Out of thin air: surveying tropical bat roosts through air sampling of eDNA

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ABSTRACT

Understanding roosting behaviour is essential to bat conservation and biomonitoring, often providing the most accurate methods of assessing bat population size and health. However, roosts can be challenging to survey, *e.g.*, physically impossible to access or presenting risks for researchers. Disturbance during monitoring can also disrupt natural bat behaviour and present material risks to the population such as disrupting hibernation cycles. One solution to this is the use of non-invasive monitoring approaches. Environmental (e)DNA has proven especially effective at detecting rare and elusive species particularly in hard-to-reach locations. It has recently been demonstrated that eDNA from vertebrates is carried in air. When collected in semi-confined spaces, this airborne eDNA can provide remarkably accurate profiles of biodiversity, even in complex tropical communities. In this study, we deploy novel airborne eDNA collection for the first time in a natural setting and use this approach to survey difficult to access potential roosts in the neotropics. Using airborne eDNA, we confirmed the presence of bats in nine out of 12 roosts. The identified species matched previous records of roost use obtained from photographic and live capture methods, thus demonstrating the utility of this approach. We also detected the presence of the white-winged vampire bat (*Diaemus youngi*) which had never been confirmed in the area but was long suspected based on range maps. In addition to the bats, we detected several non-bat vertebrates, including the big-eared climbing rat (*Ototylomys phyllotis*), which has previously been observed in and around bat roosts in our study area. We also detected eDNA from other local species known to be in the vicinity. Using airborne eDNA to detect new roosts and monitor known populations, particularly when species turnover is rapid, could maximize efficiency for surveyors while minimizing disturbance to the animals. This study presents the first applied use of airborne eDNA collection for ecological analysis moving beyond proof of concept to demonstrate a clear utility for this technology in the wild.

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INTRODUCTION

Bats and their roosts

Bat species are characterized by a wide variety of roosting ecologies (*Fenton & Ratcliffe, 2010*; *Voss et al., 2016*) utilizing caves, trees, man-made structures, cracks in rocks (*Altringham, 2011*), leaf litter (*Mormann & Robbins, 2007*), and even pitcher plants (*Grafe et al., 2011*). Some species modify the environment to create their roosts (*e.g.*, creating leaf tents (*Kunz, 1982*) or excavating roosts within termite mounds (*Esquivel et al., 2020*)) and multiple species may use the same roost (*Villalobos-Chaves et al., 2016*; *Kelm, Toelch & Jones, 2021*). Bats require safe roosts that provide protection from predators with appropriate environmental conditions related to temperature and humidity. Bats may use different roosts at night or during the day at different times of year, for breeding, migration or hibernation (*Altringham, 2011*). Roosts are additionally important for mating and raising young, playing a key role in social interactions and maintaining populations (*Humphrey, 1975*; *Kunz, 1982*). Many bat species live in fission-fusion societies meaning they will roost together during some but not all periods, with subgroups moving among roosts over time, hence roost switching is common (*Patriquin & Ratcliffe, 2016*). Roost switching supports larger social networks (*Patriquin & Ratcliffe, 2016*) but generates high individual turnover rates in roost occupancy (*Aguirre, Lenstra & Matthysen, 2003*; *Patriquin & Ratcliffe, 2016*) creating a challenge for conservation monitoring.

Understanding roosting ecology is important for bat conservation, especially as roosts are thought to be a limiting resource for some species (*Humphrey, 1975*; *Aguirre, Lenstra & Matthysen, 2003*; *Voss et al., 2016*). Roost surveys can inform decision making regarding the protection of bat habitat and roost loss prevention (*Villalobos-Chaves et al., 2016*), and can help understand and monitor community composition (*Voss et al., 2016*; *Kelm, Toelch & Jones, 2021*). Annual roost surveys using visual counts, mist nets, acoustic monitoring, and PIT tagging are conducted in many regions to estimate population health (*Kaarakka, 2020*; *Bat Conservation Trust, 2021*). Roost monitoring in temperate zones provides insight into migration stopovers and hibernation patterns (*Klüg-Baerwald et al., 2017*). Monitoring roost occupancy is one of the most effective ways to estimate the population sizes of some species (*Kunz, 2003*). Roost occupancy counts have also been key to tracking the impact of disease dynamics *e.g.*, white nose syndrome in North American populations (*Janicki et al., 2015*), particularly during hibernation.

Airborne eDNA sampling for roost surveys

Traditional methods of roost surveying such as mist netting or trapping outside of roosts, visual surveys inside roosts, and camera trapping entrances can be challenging, expensive in time and cost, and may disturb the animals. For example, standard camera traps for other mammals do not work for fast-moving species such as bats. Instead, specialized systems using infrared trip beams and high speed flashes are required, which can only partially be automated and may still disrupt bat behavior (*Rydell et al., 2022*). Similarly,

acoustic monitoring cannot be used on inactive hibernating bats and is challenging in neotropical areas where calls are not species specific. Some roosts are physically inaccessible while others are too dangerous or toxic for humans to explore, which limits manual observation counts. It can be challenging to accurately determine species composition using existing methods (*Behrens et al., 2017*), and methods that involve capture or entering roosts additionally risk disturbing bats. Methods that involve entering hibernacula can be especially detrimental to hibernating bats since they can cause arousal and unnecessary use of fat reserves (*Speakman, Webb & Racey, 1991*). A non-invasive sampling method that does not require physical access to the bats could help overcome these challenges.

One way to increase roost monitoring efficiency is the use of environmental (e)DNA. eDNA is any genetic material not collected directly from an individual (*e.g.*, hair fragments or skin cells free floating in the environment), and it has become a powerful tool in detecting organisms without physical access to individuals. Sampling eDNA from water or soil has become widespread (*Thomsen & Willerslev, 2015*) and collecting aquatic eDNA is now a common industry tool in monitoring aquatic ecosystems (*Rees et al., 2014*; *Ruppert, Kline & Rahman, 2019*). More unconventional methods in terrestrial zones have targeted eDNA from spider webs (*Gregorič et al., 2022*) and snow tracks (*Kinoshita et al., 2019*) to learn about local ecology. Cavity roosts of bats have been suggested as an ideal target for terrestrial eDNA collections (*Clare et al., 2021*). The very reason that roosts are used by bats—because they are enclosed and protected—may contribute to the longer-term preservation of environmental DNA which might otherwise degrade or be washed away (*Mena et al., 2021*) or become too dispersed to capture. While no bats were detected, soil from caves has been used to detect some cave-dwelling vertebrate species, both those that are currently present and those from recent occupation (*Hofreiter et al., 2003*), suggesting the presence of accumulating eDNA in these habitats.

Collecting and analysing airborne eDNA has been proposed as a method to monitor terrestrial animals (*Barnes & Turner, 2015*; *Ruppert, Kline & Rahman, 2019*). The first article to demonstrate this technique targeted naked mole rats in artificial burrows (*Clare et al., 2021*) because of the perceived potential for eDNA to build up in an enclosed space. Airborne eDNA detection of vertebrates, insects and general biodiversity is in its infancy, but has already proven useful in detecting plant species missed using conventional sampling (*Johnson et al., 2021*). Airborne eDNA does not require access to the individual animal, reducing the risks associated with disrupting roosting bats, potentially allowing extended sampling times in otherwise inhospitable roosts and permitting sampling in roosts that are inaccessible using existing methods. The use of airborne eDNA to detect terrestrial vertebrates has been validated both inside and outside artificial dens in zoos (*Clare et al., 2022*; *Lynggaard et al., 2022*). Passive airborne dust collection methods sampling for weeks at a time have also been able to detect recent mammal activity in natural landscapes (*Johnson et al., 2023*). More recently, *Garrett et al. (2023)* demonstrated that new prototype air sampling devices effectively detected eDNA from a diverse assemblage of Neotropical bats in an enclosed environment with remarkable accuracy.

These findings validated the use of airborne eDNA in complex communities and suggest an effective novel approach for surveying roosts.

Validating airborne eDNA for small cavity roost surveys in the neotropics

Given the potential of airborne eDNA demonstrated through previous pilot studies (*Clare et al., 2021*; *Serrao et al., 2021*; *Clare et al., 2022*; *Lynggaard et al., 2022*; *Garrett et al., 2023*), our objective was to evaluate airborne eDNA as an applied survey tool for a set of neotropical bat roosts in the first targeted deployment of airborne eDNA sampling in a truly natural setting. Our goals were to assess eDNA as a roost survey method and to develop a profile of roost use in our study area. Neotropical bat roosting behaviour is complex and understudied (*Fenton et al., 2001*; *Villalobos-Chaves et al., 2016*), and monitoring using airborne eDNA could be a game-changing approach to this field. The bat fauna in our study site has been well documented for over a decade using live capture methods (*i.e.*, mist nets, hand nets, and harp traps; *Fenton et al., 2001*; *Herrera et al., 2018*) and camera traps (*Rydell et al., 2022*) giving us *a priori* knowledge of the local bat fauna as well as baseline roosting ecology of many species. This creates an ideal study system in which to test the application of airborne eDNA during roost surveys and directly compare detections to known species inventories. Using this system, we collected airborne eDNA from a variety of natural and man-made roosts. We tested the hypothesis that airborne eDNA collects in sufficient quantities in natural roosts to document the roosting ecology of cavity-roosting neotropical bat species.

MATERIALS AND METHODS

Study site

This study was conducted in late April and early May 2022 in and around the riverine forest of Lamanai Archaeological Reserve (LAM) and the nearby forest fragment of the Ka'kabish Archeological Research Project (KKB) in the Orange Walk District of Belize. Both sites are ancient Maya cities that have become overgrown with semi-deciduous tropical forest. LAM contains excavated ruins that are open to the public and preserves approximately 450-ha of tropical forest adjacent to the freshwater New River lagoon (*Herrera et al., 2018*). The forest fragment at KKB is substantially smaller (45-ha) and is entirely surrounded by agricultural land (*Herrera et al., 2018*); it is not open to the public. Both LAM and KKB are surrounded by a matrix of agricultural fields, pastures, farms, and villages. Work in this area was conducted under Belize Forest Department permits FD/WL/1/21(12) and FD/WL/1/21(18), and Belize Institute of Archaeology permit IA/H/1/22(03).

Roost surveys

We sampled twelve known or suspected bat roosts in LAM, KKB, and nearby local farms and villages. These consisted of four tunnels carved into Maya ruins at KKB by archaeologists and looters (Figs. 1A–1C); one large cistern in LAM; one attic in a house in Indian Church Village; four hollows in large trees in the LAM; one natural cave of

Figure 1 Twelve natural and manmade roosts were surveyed using airborne eDNA. These included manmade looters tunnels in Maya ruins (A and C), archaeological excavations (B), tree roosts (D and F), and natural caves (E). Samplers were deployed inside roosts (*e.g.*, E) and left for up to 24 h to filter air (Images by Helen Haines (B), Elizabeth Clare (A, E and F), and Nancy Simmons (C and D)).

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Table 1 Sampling effort at each of the roost sites, indicating number of 12V air samplers run at one time, number of separate days they were deployed, and the runtime of each.

Type	Roost	# Samplers	# Deployments	Time sampled (h)
Natural caves	Indian Creek Cave	2	1	8
	Schoolhouse Cave	6	2	~24
Artificial tunnels (KKB)	Red Room	1	1	~24
	Plaza Tunnel	1	2	~24
	Natalus Tunnel	1	2	~24
	White Room	1	1	~24
Other human-made	Helen's House	1	1	~24
	Cistern (LAM)	2	1	6
Tree roosts (LAM)	High Temple Hollow Tree	1	2	6
	Museum Tree	1	1	6
	Sugar Mill High Tree	1	3	6
	Sugar Mill Low Tree	1	2	6

uncertain size in secondary forest (Indian Creek Cave) and one relatively small natural cave in a small cleared hill in a pasture (Schoolhouse Cave; Fig. 1E), both in the vicinity of Indian Creek (Table 1). At the Schoolhouse Cave, we placed our samplers 3 to 5 m inside and spanning the width of the cave (3 to 4 m) (Fig. 1E). Indian Creek Cave had a steep vertical drop at the entrance and the White Room roost at KKB was covered by an unstable tin roof, so for safety reasons we did not enter these roosts. In both cases we placed our samplers near the entrance. The Red Room roost (Fig. 1A) was approximately 4 to 5 m in height and 2 to 3 m in width. The White Room roost (Fig. 1B) was part of the same ruin as the Red Room roost, but on the opposite side of the structure. Two of the artificial tunnels in KKB, Plaza Tunnel, and *Natalus* Tunnel, were accessible but both are relatively narrow, about 1 to 1.5 m across (Fig. 1C) and 1.5 to 2 m tall. Three of the four hollow trees that we sampled were large Guanacaste trees around LAM (Figs. 1D and 1F), with accessible openings large enough to set up samplers inside the hollows. The Museum Tree had a much smaller opening, only a few centimetres wide and was located near the LAM museum. For this roost, the sampler was positioned facing inwards just outside the entrance slot. Sampling was done with approval from the York University Animal Care Committee (ACC), approval number: 2021-10.

We filtered roost air by deploying 12V Large samplers as described by *Garrett et al. (2023)* in each roost (Table 1 and Fig. 1). KN95 masks and gloves were worn by researchers when handling filters. Sampling time varied across roost sites based on roost size, access, and weather with some specific site access restrictions. Samplers in the LAM could only be deployed when the reserve was closed to the public, between late afternoon and dawn, limiting sampling hours at roosts in that area. Samplers also could not be left out during heavy rain in LAM as they were uncovered. In total we ran samplers for approximately 24 h in six of the roosts, for 6 h in five roosts, and for 8 h in one roost (Table 1). In the Schoolhouse Cave, we sampled for approximately 24 h starting at about 8:30 in the

morning; however, the filters were changed in the late afternoon, producing one set of daytime and one set of overnight samples. This resulted in 27 samples collected in total across 12 roost sites. Between each use, samplers were decontaminated using a 50% bleach solution followed by water to decrease instances of cross contamination between roosts. Filters were removed from samplers, folded so the exposed "disk" collection surface was on the inside, and placed in sterile bags before they were frozen at −20 °C for storage.

DNA extraction

Sample processing was performed in a decontaminated, UV sterilized Biosafety cabinet to limit sources of extraneous eDNA. The decontamination protocol involved cleaning the surfaces of the Biosafety cabinet (including the space under the grill) with 1% Virkon, followed by 70% Ethanol. A head cover, mask, lab coat, gloves and sleeve covers were used to minimize human DNA load during subsampling and DNA extraction. Prior to extraction we unfolded the filters (one at a time) and cut out a half circle from the centre of each filter disk using sterile scissors. We then cut each half circle into segments and placed these in a 5 mL Eppendorf tube with 4 mL of PBS. We soaked these overnight while incubating them at 56 °C using a rotary wheel hybridization oven. Following incubation, we transferred 1,000 µL of the PBS solution from each filter sample to a 1.5 mL DNA LoBind Eppendorf tube and spun it at 6,000 (×G) for 3 min. We pipetted the liquid into an empty 5 mL DNA LoBind Eppendorf tube, leaving any precipitate behind. We repeated this process until all the PBS was removed from the first 5 mL DNA LoBind Eppendorf tube and the precipitate was concentrated into one Eppendorf tube. For all subsequent steps we treated the precipitate as the "tissue" and DNA was extracted using a Qiagen Blood and Tissue Kit (Qiagen) following manufacturer's guidelines with the exception of the elution step, where we incubated the buffer at 56 °C and eluted the DNA in 100 µL of elution buffer. We processed extraction blanks using only the solutions in the kit as an extraction negative control. We froze the extracted DNA at −20 °C prior to PCR.

PCR and sequencing

PCR reagent preparation in 96-well plates was done in the AirClean PCR cabinet located in the ISO 7 Clean Room at NatureMetrics laboratory in Guelph, Ontario. Head covers, lab coats, gloves, sleeve covers, and boot covers were worn in the clean room to minimize human DNA contamination. PCR protocols follow those described by *Garrett et al. (2023)*. PCR setup (adding DNA to plates prepared in ISO 7 clean room) was performed in the PCR-free room in the AirClean PCR cabinet decontaminated and UV sterilized as described above, using the same PPE with the exception of boot covers. We preformed three technical replicate PCRs using the mam1 and mam2 primers (*Taylor, 1996*; *Calvignac-Spencer et al., 2013*) modified with Illumina adaptors. These primers have minimal mismatch with the target taxa (*Garrett et al., 2023*). We included negative (no template) and positive (*Pteronotus psilotis)* controls and visualized all PCR products, including all controls (positive, negative, and extraction blank) using an Invitrogen E-Gel™ 96 Agarose Gels with SYBR™ Safe DNA Gel Stain, 2%, run for 8 min on the E-Gel™ Power Snap Plus Electrophoresis System. All PCR products were sequenced on the

Illumina MiSeq by the NatureMetrics laboratory in Guelph, Ontario using the sequencing protocols of *Garrett et al. (2023)*. Reads were demultiplexed and exported as FASTQ files in preparation for bioinformatic analysis.

Bioinformatic methods

During standard bioinformatic processing of the data, we identified several ASVs (amplicon sequence variants) which showed evidence of unexpected primer combinations. The PCR products had been sequenced with an unrelated data set by pooling amplicons from different areas of the genome for barcoding with the same Illumina tag. This resulted in a small number of sequences with a forward primer of one amplicon and a reverse primer of the other, which made it impossible to automate primer removal. To correct this, we processed the data using the DADA2 pipeline as described by *Garrett et al. (2023)* but without primer removal to generate ASVs with primers still attached. We then examined these ASVs manually in BIOEDIT (*Hall, 1999*) and separated the 16S reads from non-16S (the unrelated project which shared the sequencing run) reads based on known nucleotide signatures of the two regions amplified which are quite distinct. We identified a small number of ASVs which had mixed or incomplete primers, likely from primer leftover during library building when independent projects were pooled for barcode addition. These ASVs represented less than 0.006% of the total data and they were discarded. We identified and removed the intact primers from the remaining 16S ASVs manually in BIOEDIT.

We compared the trimmed ASVs to the full NCBI nucleotide collection using BLAST. We removed all ASVs matched to human DNA and, based on full negative control filtering (*Garrett et al., 2023*), we discarded all ASVs with read counts lower than 21, the highest read count identified in any negative control replicate after removal of human DNA. These 21 reads were identified as *Pteronotus psilotus*, the species we used as our positive control. All ASVs greater than 96% identity (100% overlap) were retained for further examination. We also retained ASVs matched to the bat *Chrotopterus auritus* and primate *Alouatta* sp. based on lower percentage matches. *Chrotopterus auritus* is considered to be an unresolved cryptic species complex with at least three distinct mitochondrial lineages with as much as 16% sequence divergence between Central and South American lineages (*Clare et al., 2011*). The closest match to reference material on NCBI comes from a specimen from Peru (AMNH Mammalogy 280554) and thus a more relaxed match of 93–94% with no other similar reference was retained. Several ASVs match to *Alouatta palliata* at 92.5%. This species is not found in Belize, but the related *Alouatta pigra* is common in our research area. The taxonomy of *Alouatta* is complex and has recently undergone revisions (*Doyle et al., 2021*). It is not clear if any *Alouatta pigra* 16S references are contained in the Genbank nucleotide collection (the name does not exist in Genbank). We retain *A. pigra* for these reads as the mostly likely identification. Six of the samples (two each from Sugar Mill High Tree, Sugar Mill Low Tree, and Schoolhouse Cave) had been sequenced previously (*Garrett et al., 2023*) and were included at the analysis stage.

Sample coverage and day *vs* night detections

We could easily enter the Schoolhouse Cave (Fig. 1E) and the floor area of the roost was large, allowing for a greater sampling effort. Therefore, from this roost we estimated the effect of sampling effort on taxonomic recovery. Using a Hill number approach, we generated diversity accumulation curves at three different diversity orders of (q). These Hill numbers are equivalent to the commonly used diversity indices: species richness (q = 0), the Shannon index (q = 1), and the Simpson index (q = 2). The diversity profiles were generated with 95% confidence intervals using the iNEXT package (*Chao et al., 2014*; *Hsieh, Ma & Chao, 2022*) in RStudio (*RStudio Team, 2021*). Following the protocol described by *Chao et al. (2014)*, curves were extrapolated to double the size of the observed value. At the Schoolhouse Cave where we had greater access we also ran a test of the difference in detections in night *vs* day sampling. We performed a paired t-test on the mean number of species detected to determine if more bat species were detected at night than during the day. We also compared whether more non-bat vertebrates were detected during the day then at night. We tested the homogeneity of the data using the Bartlett test and the distribution using the Shapiro-Wilks test.

RESULTS

Species detections

Of the 207 ASVs identified, we retained 138 after removal of human DNA and filtering using controls. We identified these as coming from 23 taxa, including 11 bat taxa, four amphibian species, three non-bat native mammal taxa, and five domestic mammals (Table 2). One bat taxon (*Molossus*) could only be identified to the genus level as two local species have very similar DNA sequences (although a photograph at the roost suggests it may have been *M. alvarezi* (Fig. 2H; see discussion)).

The natural cave roosts recovered the highest overall richness of taxa from DNA with 18 species being identified. These were seven bat species, three non-bat native mammals, five domestic mammals, and three amphibian species (Table 2). In the tree roosts, we detected six bat species, two of which, *Diaemus youngi* (Sugar Mill High Tree) and *Molossus sp.* (Museum Tree), were not detected in any of the other roosts (Table 2). We also detected DNA from two non-bat native mammals and two domestic mammals in the tree roosts. We detected DNA from seven bat species, one non-bat native mammal, three amphibians, and four domestic mammals in the artificial tunnels. One of the frog species, *Dendropsophus microcephalus* (*Natalus* Tunnel), and one of the bat species, *Chrotopterus auritus* (White Room), were detected only in the artificial tunnels (Table 2). We detected DNA from one bat species and two domestic animals in the other man-made roosts. While DNA from domestic animals is almost certainly coming from the surrounding habitat, we can confirm the presence of the small mammals and bat species in the vicinity, and often in the same roosts, based on visual sightings, captures in nets nearby, and/or photographic records (Fig. 3).

Table 2 Summary of roost survey detections grouped by roost type.

Type	Roost	Bats	Other vertebrates
Natural Caves	Indian Creek Cave	*Carollia perspicillata*	*Bos taurus* (cow)
		Desmodus rotundus	
		Glossophaga mutica	
		Natalus mexicanus	
	Schoolhouse Cave	*Carollia perspicillata*	*Alouatta pigra** (Yucatan black howler monkey)
		Glossophaga mutica	*Bos taurus* (cow)
		Natalus mexicanus	*Canis* sp. (canine sp.)
		Saccopteryx bilineata	*Equus caballus* (horse)
		Sturnira parvidens	*Ototylomys phyllotis* (big eared climbing rat)
		Trachops cirrhosus	*Leptodactylus fragilis* (white-lipped frog)
			Ovis aries (sheep)
			Scinax staufferi (Stauffer's tree frog)
			Sus scrofa (pig)
			Sylvilagus floridanus (eastern cottontail)
			Trachycephalus typhonius (veined tree frog)
Artificial tunnels (KKB)	Red Room	No detections	*Canis* sp. (canine sp.)
	Plaza Tunnel	*Carollia perspicillata*	*Ototylomys phyllotis* (big eared climbing rat)
		Saccopteryx bilineata	*Sus scrofa* (pig)
			Trachycephalus typhonius (veined tree frog)
	Natalus Tunnel	*Carollia perspicillata*	*Bos taurus* (cow)
		Glossophaga mutica	*Canis* sp. (canine sp.)
		Natalus mexicanus	*Dendropsophus microcephalus* (yellow tree frog)
		Sturnira parvidens	*Ototylomys phyllotis* (big eared climbing rat)
		Trachops cirrhosus	*Ovis aries* (sheep)
			Leptodactylus fragilis (white-lipped frog)
			Sus scrofa (pig)
	White Room	*Chrotopterus auritus**	*Bos taurus* (cow)
			Ototylomys phyllotis (big eared climbing rat)
Other human-made	Helen's House	*Glossophaga mutica*	*Bos taurus* (cow)
			Canis sp. (canine sp.)
	Cistern (LAM)	No detections	*Bos taurus* (cow)
			Canis sp. (canine sp.)
Tree roosts (LAM)	High Temple Hollow Tree	No detections	No detections
	Museum Tree	*Molossus* sp.	*Alouatta pigra** (Yucatan black howler monkey)
	Sugar Mill High Tree	*Desmodus rotundus*	*Bos taurus* (cow)
		Diaemus youngi	*Ototylomys phyllotis* (big eared climbing rat)
		Saccopteryx bilineata	*Ovis aries* (sheep)
	Sugar Mill Low Tree	*Pteronotus fulvus*	
		Sturnira parvidens	No detections

Note:
Taxa with a % match in GenBank (NCBI) lower than 95% are denoted with an asterisk (*).

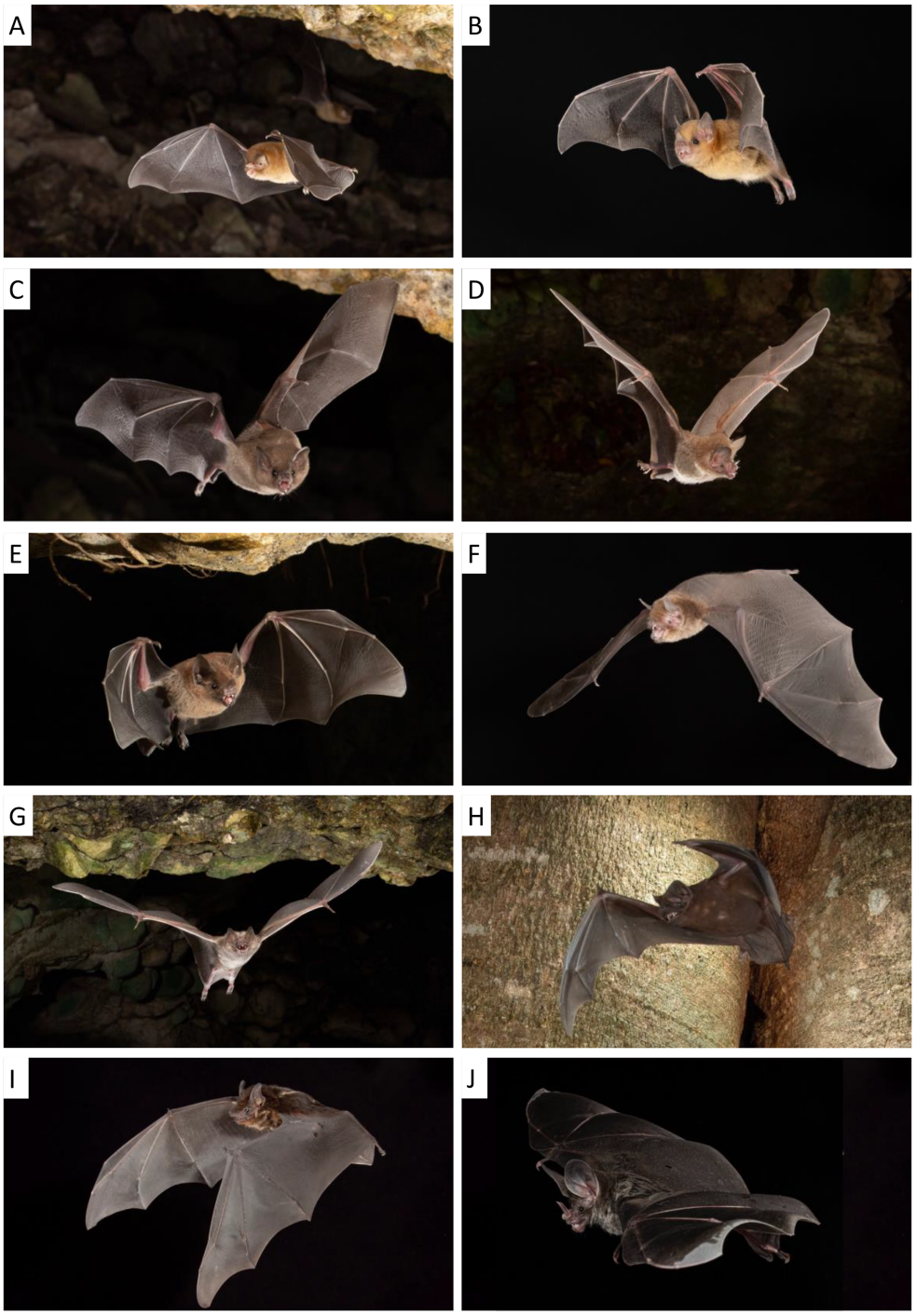

Figure 2 With the exception of *Diaemus youngi*, all bats detected using airborne eDNA have also been documented in the study area from camera traps at roost exits, from mist net captures and/or from being captured in roosts. *Natalus mexicanus* (A), *Glossophaga mutica* (C), *Carollia sp.* (E), *Desmodus rotundus* (G) and *Molossus cf. alvarezi* (H), were detected using camera traps exiting at least one of the roosts where their DNA was detected. *Trachops cirrhosis* (D) was detected with a camera trap at a different cave roost. *Pteronotus fulvus* (F), *Sturnira parvidens* (B) and *Saccopteryx bilineata* (I) were all captured regularly, while *Chrotopterus auritus* (J) has only been detected at an artificial tunnel we did not sample this year using traditional methods; photographs of these last four species were taken in a studio setting (images (A–I) by Charles M. Francis, (J) by M. Brock Fenton & Sherri Fenton).

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Figure 3 The big eared climbing rat *Ototylomys phyllotis* has been seen and photographed sharing roosting areas with bats. It has been seen in KKB (A and B) and in the area around the tree roosts of the LAM (C) in previous years and was both seen and recorded on acoustic equipment during the current field season. The DNA of *Ototylomys* was detected in both natural and man-made bat roosts and a hollow tree. This suggests widespread roost sharing behaviour for this species (images (A) and (C) by Elizabeth L. Clare, (B) by Sherri Fenton). Full-size DOI: 10.7717/peerj.14772/fig-3

Sample coverage and day *vs* night detections

The species accumulation curves of bat diversity show that within the Schoolhouse Cave, sampling effort was sufficient to detect the majority of bat species in the roost. For all orders of diversity (q = 0, 1, 2), curves reach an asymptote (Fig. 4A). In contrast, for total diversity (bats and other vertebrate taxa) species richness (q = 0) does not reach an asymptote (Fig. 4B), indicating increased sampling may add more species. However, for the other orders of diversity (q = 1, 2) curves do reach an asymptote (Fig. 4B). This diversity profile indicates that most of the common species have been captured by the sampling effort, as q = 1 can be considered the effective number of common species and q = 2 the effective number of dominant species (*Hsieh, Ma & Chao, 2022*). The mismatch between q = 0 for total diveristy, and the bat-only profile could indicate that the current sampling was insufficient for rarer non-bat vertebrates (Fig. 4). There was no statistically significant difference between the mean number of bats ($t_5 = -1.19$, $p = 0.14$) or other vertebrate species ($t_5 = 0.44$, $p = 0.34$) detected during daytime sampling compared to nighttime sampling (Fig. 5). Both the normality and homogeneity assumptions were met. See appendix 1 and 2 (Figs. S1 and S2) for detections by sampler.

DISCUSSION

In this study, we moved beyond proof-of-concept experiments and conducted the first real-world use of airborne eDNA sampling for applied ecological analysis of a wild terrestrial vertebrate community. Our goal was to document roosting ecology of neotropical bat species in small cavity roosts using non-invasive air sampling which minimizes any disturbance to the animals. In Belize, we sampled air from 12 potential roosts and were able to confirm bat occupancy in nine of these, including at least one in each roost type (natural caves, artificial tunnels, other human-made structures, and tree cavities) therefore confirming that nine of the roosts were currently occupied. Of the three roosts without detections, one had never previously been surveyed (High Temple Hollow

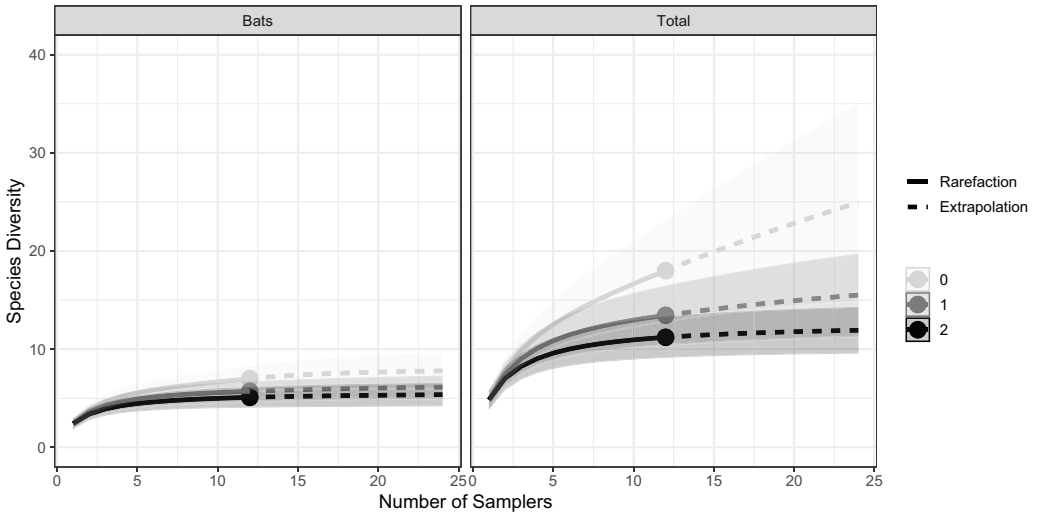

Figure 4 Accumulation curves for bat diversity and total diversity for three orders equivalent to species richness (q = 0), the Shannon index (q = 1) and the Simpson index (q = 2). Including 95% confidence intervals and extrapolated to double the observed value (solid circle).
Full-size DOI: 10.7717/peerj.14772/fig-4

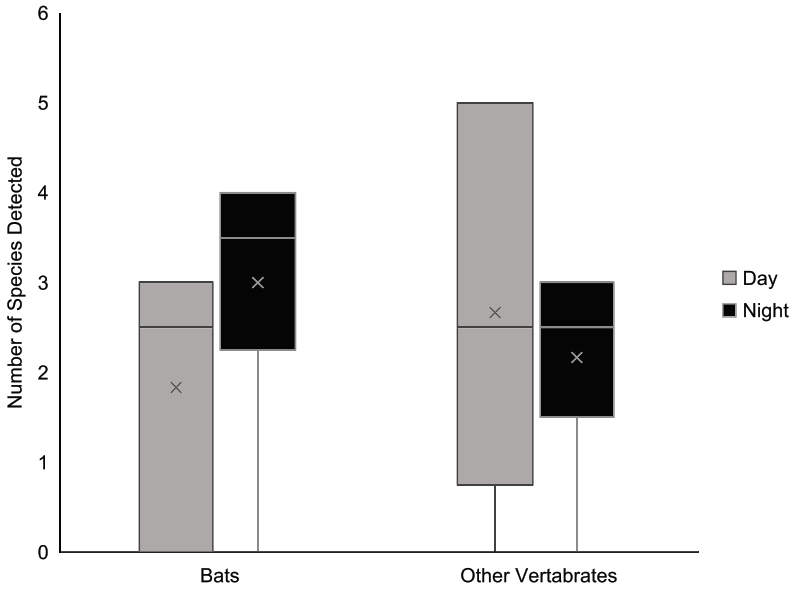

Figure 5 The mean (x) number of species detected during the day (grey, n = 6) and night (black, n = 6) did not differ significantly for bat species (t_5 = −1.19, p = 0.14) and other vertebrates (t_5 = 0.44, p = 0.34) at the Schoolhouse Cave. Graph shows the range (line), 5th, 50th (median) and 95th percentiles in the boxes.
Full-size DOI: 10.7717/peerj.14772/fig-5

Tree) and so we had no evidence it was a roost. One is only known to be used as an occasional day roost (Cistern) and one (Red Room) had no bats present at any time we visited this year and no evidence of recent occupation (*e.g.*, no guano, no acoustic recordings) although it had been occupied by bats in previous years. Overall, we detected 23 taxa including bats, co-habiting mammals, and a selection of other known local animals, demonstrating that airborne eDNA can be used to detect bats in their roosts as

well as other vertebrates in the surrounding area. In general, eDNA in tropical zones is thought to degrade faster than in temperate zones because of higher temperatures, humidity, increased UV exposure, and rainfall patterns (*Huerlimann et al., 2020*). While this has not been studied in airborne eDNA, the environmental effects are likely to have a similar or greater effect on airborne material. Thus, the ability of our methods to detect a relatively large diversity of species, including those that are rare and/or difficult to capture, in an environment where eDNA may degrade quickly, is promising. These results showcase airborne eDNA's potential to survey and monitor difficult to access locations with considerable efficiency.

Roost species assemblages

We detected 11 bat taxa using airborne eDNA, several of which were documented at the same roosts using camera traps and all but one of which have been captured in the vicinity. The exception was *D. youngi*, the white-winged vampire, which was detected in a tree roost from eDNA, but which has not previously been photographed or netted in the area over more than a decade of survey effort. Despite the absence of local records, the species' range overlaps this area, and it was considered "likely to be present" in the local key to bats (*Clare & Simmons, 2021*). Thus, its detection is a confirmation rather than a surprise. This detection was also made independently in all three samplers at the location with a large read count providing robust evidence for its presence. Using airborne eDNA we also detected a potential new roost type in the area for a well-known species *Sturnira parvidens* in an artificial tunnel and in the Schoolhouse Cave. Although regularly captured, this species had not previously been detected in cave-like roosts locally; it had only been documented in tree roosts (*Fenton et al., 2000*, under the name *S. lilium*).

The detection of rare or elusive species is a significant advantage of eDNA methods. Aquatic eDNA has been highly successful in detecting species in locations where they were not previously known to be present. For example, aquatic eDNA samples taken from caves in Croatia detected the presence of the IUCN red-listed amphibian, *Proteus anguinus*, for the first time in five different caves (*Vörös et al., 2017*). In Nova Scotia's Kejimkujik National Park and Historic Site, aquatic eDNA detected the threatened Blanding's turtle (*Emydoidea blandingii*) as well as two invasive species (chain pickerel (*Esox niger*) and smallmouth bass (*Micropterus dolomieu*)) in locations where they were not known to occur (*Loeza-Quintana et al., 2020*). As with aquatic DNA, using airborne eDNA as a detection method could expand lists of known species in areas with elusive species and help to document complex communities. Our detection of *S. parvidens* and *D. youngi* in previously unknown roosting locations is a clear demonstration of the complimentary potential of this technique for assessing roosting ecology, even in locations which have been studied extensively using other methods.

We detected 12 non-bat vertebrates using airborne eDNA. Five of these were domestic animals whose DNA likely drifted into the roosts from the surrounding farmland. This drift is best showcased by the detection of cow DNA (*Bos taurus*) in the attic roost where it was clearly not possible for the animal to be physically present. Cows are abundant in the surrounding area, thus the detection of trace eDNA from such common species is likely to

represent a consistent false positive in many monitoring activities. In our recent validation of eDNA sampling for complex tropical bat communities inside an open laboratory room (*Garrett et al., 2023*) we also detected commonly-known local non-bat species that we presumed resulted from eDNA drifting into our sample site on wind currents. These results suggest that, particularly for very common species, pinpointing the source of an eDNA signal may be difficult. A similar problem is found in aquatic surveys where signals are occasionally found far from their source. In one study of the drift potential of DNA, *Jane et al. (2015)* were able to detect trout eDNA in streams over 230 m away from the nearest source. Although there is little research investigating the extent of such dynamics using airborne eDNA, our results indicate that moderate distance transport is likely, particularly for common species. While most signals appeared quite localized, it will be difficult to trace all sources and more work on determining drift dynamics of airborne eDNA is required.

Of the remaining seven species detected in our study (four amphibians and three non-bat native mammals), it is likely that *Alouatta pigra* (the Yucatan black howler monkey) and *Sylvilagus floridanus* (eastern cottontail) were also detected from eDNA which drifted into the roosts. The four amphibian species and remaining mammal (*Ototylomys phyllotis*, big-eared climbing rat) are known or suspected to use the locations where they were detected. Thus, it is likely these represent true detections within the roosts, rather than detections of eDNA that had drifted into the site. In particular, *O. phyllotis* has been previously observed and photographed in or around some of these roosts; our data further confirms this roost-sharing behaviour in both tree and cave roosts (Fig 3). Non-target detections such as these suggest that airborne eDNA could be used not only to target one taxon, but document larger ecosystem-level community assemblages.

Roosting behaviour

Four of the bat species detected (*Carollia perspicillata, Desmodus rotundus, Glossophaga mutica*, *Trachops cirrhosus*) are known to use multiple roost types in the Neotropics in general (*Reid, 2009*) and at our study site in particular (*Herrera et al., 2018*) so their detection in multiple locations is not surprising. For example, *G. mutica* was detected in all the roost types except for the tree roosts (though we have previously caught them in Sugar Mill High Tree), and it was the only species detected in the attic roost at Helen's House. *Natalus mexicanus* was only detected in cave or cave-like roosts (artificial tunnels). This behaviour is supported by other observations of these bats preferring to roost in these roost types (*López-Wilchis, Torres-Flores & Arroyo-Cabrales, 2020*). Despite photographic documentation and captures in hand nets in our study area, *Natalus* has never been caught in a mist net at this location in a decade of surveys. *Saccopteryx bilineata* was observed roosting at the entrances of the artificial tunnels and we have observed them roosting in the sugar mill structures near the tree roost where they were detected. They have been observed emerging from tree roosts in our study area and are known to roost in hollow trees elsewhere in the neotropics (*Villalobos-Chaves et al., 2016*; *Voss et al., 2016*), thus their detection in both the artificial tunnels where they were seen, and in the hollow tree roosts, is consistent with documented roosting behaviour. While it is believed that *Sturnira*

parvidens does sometimes roost in caves (or cave-like structures) as our data suggested and often co-roosts with other bats (*Fenton et al., 2000*), this species has not previously been observed doing this in the local area. Historically, we have only found them in tree roosts. In contrast, *Pteronotus fulvus* is thought to prefer cave roosts (*Willson & Mittermeier, 2019*), but was only detected in a tree roost (Sugar Mill Low Tree) in our study. While we cannot confirm the detections of *S. parvidens* or *P. fulvus* with photographic or capture data from these particular sites, the behaviour we infer from our eDNA data would not be surprising given the roosting patterns of congeners which also utilize tree roosts occasionally (*Voss et al., 2016*). However, it is also possible we are detecting eDNA moving from the local area into the roost. More documentation of airborne eDNA movement patterns is required to determine the likelihood of detection of bats within a roost that were present in the area but not actually using the roost. We detected *Chrotopterus auritus* in only one roost, where it was the only species detected. This species, which is a large carnivorous bat, often roosts alone and while this roost was not surveyed by camera or netting during this field season because of safety concerns, the species has been caught in the same roost at that location in previous years (*Brigham et al., 2018*).

It should be noted that the *Molossus* sp. detection could not be identified to species based on eDNA. In a large survey (*Clare et al., 2011*) using DNA barcodes it was noted that while most central and South American molossids can be differentiated using mtDNA, the % divergence between species tends to be very low. Given the small fragments amplified and sequenced in this study, we could not confidently identify the species. Perfect matches might be reliable, but more assessment using short reads is necessary. In this location the most reliable external character to differentiate the two regularly captured *Molossus* species is the white fur base in *Molossus alvarezi* and dark fur base in *Molossus nigricans* (*Loureiro, Engstrom & Lim, 2020*). Based on camera trap images (*e.g.*, Fig. 2H) we suspect that the *Molossus* sp. detected in the tree roost was *M. alvarezi*. In that picture the fur has been parted by the air currents and a white base appears visible and more distinct than the pale skin under dark fur of *M. nigricans* would be.

Airborne eDNA as a roost survey tool

The use of airborne eDNA to study roosts, hollows, and burrows was cited as an ideal application in the first proof-of-concept of airborne eDNA detection of mammals (*Clare et al., 2021*). Our current study highlights the strong potential of this application with the first use of air-based bat roost surveys under natural field conditions. One of the most obvious advantages of this eDNA approach is that it enabled us to survey areas that were largely inaccessible and detect species that were not observed in our study area using other methods. The entrance to Indian Creek Cave drops steeply into the ground, making it difficult to enter the cave to survey bats. Similarly, it is not possible to enter the tree roosts (Figs. 1D and F) to visually identify species because the entrances and spaces used by the bats are, in many cases, too small to permit human entry. However, we were able to easily insert our small filter units into the entrances of these roosts and, using airborne eDNA, determine that these roosts were occupied and provide a basic list of the inhabitant species. Without this approach, we would not have been able to survey some of these roosts.

For example, we were able to survey the tin roof structure (Fig. 1A) which was deemed too unstable to enter and thus unsafe to survey using nets or even photographic equipment. It also allowed us to detect *D. youngi*, a species not previously detected in the area but predicted to be present. The ability to detect elusive species is one of the main advocated benefits of eDNA. Aquatic eDNA studies have detected rare and elusive fish (*Weltz et al., 2017*; *Nester et al., 2022*), amphibians (*Plante et al., 2021*), birds (*Neice & McRae, 2021*) and marine mammals (*Ma et al., 2016*; *Juhel et al., 2021*). Recently, eDNA left on agave flowers in Mexico and Texas (*Walker et al., 2022*) and in guano deposits in Redwood tree hollows in California (*Armstrong et al., 2022*) have been used to successfully detect roosting and migrating bats. To these methods we now add airborne eDNA detection of elusive bat species.

The use of airborne eDNA also allows for a longer sampling time than visual surveys of bat roosts. During visual surveys, usually the longer a researcher is in the roost the more likely they are to identify all the species present; however, the longer researchers stay inside a roost the more they may disturb the bats, and more manpower is needed to cover more roosts. With airborne eDNA one can leave a sampler in a roost for up to 24 h (or longer depending on the sampler type and battery) with minimal disturbance to the bats. The units used here emitted no obvious ultrasound (M. Kalcounis-Rueppell, personal communication, 2022) and are quiet at other frequencies. We observed bats roosting directly above them in multiple instances, suggesting they are very minimally disruptive to roosting bats. Such considerations make eDNA samplers ideal for a non-invasive survey approach. *Klepke et al. (2022)* found that airborne eDNA accumulates over time, suggesting that the longer a sampler is left in a roost, the more likely it is to capture the total diversity in said roost. The low cost of this sampler design (*Garrett et al., 2023*) and ability to deploy samplers unattended for 24 h or longer means a small team can survey many potential roosts simultaneously. Doing so could confirm the species present in both known and suspected roosts, and simultaneously provide preliminary occupancy estimates. This could be especially useful for broad surveys in the neotropics where bat roosts may be hard to find (*Villalobos-Chaves et al., 2016*).

Sample coverage

The number of samplers deployed in the Schoolhouse Cave was sufficient to capture the bat diversity in the roost but did not capture total species richness for all taxa in the area. Many of the other vertebrate species detected in this cave were found presumably as a result of eDNA from the surrounding area drifting into the cave and naturally settling since this cave occupies a physical low point in the natural landscape. It is likely that more sampling is needed to capture the richness of the surrounding area, if not the cave itself. But it is an interesting observation that the Schoolhouse Cave is at a low point and might be a natural site of eDNA accumulation, if airborne DNA drifts in, settles and becomes captured in these natural structures. The cow in the attic roost at Helen's House and the pigs in the caves are false positives for these roosts but not for the immediate surrounding area, indicating that eDNA may accumulate in such locations, making them a better target than open "wind swept" areas, though this hypothesis remains to be tested. While in most

cases we know from alternative data sources that our bat detections are consistent with known habitation, our data also suggests that detection should not immediately be used to conclude roost occupancy, and we cannot exclude drift from the local area. This could be true for the detection of *Sturnira parvidens* in the cave-like roosts where other capture methods have failed to indicate such a roosting behaviour in this area. Research investigating how localized airborne eDNA signals are—and how eDNA may move through the environment on wind currents, *etc.*—will help address such questions and aid in study design.

Day *vs* night detections

We observed a non-significant but suggestive pattern of diurnal *vs* nocturnal detections. Patterns of non-uniform DNA shedding have been observed in aquatic eDNA studies (*Klymus et al., 2015*; *Sassoubre et al., 2016*; *Thalinger et al., 2021*) and we suspect a similar pattern here. Bats are more active at night, which may increase eDNA shedding during that time, and thus detection rates may be greater at night. The opposite may be true for farm animals that are diurnal. While our data is based on a single roost (Schoolhouse Cave) over a single 24 h period, where we had paired day and night measures, we observed a distinct pattern of a greater number of bat detections at night and slightly more non-bat detections in samples collected during the day. We treat this observation with caution, however; while the trend is interesting, the difference observed was not significant and sampling times were not equal (the "nocturnal" samples were a few hours longer than the diurnal samples). We would not normally report and discuss this non-significant finding but include it here as it may be an important consideration in future sampling designs. Our comparisons are based on six day and six night filters which may not be independent (sampling encompassed only one actual day with six air samplers deployed at the same time) but the emerging pattern is cause for careful consideration of how and when sampling should be conducted. The patterns we observed may indicate that eDNA is a very short-term signal in air, either because of degradation or because it falls out of the air quickly. If eDNA signals in air are of a short duration and distance, it may might provide an accurate indication of recent activity. This contrasts with the potential of long-range drift we suspect from the cow eDNA that we detected in some samples. The matrix surrounding these areas of secondary forests includes fields with high cow biomass, a significant and unusual source for DNA in the landscape. Clearly more research is needed to evaluate the role of drift in studies of airborne eDNA.

Future directions

The use of airborne eDNA for applied ecology is in its infancy. Our study is the first application we are aware of that goes beyond proof of concept to study an actual ecological objective, in our case surveying bat roosts. As a new field, there are many unresolved questions required for full validation of the technique. While many have been reviewed elsewhere, we raise a number of specific issues relevant to our study that require additional investigation. First, the movement of eDNA through the air is unclear and how far it can drift is largely unknown. While eDNA in air does not appear to be a homogeneous soup

(*Johnson et al., 2023*) and may have a short window of detection (*Garrett et al., 2023*), we also see evidence of some drift, as indicated by cow DNA in the roof roost and farm animal DNA in a cave roost. Some detections only occur in one location, like *D. youngi*, while others are common (*e.g.*, *Sturnira*). While it is likely these detections indicate animals present in the roosts, we cannot discount the possibility that some of these detections represented drift from the local environment. One potential method to test for drift, would be to run a transect away from roosts to test whether detections decrease with distance away from the roost (which would suggest the roost was the source). Further work is also needed to determine the ideal sampling time and frequency. Our sampling was constrained by local restrictions and our sampling design sought to maximize sampling time and thus detections under those limitations. Having demonstrated that this approach was successful, it would now be useful to integrate this method with an occupancy modelling framework. By doing standardised repeated surveys at each roost, we could understand differences between detection and occupancy probabilities. This would give a more nuanced understanding of how bats are utilising the roosts while accounting for false detections. Addressing these questions will both refine this method of detection and allow for better sampling design of future applications in a broader ecological context.

CONCLUSIONS

We used airborne eDNA to detect vertebrates both inside bat roosts and from the areas surrounding roosts, indicating that airborne eDNA is a potential game-changing tool for non-invasive surveys of caves, hollows, and other bat roosts. However, more research is needed to understand the ecology of airborne eDNA, including how much eDNA may be drifting into roosts from the surrounding areas, and to determine the best sampling strategy for roost surveys, particularly with respect to sampling intensity, duration, and timing. Our study showcases airborne eDNA as a roost survey tool that could be especially useful in surveying difficult to access locations and determining roost occupancy over periods beyond that of a single visual inventory or camera trapping campaign.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

Natalia Ivanova & Amanda Naaum are employed by Nature Metrics North America Ltd. Andrew Briscoe is employed by Nature Metrics Ltd.

Author Contributions

- Nina R. Garrett conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Jonathan Watkins performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Charles M. Francis analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.
- Nancy B. Simmons analyzed the data, authored or reviewed drafts of the article, and approved the final draft.
- Natalia Ivanova performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Amanda Naaum performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Andrew Briscoe performed the experiments, authored or reviewed drafts of the article, and approved the final draft.
- Rosie Drinkwater analyzed the data, authored or reviewed drafts of the article, and approved the final draft.

- Elizabeth L. Clare conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the article, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (*i.e.*, approving body and any reference numbers):

This research was approved by the York University Animal Care Committee (ACC) (2021-10).

Field Study Permissions

The following information was supplied relating to field study approvals (*i.e.*, approving body and any reference numbers):

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Data Availability

The following information was supplied regarding data availability:

The DNA sequences are available at GenBank: SAMN31782501 to SAMN31782550.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.14772#supplemental-information.

REFERENCES

Aguirre LF, Lenstra L, Matthysen E. 2003. Patterns of roost use by bats in a neotropical savanna: implications for conservation. *Biological Conservation* **111(3)**:435–443 DOI 10.1016/S0006-3207(02)00313-0.

Altringham JD. 2011. Roosting and feeding ecology. In: *Bats: From Evolution to Conservation*. New York: Oxford University Press, 138–150.

Armstrong AJ, Walker FM, Sanville C, Martin S, Szewczak JM. 2022. Bat use of hollows in California's old-growth redwood forests: from DNA to ecology. *Animals* **12(21)**:2950 DOI 10.3390/ani12212950.

Barnes MA, Turner CR. 2015. The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics* **17(1)**:1–17 DOI 10.1007/s10592-015-0775-4.

Bat Conservation Trust. 2021. National bat monitoring programme annual report 2021. London.

Behrens P, Kiefte-de Jong JC, Bosker T, Rodrigues JFD, de Koning A, Tukker A. 2017. Evaluating the environmental impacts of dietary recommendations. *Proceedings of the National Academy of Sciences of the United States of America* **114(51)**:13412–13417 DOI 10.1073/pnas.1711889114.

Brigham RM, Broders HG, Toth CA, Reimer JP, Barclay RMR. 2018. Observations on the roosting and foraging behavior of woolly false vampire bats, *Chrotopterus auritus*, in Belize. *Caribbean Naturalist* **47**:1–7.

Calvignac-Spencer S, Merkel K, Kutzner N, Kühl H, Boesch C, Kappeler PM, Metzger S, Schubert G, Leendertz FH. 2013. Carrion fly-derived DNA as a tool for comprehensive and

cost-effective assessment of mammalian biodiversity. *Molecular Ecology* **22(4)**:915–924 DOI 10.1111/mec.12183.

Chao A, Gotelli N, Hsieh T, Sande E, Ma K, Colwell R, Ellison A. 2014. Rarefaction and extrapolation with Hill numbers: a framework for sampling and estimation in species diversity studies. *Ecological Monographs* **84**:45–67 DOI 10.1890/13-0133.1.

Clare EL, Economou CK, Bennett FJ, Dyer CE, Adams K, McRobie B, Drinkwater R, Littlefair JE. 2022. Measuring biodiversity from DNA in the air. *Current Biology* **32(3)**:693–700 DOI 10.1016/j.cub.2021.11.064.

Clare EL, Economou CK, Faulke CG, Gilbert JD, Bennett F, Drinkwater R, Littlefair JE. 2021. eDNAir: proof of concept that animal DNA can be collected from air sampling. *PeerJ* **9**:e11030 DOI 10.7717/peerj.11030.

Clare E, Lim B, Fenton M, Herbert P. 2011. Neotropical bats: estimating species diversity with DNA barcodes. *PLOS ONE* **6(7)**:e22648 DOI 10.1371/journal.pone.0022648.

Clare EL, Simmons NB. 2021. A field key to bats of Lamani, Belize V.5. *BioRxiv preprint* DOI 10.1101/2022.11.07.515479.

Doyle ED, Prates I, Sampaio I, Koiffmann C, Silva WA Jr, Carnaval AC, Harris EE. 2021. Molecular phylogenetic inference of the howler monkey radiation (Primates: *Alouatta*). *Primates* **62(1)**:177–188 DOI 10.1007/s10329-020-00854-x.

Esquivel DA, Peña S, Aya-Cuero C, da Tavares VC. 2020. Bats and termite nests: roosting ecology of *Lophostoma brasiliense* (Chiroptera: Phyllostomidae) in Colombia. *Mastozoologia Neotropical* **27(1)**:72–80 DOI 10.31687/saremMN.20.27.1.0.11.

Fenton MB, Bouchard S, Hollis L, Johnston DS, Lausen CL, Ratcliffe JM, Riskin DK, Taylor JR, Zigouris J. 2001. The bat fauna of Lamanai, Belize: roosts and trophic roles. *Journal of Tropical Ecology* **17(4)**:511–524 DOI 10.1017/S0266467401001389.

Fenton MB, Ratcliffe JM. 2010. Bats. *Current Biology* **20(24)**:R1060–R1062 DOI 10.1016/j.cub.2010.10.037.

Fenton MB, Vonhof MJ, Bouchard S, Gill SA, Johnston DS, Reid FA, Riskin DK, Standing KL, Taylor JR, Wagner R. 2000. Roosts used by *Sturnira lilium* (Chiroptera/Phyllostomidae) in Belize. *Association for Tropical Biology and Conservation* **32**:729–733 DOI 10.1111/j.1744-7429.2000.tb00521.x.

Garrett NR, Watkins J, Simmons NB, Fenton MB, Maeda-Obregon A, Sanchez DE, Froehlich EM, Walker FM, Littlefair JE, Clare EL. 2023. Airborne eDNA documents a diverse and ecologically complex tropical bat and other mammal community. *Environmental DNA* Epub ahead of print 11 January 2023.

Grafe TU, Schöner CR, Kerth G, Junaidi A, Schöner MG. 2011. A novel resource—Service mutualism between bats and pitcher plants. *Biology Letters* **7(3)**:436–439 DOI 10.1098/rsbl.2010.1141.

Gregorič M, Kutnjak D, Bačnik K, Gostinčar C, Pecman A, Ravnikar M, Kuntner M. 2022. Spider webs as eDNA samplers: biodiversity assessment across the tree of life. *Molecular Ecology Resources* **22(7)**:2534–2545 DOI 10.1111/1755-0998.13629.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. *Nucleic Acids Symposium Series* **41**:95–98.

Herrera JP, Duncan N, Clare E, Fenton MB, Simmons N. 2018. Disassembly of fragmented bat communities in Orange Walk District, Belize. *Acta Chiropterologica* **20(1)**:147–159 DOI 10.3161/15081109ACC2018.20.1.011.

Hofreiter M, Mead JI, Martin P, Poinar HN. 2003. Molecular caving. *Current Biology* **13(18)**:R693–R695 DOI 10.1016/j.cub.2003.08.039.

Hsieh T, Ma K, Chao A. 2022. iNEXT: interpolation and extrapolation for species diversity. *Available at http://chao.stat.nthu.edu.tw/wordpress/software_download/*.

Huerlimann R, Cooper MK, Edmunds RC, Villacorta-Rath C, Port AL, Robson HLA, Strugnell JM, Burrows D, Jerry DR. 2020. Enhancing tropical conservation and ecology research with aquatic environmental DNA methods: an introduction for non-environmental DNA specialists. *Animal Conservation* **23(6)**:632–645 DOI 10.1111/acv.12583.

Humphrey SR. 1975. Nursery roosts and community diversity of nearctic bats. *Journal of Mammalogy* **56(2)**:321–346 DOI 10.2307/1379364.

Jane SF, Wilcox TM, McKelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR. 2015. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources* **15(1)**:216–227 DOI 10.1111/1755-0998.12285.

Janicki AF, Frick WF, Kilpatrick AM, Parise KL, Foster JT, McCracken GF. 2015. Efficacy of visual surveys for white-nose syndrome at bat hibernacula. *PLOS ONE* **10(7)**:e0133390 DOI 10.1371/journal.pone.0133390.

Johnson M, Barnes MA, Garrett N, Clare E. 2023. Answers blowing in the wind: detection of birds, mammals, and amphibians with airborne environmental DNA in a natural environment. *Environmental DNA* Epub ahead of print 20 January 2023.

Johnson M, Fokar M, Cox RD, Barnes MA. 2021. Airborne environmental DNA metabarcoding detects more diversity, with less sampling effort, than a traditional plant community survey. *BMC Ecology and Evolution* **21(1)**:218 DOI 10.1002/edn3.388.

Juhel J-B, Marques V, Fernández AP, Borrero-Pérez GH, Martinezguerra MM, Valentini A, Dejean T, Manel S, Loiseau N, Velez L, Hocdé R, Letessier TB, Richards E, Hadjadj F, Bessudo S, Ladino F, Albouy C, Mouillot D, Pellissier L. 2021. Detection of the elusive Dwarf sperm whale (*Kogia sima*) using environmental DNA at Malpelo island (Eastern Pacific, Colombia). *Ecology and Evolution* **11(7)**:2956–2962 DOI 10.1002/ece3.7057.

Kaarakka H. 2020. Roost monitoring report. Wisconsin Bat Program, Wisconsin Department of Natural Resources. *Available at https://wiatri.net/inventory/bats/volunteer/roosts/*.

Kelm DH, Toelch U, Jones MM. 2021. Mixed-species groups in bats/non-random roost associations and roost selection in neotropical understory bats. *Frontiers in Zoology* **18(1)**:205 DOI 10.1186/s12983-021-00437-6.

Kinoshita G, Yonezawa S, Murakami S, Isagi Y. 2019. Environmental DNA collected from snow tracks is useful for identification of mammalian species. *Zoological Science* **36(3)**:198–207 DOI 10.2108/zs180172.

Klepke MJ, Sigsgaard EE, Jensen MR, Olsen K, Thomsen PF. 2022. Accumulation and diversity of airborne, eukaryotic environmental DNA. *Environmental DNA* **4(6)**:1–17 DOI 10.1002/edn3.340.

Klymus KE, Richter CA, Chapman DC, Paukert C. 2015. Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation* **183(2555)**:77–84 DOI 10.1016/j.biocon.2014.11.020.

Klüg-Baerwald BJ, Lausen CL, Willis CKR, Brigham RM. 2017. Home is where you hang your bat: winter roost selection by prairie-living big brown bats. *Journal of Mammalogy* **98(3)**:752–760 DOI 10.1093/jmammal/gyx039.

Kunz TH. 1982. Roosting ecology of bats. In: *Ecology of Bats*. New York: Plenum Press, 1–56.

Kunz TH. 2003. *Censusing bats: challenges, solutions, and sampling biases*. Reston: U.S. Geological Survey.

Loeza-Quintana T, Crookes S, Li PY, Reid DP, Smith M, Hanner RH. 2020. Environmental DNA detection of endangered and invasive species in Kejimkujik National Park and Historic Site. *Genome* **64(3)**:172–180 DOI 10.1139/gen-2020-0042.

Loureiro LD, Engstrom MD, Lim BK. 2020. Single nucleotide polymorphisms (SNPs) provide unprecedented resolution of species boundaries, phylogenetic relationships, and genetic diversity in the mastiff bats (*Molossus*). *Molecular Phylogenetics and Evolution* **143(1)**:106690 DOI 10.1016/j.ympev.2019.106690.

López-Wilchis R, Torres-Flores JW, Arroyo-Cabrales J. 2020. *Natalus mexicanus* (Chiroptera: Natalidae). *Mammalian Species* **52(989)**:27–39 DOI 10.1093/mspecies/seaa002.

Lynggaard C, Bertelsen MF, Jensen CV, Johnson MS, Frøslev TG, Olsen MT, Bohmann K. 2022. Airborne environmental DNA for terrestrial vertebrate community monitoring. *Current Biology* **32(3)**:701–707 DOI 10.1016/j.cub.2021.12.014.

Ma H, Stewart K, Lougheed S, Zheng J, Wang Y, Zhao J. 2016. Characterization, optimization, and validation of environmental DNA (eDNA) markers to detect an endangered aquatic mammal. *Conservation Genetics Resources* **8(4)**:561–568 DOI 10.1007/s12686-016-0597-9.

Mena JL, Yagui H, Tejeda V, Bonifaz E, Bellemain E, Valentini A, Tobler MW, Sáncez-Vendizú P, Lyet A. 2021. Environmental DNA metabarcoding as a useful tool for evaluating terrestrial mammal diversity in tropical forests. *Ecological Applications* **31(5)**:e02335 DOI 10.1002/eap.2335.

Mormann BM, Robbins LW. 2007. Winter roosting ecology of eastern red bats in southwest Missouri. *The Journal of Wildlife Management* **71(1)**:213–217 DOI 10.2193/2005-622.

Neice AA, McRae SB. 2021. An eDNA diagnostic test to detect a rare, secretive marsh bird. *Global Ecology and Conservation* **27(4)**:e01529 DOI 10.1016/j.gecco.2021.e01529.

Nester GM, Heydenrych MJ, Berry TE, Richards Z, Wasserman J, White NE, Brauwer MD, Bunce M, Takahashi M, Claassens L. 2022. Characterizing the distribution of the critically endangered estuarine pipefish (*Syngnathus watermeyeri*) across its range using environmental DNA. *Environmental DNA* **35(1)**:69 DOI 10.1002/edn3.365.

Patriquin KJ, Ratcliffe JM. 2016. Should I Stay or Should I Go? Fission-fusion dynamics in bats. In: *Sociality in Bats*. Switzerland: Springer Cham, 65–103.

Plante F, Bourgault P, Dubois Y, Bernatchez L. 2021. Environmental DNA as a detection and quantitative tool for stream-dwelling salamanders: a comparison with the traditional active search method. *Environmental DNA* **3(6)**:1128–1141 DOI 10.1002/edn3.233.

Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC. 2014. The detection of aquatic animal species using environmental DNA—A review of eDNA as a survey tool in ecology. *Journal of Applied Ecology* **51(5)**:1450–1459 DOI 10.1111/1365-2664.12306.

Reid FA. 2009. Chiroptera. In: *A Field Guide to the Mammals of Central America and Southeast Mexico*. New York: Oxford University Press, 73–177.

RStudio Team. 2021. RStudio: integrated development environment for R. *Available at http://www.rstudio.com/*.

Ruppert KM, Kline RJ, Rahman MS. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: a systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation* **17**:e00547 DOI 10.1016/j.gecco.2019.e00547.

Rydell J, Russo D, Sewell P, Seamark ECJ, Francis CM, Fenton SL, Fenton MB. 2022. Bat selfies: photographic surveys of flying bats. *Mammalian Biology* **52(7)**:105 DOI 10.1007/s42991-022-00233-7.

Sassoubre LM, Yamahara KM, Gardner LD, Boehm AB. 2016. Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. *Environmental Science & Technology* **50(19)**:10456–10464 DOI 10.1021/acs.est.6b03114.

Serrao NR, Weckworth JK, McKelvey KS, Dysthe JC, Schwartz MK. 2021. Molecular genetic analysis of air, water, and soil to detect big brown bats in North America. *Biological Conservation* **261(4)**:109252 DOI 10.1016/j.biocon.2021.109252.

Speakman JR, Webb PI, Racey PA. 1991. Effects of disturbance on the energy expenditure of hibernating bats. *Journal of Applied Ecology* **28(3)**:1087–1104 DOI 10.2307/2404227.

Taylor PG. 1996. Reproducibility of ancient DNA sequences from extinct Pleistocene fauna. *Molecular Biology and Evolution* **13(1)**:283–285 DOI 10.1093/oxfordjournals.molbev.a025566.

Thalinger B, Rieder A, Teuffenbach A, Pütz Y, Schwerte T, Wanzenböck J, Traugott M. 2021. The effect of activity, energy use, and species identity on environmental DNA shedding of freshwater fish. *Frontiers in Ecology and Evolution* **9**:1 DOI 10.3389/fevo.2021.623718.

Thomsen PF, Willerslev E. 2015. Environmental DNA—An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation* **183(1)**:4–18 DOI 10.1016/j.biocon.2014.11.019.

Villalobos-Chaves D, Murillo JV, Valerio ER, Keeley BW, Rodríguez-Herrera B. 2016. Understory bat roosts, availability and occupation patterns in a Neotropical rainforest of Costa Rica. *Revista de Biología Tropical* **64(3)**:1333–1343 DOI 10.15517/rbt.v64i3.21093.

Voss RS, Fleck DW, Strauss RE, Velazco PM, Simmons NB. 2016. Roosting ecology of Amazonian bats: evidence for guild structure in hyperdiverse mammalian communities. *American Museum Novitates* **3870(3870)**:1–43 DOI 10.1206/3870.1.

Vörös J, Márton O, Schmidt BR, Gál JT, Jelić D. 2017. Surveying Europe's only cave-dwelling chordate species (*Proteus anguinus*) using environmental DNA. *PLOS ONE* **12(1)**:e0170945 DOI 10.1371/journal.pone.0170945.

Walker FM, Sanchez DE, Froehlich EM, Lyman JA, Owens MD, Lear K. 2022. Endangered nectar-feeding bat detected by environmental DNA on flowers. *Animals* **12(22)**:3075 DOI 10.3390/ani12223075.

Weltz K, Lyle JM, Ovenden J, Morgan JAT, Moreno DA, Semmens JM. 2017. Application of environmental DNA to detect an endangered marine skate species in the wild. *PLOS ONE* **12(6)**:e0178124 DOI 10.1371/journal.pone.0178124.

Willson DE, Mittermeier RA. 2019. Pteronotus fulvus. In: *Handbook of the Mammals of the World: Mormoopidae.* Vol. 424–443. Barcelona: Lynx Edicions, 438.