	0
CA	8	2	0	2	0	0	0	5	1	5	1	0
CH	60	25	6	2	10	5	14	26	3	18	11	0
GE	29	11	1	2	3	6	1	16	1	16	1	0
GW	7	6	0	4	0	1	1	1	0	0	1	0
MA	1	0	0	0	0	0	0	1	0	1	0	0
MO	3	0	0	0	0	0	0	2	1	3	0	0
NA	4	3	0	1	0	1	1	0	1	0	1	
PQ	56	23	2	1	8	13	3	23	3	19	7	5
RO	91	37	0	22	0	7	8	45	1	11	35	8
WO	1	0	0	0	0	0	0	1	0	0	1	0

Table 3. Number of samples with observations of each prey taxon among fecal samples from 262 *Eptesicus fuscus* (Big Brown Bat) collected within the District of Columbia, Maryland, Pennsylvania, and Virginia, 2017–2018. Operational taxonomic unit (OTU) samples indicate the number of observations with the particular terminal taxonomic identification for that OTU. OTU percent is the number of OTU samples divided by the total OTUs. Higher-order samples are the number of samples for orders and genera based on taxonomic hierarchy. Previous detection indicates references to previous studies that either detected that taxon or (in parentheses) the higher-level identifications within unknown lower taxa. Samples and OTUs were determined through our stringent filtering criteria. All studies were morphologically based, except for Long et al. (2003), who performed genetic analysis on insect parts. Key: H = Hamilton 1933, P = Phillips 1966, W1 = Whitaker 1972, GG = Griffith and Gates 1985, BS = Brigham and Saunders 1990, W2 = Whitaker 1995, HB = Hamilton and Barclay 1998, M = Menzel et al. 2000, AM = Agosta and Morton 2003, A = Agosta et al. 2003, and L = Long et al. 2013. Note that Megaloptera were formerly within Neuroptera. [Table continued on following page.]

Class	Order	Family	Genus	Species	OTU samples	OTU %	Higher-order samples	Previous detection
Insecta	Blattodea	Ectobiidae	*Parcoblatta*	-	48	5.8%	-	M, W1, (order)
Insecta	Coleoptera	-	-	-	156	18.8%	238	M, BS, P, H, W2, W1, AM, GG, HB, A,
Insecta	Coleoptera	Brentidae	*Arrhenodes*	*Arrhenodes minutus*	10	1.2%	-	
Insecta	Coleoptera	Carabidae	-	-	-	-	77	L, W1, AM, W2,
Insecta	Coleoptera	Carabidae	*Agonum*	*Agonum punctiforme*	7	0.8%	-	
Insecta	Coleoptera	Carabidae	*Amphasia*	*Amphasia sericea*	5	0.6%	-	
Insecta	Coleoptera	Carabidae	*Calosoma*	*Calosoma wilcoxi*	3	0.4%	-	
Insecta	Coleoptera	Carabidae	*Chlaenius*	*Chlaenius tricolor*	5	0.6%	-	
Insecta	Coleoptera	Carabidae	*Harpalus*	*Harpalus pensylvanicus*	32	3.9%	-	L
Insecta	Coleoptera	Carabidae	*Notiobia*	*Notiobia terminata*	25	3.0%	-	L
Insecta	Coleoptera	Carabidae	*Selenophorus*	*Selenophorus opalinus*	16	1.9%	-	
Insecta	Coleoptera	Carabidae	*Stenolophus*	*Stenolophus ochropezus*	16	1.9%	-	L
Insecta	Coleoptera	Cerambycidae			-	-	36	L
Insecta	Coleoptera	Cerambycidae	*Astylopsis*	*Astylopsis sexguttata*	2	0.2%	-	
Insecta	Coleoptera	Cerambycidae	*Graphisurus*	*Graphisurus fasciatus*	7	0.8%	-	
Insecta	Coleoptera	Cerambycidae	*Orthosoma*	-	27	3.3%	-	
Insecta	Coleoptera	Elateridae	-	-	-		123	AM
Insecta	Coleoptera	Elateridae	*Athous*	*Athous brightwelli*	6	0.7%	-	
Insecta	Coleoptera	Elateridae	*Elater*	*Elater abruptus*	7	0.8%	-	
Insecta	Coleoptera	Elateridae	*Hemicrepidius*		-	-	42	L, W1, AM
Insecta	Coleoptera	Elateridae	*Hemicrepidius*	*Hemicrepidius brevicollis*	3	0.4%	-	
Insecta	Coleoptera	Elateridae	*Hemicrepidius*	*Hemicrepidius memnonius*	39	4.7%	-	L
Insecta	Coleoptera	Elateridae	*Melanotus*	-	13	1.6%	92	L

Table 3, continued

Class	Order	Family	Genus	Species	OTU samples	OTU %	Higher-order samples	Previous detection
Insecta	Coleoptera	Elateridae	*Melanotus*	*Melanotus decumanus*	6	0.7%	-	L, (genus)
Insecta	Coleoptera	Elateridae	*Melanotus*	*Melanotus morosus*	21	2.5%	-	L, (genus)
Insecta	Coleoptera	Elateridae	*Melanotus*	*Melanotus similis*	67	8.1%	-	L
Insecta	Coleoptera	Hydrophilidae	-	-	4	0.5%	-	H
Insecta	Coleoptera	Lucanidae	*Ceruchus*	*Ceruchus piceus*	12	1.4%	-	
Insecta	Coleoptera	Pyrochroidae	*Dendroides*	*Dendroides canadensis*	10	1.2%	-	
Insecta	Coleoptera	Scarabaeidae	-	-	-	-	61	P, W2, W1, AM, GG
Insecta	Coleoptera	Scarabaeidae	*Nipponoserica*	*Nipponoserica peregrina*	34	4.1%		
Insecta	Coleoptera	Scarabaeidae	*Phyllophaga*	-	12	1.4%	18	P
Insecta	Coleoptera	Scarabaeidae	*Phyllophaga*	*Phyllophaga drakii*	6	0.7%	-	P (genus)
Insecta	Coleoptera	Scarabaeidae	*Serica*	*Serica atracapilla*	14	1.7%	-	
Insecta	Coleoptera	Tenebrionidae	-	-	-	-	28	
Insecta	Coleoptera	Tenebrionidae	*Alobates*	*Alobates pensylvanicus*	18	2.2%	-	
Insecta	Coleoptera	Tenebrionidae	*Hymenorus*	*Hymenorus picipennis*	10	1.2%	-	
Insecta	Hemiptera	Cicadidae	*Tibicen*	*Tibicen lyricen*	3	0.4%	-	M, W2, AM, A, H (order)
Insecta	Hemiptera	Miridae	*Lygus*	*Lygus lineolaris*	15	1.8%	-	M, W2, AM, A, H (order)
Insecta	Hemiptera	Pentatomidae	-	-	-	-	60	W2, GG, P; M, BS, AM (order)
Insecta	Hemiptera	Pentatomidae	*Acrosternum*	*Acrosternum hilare*	38	4.6%	-	AM, W2, A (genus); P, GG (family)
Insecta	Hemiptera	Pentatomidae	*Halyomorpha*	*Halyomorpha halys*	27	3.3%	-	P, GG (family); AM, W2 (order)
Insecta	Hymenoptera	Formicidae	*Camponotus*	-	19	2.3%	-	W2, P, GG, A, W1, M (family); AM, H, HB (order)
Insecta	Lepidoptera					0.0%		
Insecta	Lepidoptera	Limacodidae	*Isa*	*Isa textula*	2	0.2%	-	M, BS, W2, W1, GG, AM, HB, A, P, (order)
Insecta	Lepidoptera	Tineidae	*Acrolophus*	*Acrolophus popeanella*	17	2.0%	-	M, BS, W2, W1, GG, AM, HB, A, P (order)
Insecta	Megaloptera	Corydalidae	*Chauliodes*	*Chauliodes pectinicornis*	4	0.5%	-	W2 (order)
Insecta	Trichoptera	Hydropsychidae	*Hydropsyche*	-	-	-	10	W2, BS, W1, GG, H, HB (order)
Insecta	Trichoptera	Hydropsychidae	*Hydropsyche*	*Hydropsyche hageni*	10	1.2%	10	W2, BS, W1, GG, H, HB (order)
Insecta	Trichoptera	Hydropsychidae	*Hydropsyche*	*Hydropsyche hoffmani*	3	0.4%	-	W2, BS, W1, GG, H, HB (order)
Mammalia	Chiroptera	Vespertilionidae	*Eptesicus*	*Eptesicus fuscus*	51	6.1%	-	GG (order)

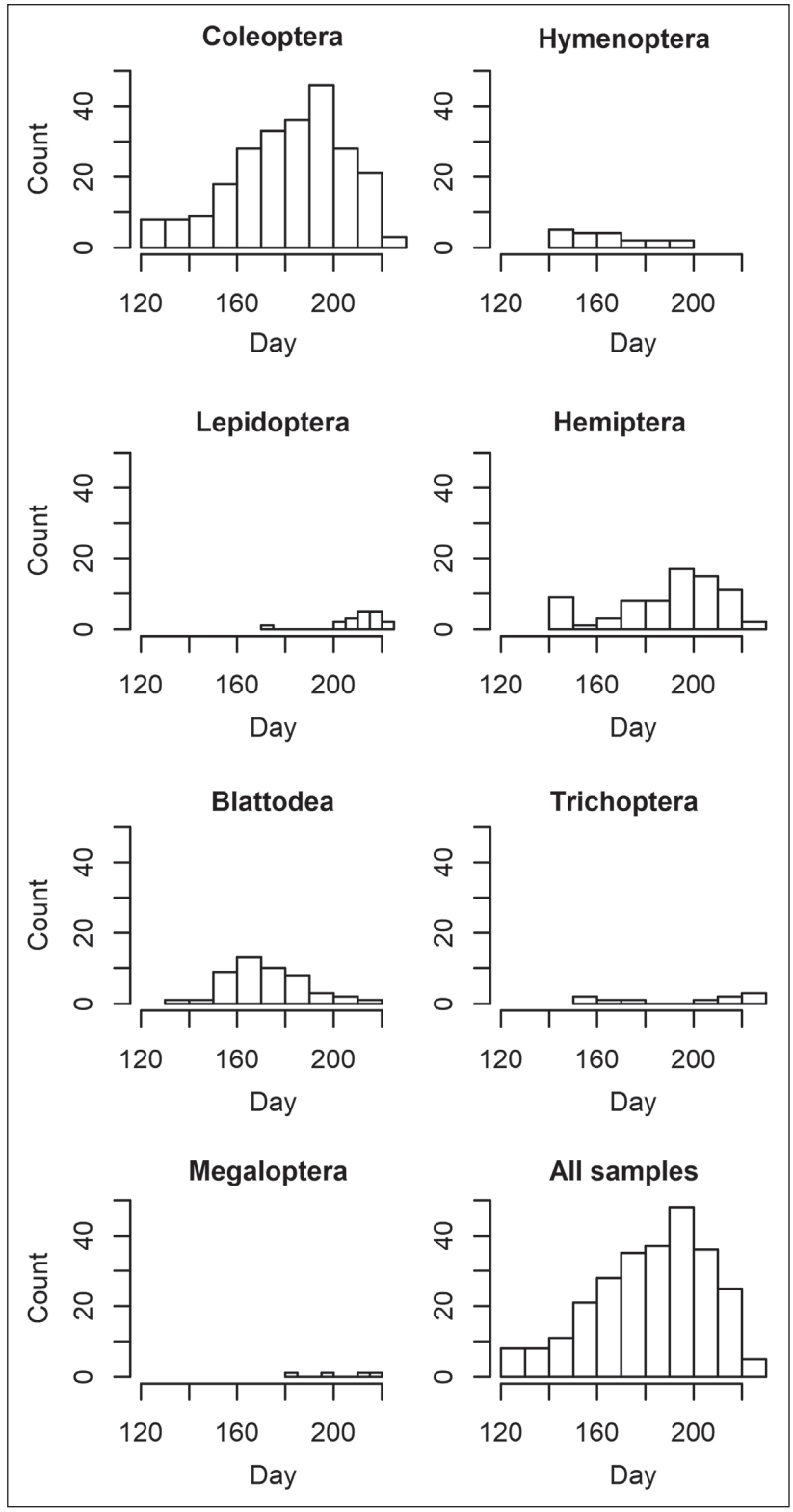

Figure 2. Number of samples in listed orders by day of year (Day) within 262 *Eptesicus fuscus* (Big Brown Bat) fecal samples collected in the District of Columbia, Maryland, Pennsylvania, and Virginia from 2017 to 2018. Operational taxonomic units and taxa were determined through our stringent filtering criteria.

N1935.s1); Elateridae were in the highest proportion for samples for CA, CH, GW (in the same proportion as Carabidae), PQ, RO, and WO (at the same number as Scarabaeidae).

Order-level collector's curves indicated that a plateau in taxa occurred only as the full number of samples were included (Fig. 3). Although some family- and genus-level collector's curves appeared to show a flattening slope, they did not reach an asymptote. Therefore, the unequal sample size across areas, periods, sex, and reproductive status limited our ability for statistical comparisons. Within Coleoptera, we noted differences in the presence of families among the GE, RO, and PQ areas across time (see Supplemental File 1). Although pregnant bats showed dietary richness similar to male bats, lactating and post-lactating females showed somewhat lower dietary richness (Fig. 4). Juvenile bats consumed no OTUs that were not also consumed by adults.

Discussion

Our results showed most of the same orders of Big Brown Bat prey that were observed using visual prey identification methods (Table 3; Agosta and Morton 2003; Griffith and Gates 1985; Hamilton 1933; Hamilton and Barclay 1998; Whitaker 1972, 1995). However, in contrast, we did document heretofore undocumented putative consumption of families Brentidae (*Arrhenodes minutus* [Oak Timberworm]), Pyrochroidae (*Dendroides canadensis* [Fire-colored Beetle]), Tenebrionidae (*Alobates pensylvanicus* [False Mealworm Beetle] and *Hymenorus picipennis*), and Hydropsychidae (*Hydropsyche hageni* and *Hydropsyche hoffmani*)

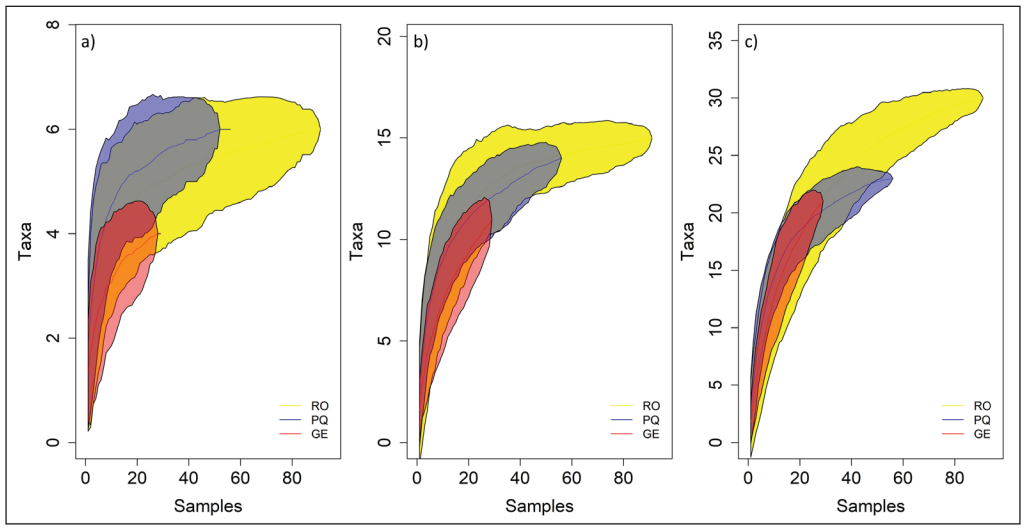

Figure 3. Collector's curves (see text) with standard-error buffers for prey taxa observed in *Eptesicus fuscus* (Big Brown Bat) fecal samples collected 2017–2018 at the (a) order, (b) family, and (c) genus levels. Sampled areas include Gettysburg National Battlefield (GE; red), Prince William Forest Park and Marine Corps Base Quantico (PQ; blue) and Rock Creek Park (RO; yellow). Operational taxonomic units and taxa were determined through our stringent filtering criteria.

(Table 2). Similar to the results of previous studies using visually based methods (Griffith and Gates 1985; Hamilton 1933; Hamilton and Barclay 1998; Whitaker 1972, 1995), we found that members of Order Coleoptera were the most common

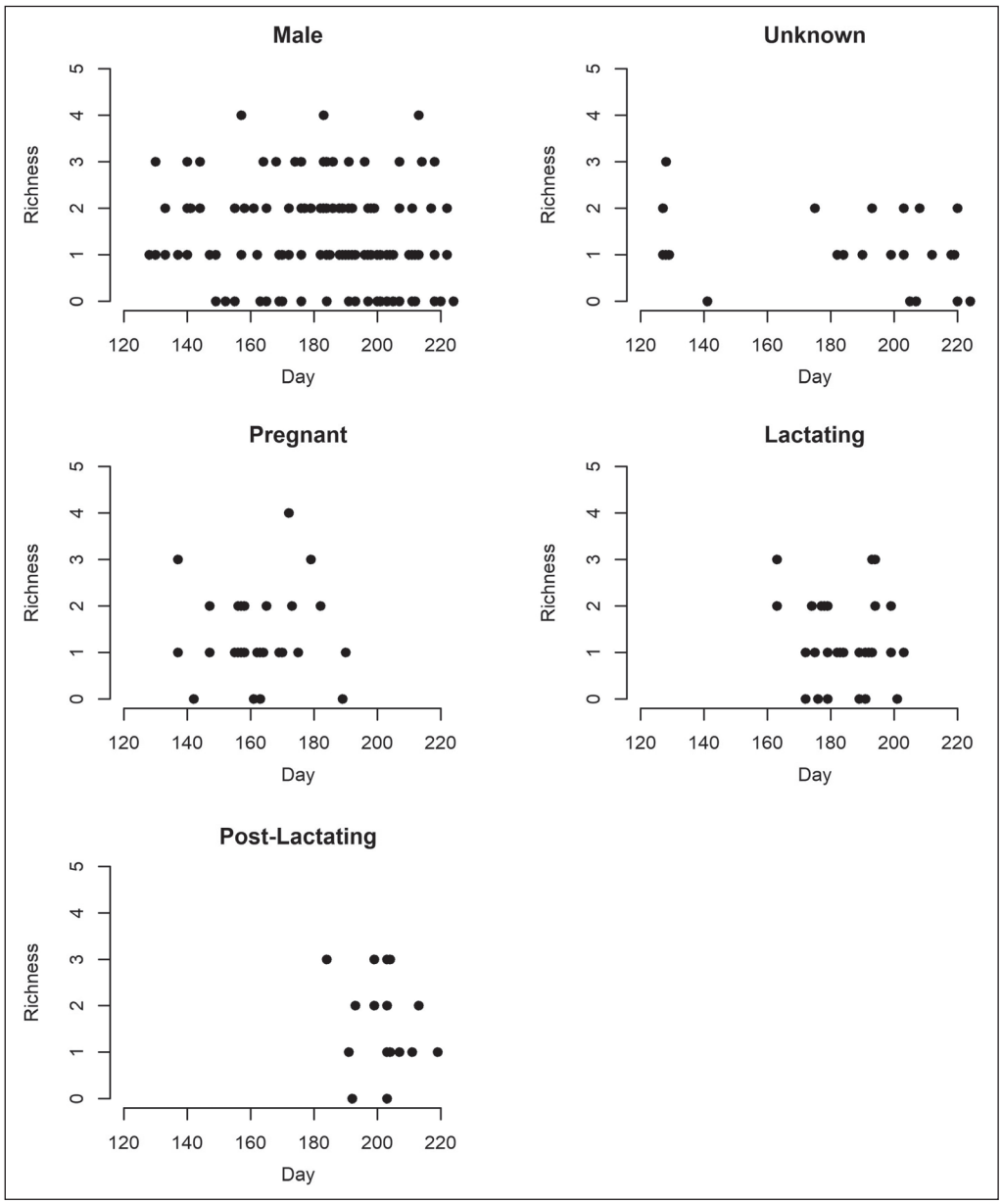

Figure 4. Richness of Coleopteran families by bat sex and reproductive condition within 249 *Eptesicus fuscus* (Big Brown Bat) fecal samples collected in the District of Columbia, Maryland, Pennsylvania, and Virginia from 2018 to 2019. Males with descended testes and in a non-reproductive status were combined (Male). The remaining categories represent female reproductive conditions. Operational taxonomic units and taxa were determined by applying our stringent operational taxonomic unit filtering criteria.

in terms of both abundance and frequency of occurrence across samples. Our results also were similar to those of other DNA-sequencing studies of Big Brown Bat diet, including a DNA barcoding-based assessment conducted by Long et al. (2013) in Michigan and a metabarcoding survey by Clare et al. (2014) in Canada. However, unlike our results, Long et al. (2013) and Clare et al. (2014) found family Carabidae more common than Elateridae, which may be reflective of regional insect abundance and consequent diet differences between Big Brown Bats in the temperate mid-Atlantic biome versus the northern transition zone or in differences in sequencing techniques. Big Brown Bat OTUs were observed in fewer samples than might be expected. We attribute this low frequency to use of a primer pair designed to amplify insect DNA, with any observations of bat DNA due to low annealing temperatures in the PCR amplifications.

Notably, we observed that OTU inclusion standards affected the perceived distribution of prey items in the diet of Big Brown Bats. Whereas our criteria were appropriate for a conservative assessment of diet, it excluded smaller-bodied and less-often consumed OTUs and taxa. Although we should not assume that reads reflect relative abundance of a particular prey item (Pompanon et al. 2012), it is revealing that read numbers of likely prey items were low within many samples. In particular, Diptera were consumed by most bats (using the less-strict ≥97% confidence criterion; Table 1), indicating that almost all bats are consuming items from the order. An issue with strict per-OTU filtering thresholds is that signal may be obscured. Diptera are speciose, and as Big Brown Bats consume generally, we would expect a high richness of OTUs, but a low number of individuals per OTU, as was reflected in the low number of reads and large number OTUs present in just 1 sample. Additionally, the use of particular PCR primers and amplification conditions also may have affected our findings. Primer pair ANML amplified fewer dipteran taxa in dietary samples than other primer pairs (Novella-Fernandez et al. 2020).

We did not observe, in the results from our stringent filtering criteria, the diversity within bat diets that Clare et al. (2014) documented at all taxonomic levels with their analysis. Although Big Brown Bat diet may differ between Ontario and the mid-Atlantic, by setting our criterion for identification of a taxon in the diet based on the relative abundance of taxa of environmental origin, we excluded rarer, as well as smaller, prey taxa. For example, unlike other visual and DNA-based analyses of Big Brown Bat feces (Agosta and Morton 2003, Clare et al. 2014, Hamilton 1933, Long et al. 2013, Whitaker 1995), our more-stringent selection criteria resulted in the exclusion of Diptera. Additionally, Agosta and Morton (2003) documented taxa from a mine near our northern CH sites via visual identification that our stringent filtering criteria excluded (e.g., Order Diptera and Family Curculionidae).

We believe that our standards for retention of data were sufficiently strict, as all carabid species that we documented, except *Calosoma wilcoxi*, also were identified in a 9-year study of Carabidae within GW (Steury 2014). Additionally, along the Potomac River at GW, Flint (2008, 2011) documented the 2 caddisfly species (Family Hydropsychidae, Order Trichoptera) that we identified in bat diets at CH

and RO. The Hydropsychidae are an abundant caddisfly group typically found in wetland areas; therefore, their consumption by bats within 2 units adjacent to wetland areas and in proximity to that river would not be surprising, as Big Brown Bats use riparian areas for foraging (Ford et al. 2005, 2006). Additionally, in the CH mine survey, Agosta and Morton (2003) also identified *Acrosternum* spp. (stinkbugs).

Perhaps of more importance to resource managers, we documented pest families, genera, and species in bat diets throughout the study area, including within both highly developed and fragmented areas (e.g., NA) and highly forested areas (e.g., CA). For example, we found 2 genera of click beetles that are agricultural pests: *Melanotus* and *Hemicrepidius* (Belcher and Tenne 1987, Riley and Keaster 1979). The presence of these species in our samples may be the result of Big Brown Bats foraging in the suburban and agricultural habitats surrounding the sampling area's more contiguous forest stands. Hemipteran pest species included *Lygus lineolaris* (Tarnished Plant Bug), which damages crops such as *Fragaria* x*ananassa* (Strawberry; Mailloux and Bostanian 1988); *Acrosternum hilare* (Green Stink Bug), a significant pest on *Glycine max* (Soybean; McPherson et al. 1979); and *Halyomorpha halys* (Brown Marmorated Stink Bug), an invasive species within the mid-Atlantic region that damages a wide array of tree and crop species (Nielsen and Hamilton 2009). Within Family Carabidae, we documented crop pests such as *Harpalus pensylvanicus* (Pennsylvania Ground Beetle) and the tree pest Oak Timberworm (Family Brentidae; Buchanan 1960, Esau and Peters 1975, Kirk 1973).

Our collector's curves (Fig. 3) indicated that despite the large sampling effort in PQ and RO, we likely did not capture the full extent of prey within any area. Therefore, any comparisons of diet between areas, reproductive conditions, and times must be caveated by sample size. We suggest that diet assessments such as ours continue to determine whether sampling is fully adequate before categorical comparisons of diet are made over space, time, or between demographic groups. Though our ability for complex analysis was limited, our results indicated that male bats had higher diet diversity than reproductive females, similar to the findings of Czenze et al. (2018). As expected, diet changed between periods, likely reflective of seasonal insect phenology and population dynamics on the landscape. For example, Pennsylvania Ground Beetles emerge from the soil as adults early in the summer with populations increasing until mating and egg oviposting in the late summer (Kirk 1973), a pattern we observed from Big Brown Bat fecal samples.

Our methods identified prey at finer taxonomic levels (such as species-level) than visual identification had previously achieved, supporting additional inference of depredation of interest to both ecologists and land managers, such as predation upon pest species. However, we suggest using a thoughtful approach to interpreting metabarcoding data for identifying prey items from bat fecal samples and adoption of consistent criteria for comparing results among metabarcoding studies. Big Brown Bats may have consumed DNA from Chordata and non-prey insect taxa by drinking water, by exposure to biological material at roosting locations, or incidental capture, but the possibility of sample contamination cannot be discounted.

Metabarcoding may provide insights into host population health; for example, exposure to DNA from mite and tick parasites can aid in assessments of pathogen pathways for bats and other species. Likewise, less-strict screening criteria also may provide insights into other behaviors: the Gray Squirrel reads that served as the basis for our conservative screening imply that bats used day-roost sites previously inhabited by squirrels.

If conducting a conservative "presence-only" assessment of diet, we suggest filtering OTUs by confidence level and then conducting post-hoc assessments of the results to reach inferences regarding both prey and predator habitat associations relative to the individual research question being addressed. Our results highlight the analytical difficulty in distinguishing OTUs present due to depredation as opposed to incidental consumption. Therefore, if the intent of a study is to assess diet only, then a combination of OTU confidence level and read numbers can be used to develop a conservative taxonomic list, whereas if using findings as a tool to monitor invasive insects, a more liberal OTU confidence level might be appropriate. Regardless, metabarcoding technology provides a valuable new tool for identifying dietary items and other items due to environmental exposure that represents a tremendous improvement over traditional techniques using visual identification.

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Appendix 1. Table of taxa mentioned in the manuscript, including common names and authorities.

Species	Authority	Common name
Arrhenodes minutus	(Drury)	Oak Timberworm
Agonum punctiforme	(Say)	Gound beetle species
Amphasia sericea	(Harris)	Gound beetle species
Calosoma wilcoxi	LeConte	Gound beetle species
Chlaenius tricolor	Dejean	Gound beetle species
Harpalus pensylvanicus	(De Geer)	Pennsylvania Ground Beetle
Notiobia terminata	(Say)	Gound beetle species
Selenophorus opalinus	(LeConte)	Gound beetle species
Stenolophus ochropezus	(Say)	Gound beetle species
Astylopsis sexguttata	(Say)	Long-horned Beetles
Graphisurus fasciatus	(De Geer)	Longhorn beetle species
Athous brightwelli	(Kirby)	Click beetle species
Elater abruptus	Say	Click beetle species
Hemicrepidius brevicollis	(Candèze)	Click beetle species
Hemicrepidius memnonius	Herbst	Click beetle species
Melanotus decumanus	(Erichson)	Click beetle species
Melanotus morosus	Candèze	Click beetle species
Melanotus similis	(Kirby)	Click beetle species
Ceruchus piceus	(Weber)	Stag beetle species
Dendroides canadensis	Latreille	Fire-Colored Beetle
Nipponoserica peregrina	(Chapin)	Scarab beetle species
Phyllophaga drakii	(Kirby)	Scarab beetle species
Serica atracapilla	(Kirby)	Scarab beetle species
Alobates pensylvanicus	(De Geer)	False Mealworm Beetle
Hymenorus picipennis	Casey	Darkling beetle species
Tibicen lyricen	(De Geer)	Lyric Cicada
Lygus lineolaris	(Palisot)	Tarnished Plant Bug
Acrosternum hilare	(Say)	Green Stink Bug
Halyomorpha halys	Stål	Brown Marmorated Stink Bug
Isa textula	(Herrich-Schäffe)	Crowned Slug Moth
Acrolophus popeanella	(Clemens)	Clemens' Grass Tubeworm Moth
Chauliodes pectinicornis	(L.)	Summer Fishfly
Hydropsyche hageni	Banks	Netspinning caddisfly species
Hydropsyche hoffmani	Ross	Netspinning caddisfly species
Eptesicus fuscus	(Palisot de Beauvois)	Big Brown Bat
Sciurus carolinensis	Gmelin	Gray Squirrel
Glycine max	(L.) Merr.	Soybean
Fragaria x *ananassa*	(Weston) Duschene ex Rozier	Stawberry