Measuring neotropical bat diversity using airborne eDNA

NINA R. GARRETT

A Thesis submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Master of Science

Graduate Program in Biology,

York University,

Toronto, Ontario

July, 2023

GENERAL ABSTRACT

This thesis develops the novel technique of capturing airborne eDNA for the detection of tropical bat species. In chapter two, I use an artificial mixed species community to test three prototype samplers and validate airborne eDNA as a survey method. I demonstrate that airborne eDNA can accurately characterize a mixed species community with varying abundances and that the type of sampler does not impact DNA concentration or read count. In chapter three, I used airborne eDNA to survey 12 known or suspected bat roosts in Orange Walk District, Belize. I identified 23 taxa, 11 of which were bats. This thesis adds to the body of research that seeks to better understand airborne eDNA and its potential applications. Based on the data presented as well as those from other studies, airborne eDNA could be a valuable tool in the monitoring of biodiversity.

ACKNOWLEDMENTS

There are many people that made this thesis possible. First, I would like to thank my supervisor Dr. Elizabeth Clare for all her guidance, support, feedback on many, many drafts and all our adventures in the field. I would like to thank Dr. Brock Fenton for not only making my field work possible and giving me feedback on manuscripts but also for introducing me to Dr. Clare. Without him, I would not have found the Clare lab. I would like to thank Dr. Nancy Simmons for organizing the field season and coordinating the 40+ bat biologist that made my research possible and for offering feedback and guidance during my projects. I would like to thank the staff at Lamanai Outpost Lodge for their all their support. From fantastic food and more than comfortable accommodations to guiding us and sharing their vast knowledge of the local environment, this work would not be possible without them.

I would like to thank my lab mates Katherine Lunn, Nicole Borsato and Alejandro Biganzoli Rangel for helping me take a break from the research and the writing to have fun and for being such great sounding boards. I would like to thank Mehdi Movahed for his help cutting filters and making PCR standards in the lab. I would like to thank York University and NSERC for funding my research through a York Graduate Scholarship, the Vernon Oliver Stong Graduate Scholarship in Science, the Academic Excellence fund and an NSERC of Canada CGS-M.

I would like to thank my friends outside of academia, especially the Crows and Daniella Castillo, for always being willing to hear about my work and helping me disconnect from time to time. I would like to thank my parents, Aileen and Crae Garrett who always believe in me and have supported me in immeasurable ways throughout my schooling and beyond. Thank you for teaching me to push myself and to always be curious. Finally, I would like to thank my big sister, Lucy Garrett, for being my biggest cheerleader and for always being in my corner. Thank you for reminding me what I am capable of and for never doubting me.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Chapter 3

Appendix 5

Appendix 7

List of Tables

Chapter 3

Chapter 1: Introduction

History and Background of eDNA

Environmental DNA or eDNA was first referenced in 1987 and refers to fragments of DNA found outside of living organisms, for example in the water, soil, and air (Taberlet et al., 2012). It can be in the form of whole cells from living organisms or existing as extracellular DNA (Sassoubre et al., 2016) (Figure 1.1). As a research tool it was first used primarily by microbiologists analyzing soil and water samples to identify microbial taxa, identify important biochemical functions through gene analysis and assemble whole genomes of uncultivated microorganisms (Taberlet et al., 2012) a process made famous for microbial environmental genomics by shotgun sequencing genomes from the Sargasso sea (Venter et al., 2004). The use of “environmental DNA” outside of microbes is accredited to the reconstruction of paleo environments in the late 1990s and early 2000s (Willerslev et al., 1999) where it gained widespread from the ecological research community by the reconstruction of ancient paleo communities using eDNA found in ice cores. The structure of paleo-plant communities has been further analysed using eDNA in frozen sediments (sedDNA) (Sønstebø, et al., 2010) and sedDNA has become tool in updating extinction timelines, increasing detail, and narrowing down the windows where species became locally extinct (Haile et al., 2009; Thomsen & Willerslev, 2015). Throughout the 2000s, the use of eDNA as a tool in ecological analysis gained traction with the introduction of Next-generation Sequencing Technologies (NGS) and since its application has expanded rapidly (Shadi et al., 2012; Taberlet et al., 2012).

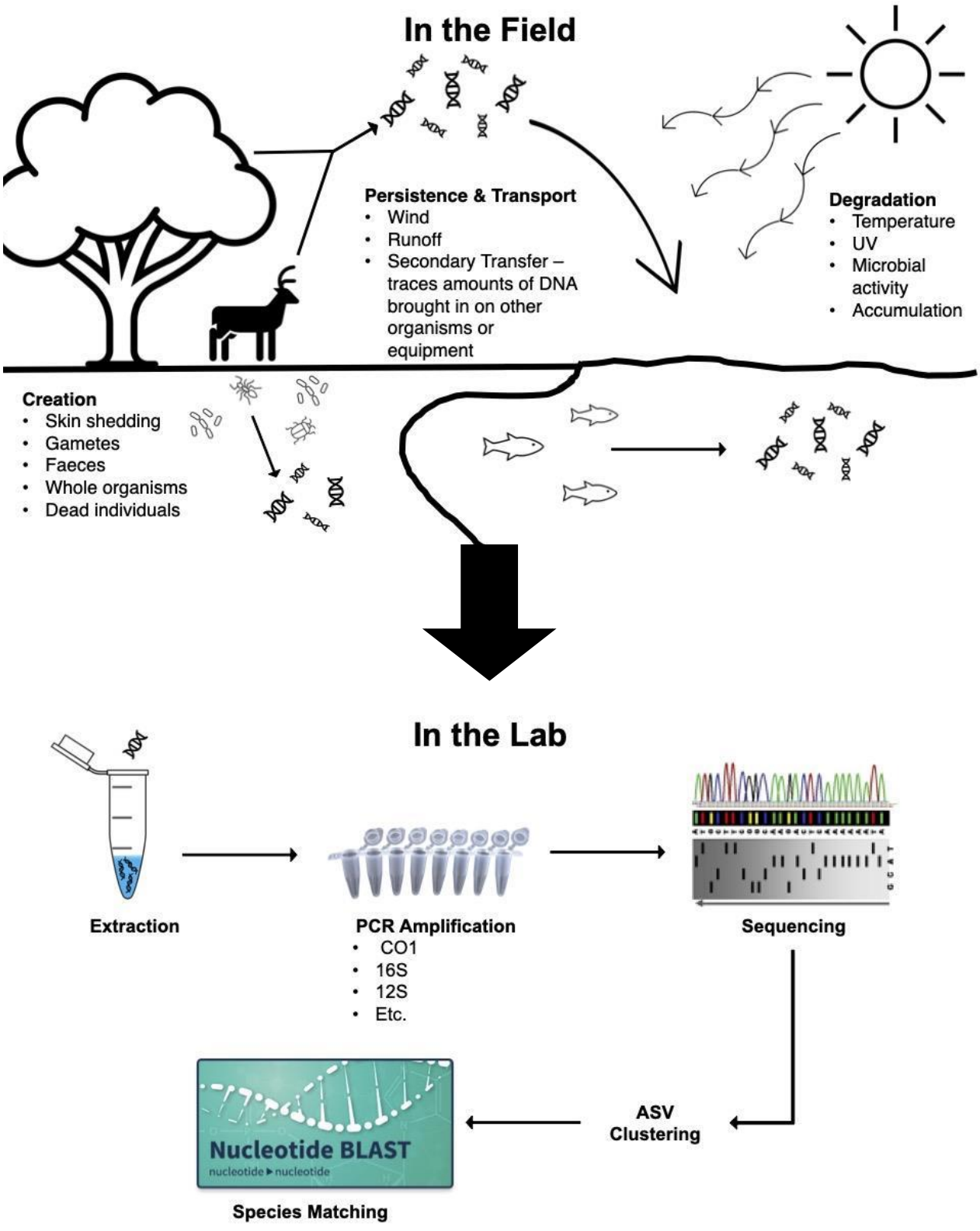


Figure 1.1 Sources of eDNA, persistence, transport, and degradation in the terrestrial environment and lab workflow during sample processing.

Using eDNA for species detection and biodiversity estimates presents several benefits. In the context of paleo-earth, ancient eDNA can eliminate the need for large, intact fossils when trying to reconstruct ancient species composition as it can be found in ice cores and frozen sediment samples (Thomsen & Willerslev, 2015). eDNA sampling is often less time intensive than traditional survey methods (M. D. Johnson et al., 2021; Plante et al., 2021) and it can be less invasive than other methods as it does not require direct access to wildlife for successful detection and identification (Thomsen & Willerslev, 2015). Its ability to operate semi-remotely makes it possible to access locations largely inaccessible to traditional methods and potentially decreases the manpower needed to survey an area (Littlefair et al. *In Press*). As it does not rely on specialized identification knowledge to ID species, eDNA sampling is often more accurate in identification and because eDNA traces are left behind after an animal leaves an area it can detect rarer species more consistently than traditional survey methods which require the animal to be captured on a camera or by a live observer (Hallam et al., 2021; Plante et al., 2021).

Despite these obvious advantages, there are significant limitations to the tool. It is nearly impossible to estimate the quantity of individuals (though relative abundance may be possible) in a study area based on eDNA concentrations in samples (Plante et al., 2021; Thomsen et al., 2012). This is in part because numerous physical processes impact the rate at which eDNA accumulates and degrades in the environment. For example, in aquatic environments, factors like turbidity can interfere with sampling and detection, and in saltwater, pH can also decrease the effectiveness of using eDNA for species detection and identification (Hallam et al., 2021). While soil pH does not appear to inhibit detection, high organic content can interfere with DNA amplification and thus interfere with species

detection (Ruppert et al., 2019). Other information like population structure and sex and age ratios also cannot be obtained using eDNA (though eRNA is being trialed for this). eDNA's tendency to move through the environment can make it difficult to determine its spatial origins. It can travel through strata depending on soil structure and texture and in the water and air, it can quickly travel long distances which creates challenges when trying to estimate its origin (Ruppert et al., 2019). eDNA also cannot be dated directly. As such, when sedDNA (sediment DNA) is used to explore paleo-earth, it must be used with other dated sources to construct accurate timelines of ancient earth (Haile et al., 2009). The tremendous potential for eDNA to persist, particularly in frozen environments, can also leave signals of species presence long after local extinction and migration events generating false positive estimates of local biodiversity if current or recent occupation is the goal.

In contrast to the potential issue of long-term persistence, eDNA is also very fragile and prone to degradation, contamination and being swamped by stronger signals in lab analysis. During DNA extraction, eDNA is generally found in forensically small amounts and is highly vulnerable to contamination (Thomsen & Willerslev, 2015). Thus, strict steps must be taken to limit this contamination as the stochastic nature of amplification by PCR can generate false positives from background laboratory contamination and significantly alter the relative prominence and abundance of certain species in a sample (Thomsen & Willerslev, 2015). As a consequence, even with strict controls, eDNA tends to produce presence absence profiles of uncertain temporal time frame rather than abundance-based analysis. There are also concerns surrounding both PCR bias and bias in databases. As PCR is an exponential process, it can amplify a handful of tax to a greater

degree than others, resulting in a species profile that does not reflect the composition in the original sample. Reference databases can also show bias towards certain taxonomic groups and may not be extensive enough to fully identify species in samples especially if the target taxon is understudied (Thomsen & Willerslev, 2015). Despite these limitations, eDNA can still be used to accurately measure biodiversity especially when used alongside traditional methods (Ruppert et al., 2019).

Modern Uses of eDNA for Surveying Biodiversity and Biomonitoring

As using eDNA in ecology has become more widespread and its limitations mitigated, its application has expanded from microbes (Venter et al., 2004) to large-scale aquatic vertebrate surveys (Cristescu & Hebert, 2018; Deiner et al., 2017), diet analysis (Hemprich-Bennett et al., 2021; Schnell et al., 2012) and several other applications (Bohmann et al., 2014). For modern ecological analysis eDNA is commonly collected from a variety of sources. Aquatic eDNA in both freshwater and saltwater systems (Díaz-Ferguson & Moyer, 2014; Thomsen & Willerslev, 2015) is a well-researched source of eDNA particularly for biodiversity monitoring and endangered species management (Hallam et al., 2021; Plante et al., 2021; Thomsen et al., 2012; Vörös et al., 2017) and has become common place in most aquatic biomonitoring settings including in government monitoring and the commercial sector (Rees et al., 2014; Ruppert et al., 2019). Along with surveying fish and amphibian diversity, aquatic eDNA can identify invasive speicies (Loeza-Quintana et al., 2020), often before exisiting methods can. A more recent less conventional use of aquatic eDNA is to indirectly to measure local terrestrial life whose DNA washes into rivers (Mena et al., 2021; Serrao et al., 2021). eDNA in the soil has also been used in biodiversity surveys for terrestrial invertebrates

(Thomsen & Willerslev, 2015), vertebrates (Andersen et al., 2012) and plants (Taberlet et al., 2012). It has proven to be particularly useful in determining the species in caves, which are often hard to survey because of their locations and physical conditions (Hofreiter et al., 2003). Diet analysis has become easier with the introduction of eDNA and metabarcoding with a dual effect of tracking changes in predator-prey interactions (Hemprich-Bennett et al., 2021) as well as conduct biodiversity surveys using invertebrates (e.g. leeches) as samplers (Drinkwater, Jucker, et al., 2021; Drinkwater, Williamson, et al., 2021). In recent years, unconventional mediums such as the surfaces of plants and snow prints (Franklin et al., 2019) have also been explored. For example, eDNA on the surfaces of has been used to detect and characterize pollinator interactions (Walker et al., 2022).

Airborne eDNA

While eDNA collection from water has become common, even being as used a regulatory mechanism and a commercially viable business in many countries, similar approaches to terrestrial biomonitoring have not settled on a common method for eDNA collection or a reliable source. In fact, terrestrial life is often measured indirectly from local waterways where runoff carries mammal, bird and invertebrate eDNA into the aquatic system where it can quite accurately assay local life (Mena et al., 2021). The most obvious equivalent source for eDNA on land is air but, until recently, the air has been a largely untapped source of eDNA used primarily for the study of allergens and microbes (Abrego et al., 2018; Korpelainen & Pietiläinen, 2017; Kraaijeveld et al., 2015; Longhi et al., 2009;

Mohanty et al., 2017), despite various review papers pointing to airborne eDNA as being the next area of interest (Barnes & Turner, 2015; Ruppert et al., 2019).

Clare et al., 2021 published a paper proving a proof-of-concept demonstration that eDNA from mammals can be collected from air samples taken in enclosed spaces demonstrating the potential for air as an eDNA source. Another study by Serrao et al., 2021, explored airborne eDNA's potential to survey terrestrial mammal biodiversity as a part of comprehensive study looking at surveying big brown bats (*Eptesicus fuscus*) using eDNA in three mediums (water, soil, and air). As in Clare et al., 2021's study, airborne eDNA samples were collected in closed and controlled environment and were successful in extracting bat eDNA from the air. While the eDNA concentrations were low in this study, Serrao et al., 2021 suggest that an optimized sampling method for airborne eDNA could be a better approach to using aquatic eDNA when sampling bats. Later Clare et al., 2022, deployed airborne eDNA samplers at the Hamerton Zoo Park in Huntingdonshire, UK to determine if terrestrial vertebrate eDNA could also be collected in a more natural environment. Using a known and non-native local zoo fauna, Clare et al. 2022 were able identify species and estimate travel distance of eDNA in air given the known relative positive of the zoo fauna. In this study, airborne eDNA successfully detected many of the known zoo species, as well as some local species of special interest and showed potential for detecting predator-prey interactions (i.e., high concentrations of prey species DNA in predator enclosures) (Clare et al. 2022). The data were used to determine that traces of DNA could be detected at least 250m away from its known source (Clare et al. 2022). An almost identical study was conducted in parallel at the Copenhagen Zoo in Denmark (Lynggaard et al., 2022). Lynggaard et al. (2022) findings independently confirmed those

of Clare et al. 2022 providing strong support for the proposal that sampling eDNA from air is a viable terrestrial survey method.

The detection of species through airborne eDNA has several potential applications. It has already showed promise in estimating plant (Johnson et al., 2019, 2021), fungi (Abrego et al., 2018) and prokaryotic diversity (Núñez et al., 2017). Early work targeting insects has also shown airborne eDNA to be a powerful detection tool for terrestrial invertebrates (Roger et al., 2022). Studies which used passive sampling techniques like dust traps (M. Johnson et al., 2023), open containers of water (Klepke et al., 2022) and even spider webs were able to detect terrestrial vertebrate diversity. It has the potential to be used for other biomonitoring applications such as tracking invasive species or detecting species composition changes (Clare, et al., 2021). It could be used to survey hard to sample locations such as caves and burrows or locations where it is unsafe for humans to spend long periods of time (i.e., toxic bat roosts) (Clare, et al., 2021). Airborne eDNA also has potential applications outside of ecology. It could be used in criminal forensics or tracking disease transmission and forensic anthropology (Fantinato et al., 2022; Mercer et al., 2023).

The existing work done using airborne eDNA as terrestrial vertebrate survey tool have only focused on simple assemblages and have yet to determine if this method is successful in characterising complex, multispecies communities under natural conditions. Studies have largely focused on vertebrates confined in spatially separated locations in low abundances and diversity (Clare et al., 2022; Lynggaard et al., 2022). As such it is unclear how successful airborne eDNA will be at detecting species in more realistic

ecological communities where there is high species diversity and mixed abundances and without controlled environmental settings. This thesis seeks to address these gaps and expand on existing knowledge of airborne eDNA's potential as a biomonitoring tool.

Thesis Structure

This thesis is composed of two data chapters. In Chapter two, I tested three newly modified prototype air samplers and a novel filter material for the active collection of airborne eDNA with a focus on bat diversity. I deployed samplers in a classroom which was used as a field laboratory where bats were processed, and captures were recorded each night for 12 nights. The classroom resembled an artificial roost with multiple species present in mixed abundances and the community's composition changed each night. The objectives of this project were to determine: 1) if eDNA can accurately characterize species inventories in a diverse mixed mammal community, 2) whether common species overwhelm eDNA inventories, obscuring the presence of rare species in the assemblage and 3) whether filter design impacts DNA collection and taxonomic recovery.

Using the same prototypes, in Chapter three I sought 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. I deployed samplers in 12 roosts in our study area to test the hypothesis that airborne eDNA can be collected in sufficient quantities in natural roosts to document roosting ecology of cavity-roosting neotropical bat species.

Chapter 2:

Published in Environmental DNA as follows:

Garrett, N. R., Watkins, J., Simmons, N. B., Fenton, B., Maeda-Obregon, A., Sanchez, D. E., Froehlich, E. M., Walker, F. M., Littlefair, J. E., & Clare, E. L. (2023). Airborne eDNA documents a diverse and ecologically complex tropical bat and other mammal community. *Environmental DNA*, 5, 350– 362. https://doi.org/10.1002/edn3.385

Chapter Summary

Environmental (e)DNA has rapidly become a powerful biomonitoring tool, particularly in aquatic ecosystems. This approach has not been as widely adopted in terrestrial communities where the methods of vertebrate eDNA collection have varied from the use of secondary collectors such as blood feeding parasites and spider webs, to washing surfaces of leaves and soil sampling. Recent studies have demonstrated the potential of direct collection of eDNA from air sampling, but none have tested how effective airborne eDNA sampling might be in a biodiverse environment. We used three prototype samplers to actively sample a mixed neotropical bat community in a partially controlled environment. We assess whether airborne eDNA can accurately characterize a high diversity community with skewed abundances and to determine if filter design impacts DNA collection and taxonomic recovery. Our study provides evidence for the accuracy of airborne eDNA as a detection tool and highlights its potential for monitoring high density, diverse assemblages such as bat roosts. Analysis of air samples recovered >91% of the species present and some limited relationship between species abundance and read count. Our data suggests this method can accurately depict a diverse mixed-mammal community, particularly when the location is contained (e.g., a roost, den or burrow) but also highlights the potential for secondary transfer of eDNA material on clothing and

equipment. Our results also demonstrate that simple, inexpensive, battery-operated homemade air samplers can collect an abundance of eDNA from the air, opening the opportunity for sampling in remote environments.

Introduction

Environmental (e)DNA was first used to reconstruct plant and microbial communities based on 2000–4000 year old ice core sections, which were melted and filtered to collect DNA (Willerslev et al., 1999). eDNA has rapidly become a powerful tool for modern biomonitoring, often complimenting traditional methods to track invasive species (M. D. Johnson et al., 2021; Thomsen & Willerslev, 2015), monitor biodiversity and detect rare species (Plante et al., 2021; Thomsen et al., 2012). In many cases, eDNA is reported to outperform traditional approaches (Hallam et al., 2021) either in efficiency (i.e., less time intensive), or taxonomic recovery (i.e., detects species traditional methods do not) and can be less invasive for the detection of rare species, (Thomsen & Willerslev, 2015). The collection of eDNA does not rely on specialized knowledge for species ID, eliminating some of the uncertainty associated with field data (Plante et al., 2021).

Environmental DNA is frequently leveraged in aquatic systems where its use for biomonitoring is widespread, and is often employed to meet regulatory obligations, which arise both commercially and from public bodies (Deiner et al., 2016; Ficetola et al., 2008; Rees et al., 2014; Ruppert et al., 2019). Aquatic eDNA is commonly used to survey diversity in freshwater and marine ecosystems (Díaz-Ferguson & Moyer, 2014; Thomsen & Willerslev, 2015), and has been applied in more complex ecological analyses such as occupancy modeling (Schmelzle & Kinziger, 2016), monitoring protected area efficacy (Ji

et al., 2022) and understanding depth partitioning (e.g., in freshwater lakes fish eDNA appears stratified reflecting thermal niches, Littlefair et al., 2020, and kelp forests, Monuki et al., 2021).

Terrestrial Vertebrate Monitoring using eDNA

Unlike aquatic systems where eDNA is sampled primarily from water or sediment, there is no widely accepted collection source for eDNA in terrestrial biomonitoring and a variety of approaches have been explored. eDNA trapped in frozen sediments (Sønstebø, et al., 2010; Willerslev et al., 2003) and soils (Andersen et al., 2012; Hofreiter et al., 2003; Ryan et al., 2022; Taberlet et al., 2012; Thomsen & Willerslev, 2015) has been used to target plants, invertebrates and vertebrates. Sampling terrestrial vertebrates using eDNA often involves targeting indirect sources; for example, local waterways where runoff has carried terrestrial eDNA into the aquatic system (Mena et al., 2021; Serrao et al., 2021) and invertebrates, which sample other animals by virtue of their feeding ecologies including dung beetles (Drinkwater, Williamson, et al., 2021), leeches (Drinkwater, Jucker, et al., 2021), and carrion flies (Calvignac-Spencer, Merkel, et al., 2013). More recently, swabbing vegetation (Lyman et al., 2022) and spider webs (Gregorič et al., 2022) has shown potential for detecting vertebrates. Many of these indirect methods of eDNA collection rely on an intermediate “vector” (leeches, flower parts, etc.) to first collect the eDNA, and in most cases these “vector” methods target only one or a small number of taxa at any given time. For example, a leech, carrion fly, or dung beetle meal results from recent interactions with one or a small number of prey or food sources, thus the sample complexity is low, and we might expect a single species to be detected compared to an environmental sample, which might have dozens, or hundreds of taxa represented. As a

consequence, these terrestrial eDNA approaches often pool multiple biological samples to increase the probability of a positive detection (Drinkwater et al., 2019). This contrasts sharply with the extreme diversity of species encountered in many aquatic eDNA samples.

Airborne eDNA

For monitoring terrestrial vertebrates on land, air is an obvious direct source for eDNA, but until recently, has primarily been used to study allergens and pathogens (Abrego et al., 2018; Korpelainen & Pietiläinen, 2017; Kraaijeveld et al., 2015; Longhi et al., 2009; Mohanty et al., 2017), which may survive longer in air than vertebrate eDNA and cells. A proof-of-concept paper showed that terrestrial vertebrate eDNA could be actively collected from air using a pump that drew air through a filter (Clare et al., 2021). This study successfully detected Naked-mole rats (*Heterocephalus glaber*) both inside their artificial burrows and the room in which they were housed. Several other studies have now explored airborne eDNA as a terrestrial biodiversity monitoring tool for vertebrates, insects, and plants. Serrao et al., 2021 were able to detect Big brown bats (*Eptesicus fuscus*) using airborne eDNA in a captive colony, but highlighted the need for an optimized sampling method. Clare et al. (2022) actively sampled the air at a zoological park in the UK and detected target zoo resident species such as the Javan binturong (*Arctictis binturong*) and local wildlife such as squirrels (*Sciurus* spp.) and ducks (*Anatidae*). Coincidentally, an almost identical study independently made similar observations of air sampled at a zoo in Denmark (Lynggaard et al., 2022) detecting species such as okapi and armadillo. Both studies also detected airborne eDNA transported around the zoo with detection distances estimated as up to 250 m from the most likely source (Clare et al. 2022; Lynggaard et al., 2022).

Under field conditions, sampling for airborne eDNA using powered “active” sampling (i.e., vacuums and pumps, which draw air through a filter) has successfully collected eDNA from insects, amphibians, birds, and mammals (Roger et al., 2022). Passively sampling material from the air (i.e., unpowered dust traps, material settling on surfaces) has shown promise as an effective method for collecting airborne eDNA from vertebrates, invertebrates, fungi, and plants (Johnson et al., 2021; Klepke et al., 2022). For example, Johnson et al. (2021) detected 91 plant species, 11 more than were detected using traditional transect-based, line-point intercept and visual surveys. From the same dust samples, Johnson, et al. (2023) also detected multiple mammals and birds and, over the course of a year's collections, observed that detections differed temporally and seemed closely related to known animal activity in the landscape, demonstrating the potential for airborne eDNA to rapidly track animal activity.

Applications to complex terrestrial communities

A major limitation of existing airborne eDNA studies is that most have only tackled simple assemblages of vertebrates (Klepke et al., 2022; Roger et al., 2022) or places where animals are confined in low diversity and abundance, and in spatially separated locations. For example, among zoo experiments, most animals were not housed in mixed species communities, simplifying the detections to one primary signal (Clare et al. 2022). To date, no study has addressed the considerable challenge of sampling air in an environment of high biodiversity and highly skewed species abundances. As such, it is unclear how airborne eDNA analysis will function in more realistic ecological communities. Some studies have begun to explore settings, which resemble these conditions (Johnson et al., 2021, Klepke et al. 2022; Roger et al., 2022). For example, targeting plants (Johnson

et al., 2021) in range land, where community turnover is low and slow, and/or invertebrates (Klepke et al. 2022; Roger et al., 2022), where community composition and dynamics are very different from vertebrates.

We address this knowledge gap using Neotropical bats as focal taxa in an artificially controlled experiment that replicates a natural community. On an annual research trip in the Orange Walk District in Belize, a team routinely brings captured bats from ≈40 species into a controlled classroom setting (a space used as a field laboratory, approximately 12 m × 6 m × 15 m) with species diversity varying from night to night depending on capture success. Identifications and detailed taxonomic records of species richness and abundance are made by expert bat taxonomists over the course of a 2-week intensive research program. Because the captures reflect local community richness and use traditional field sampling techniques for bats (i.e., mist nets and harp traps), the assemblage is naturally highly skewed with a few very abundant species combined with a few individuals of rarer species. While the collections reflect a natural community, the controlled location of a classroom allows us precise knowledge of actual species richness and abundance so that relative recovery of true biodiversity can be assessed. Detailed bat taxonomic assessments and surveys have been conducted at this site annually for more than a decade, resulting in an extremely well-known local fauna and excellent existing reference libraries for both morphological and molecular sequence data. Extremely detailed record keeping coupled with a high density of individuals in a semi-confined space makes it an excellent location to assess new sampling prototypes and test the hypothesis that airborne eDNA can accurately characterize complex mammal

communities. This design replicates an empty cave or roost where a perfectly known group of animals comes and goes every night over the course of a 2-week field campaign.

Using this design, we tested three newly modified prototype active air samplers and a novel filter material for the active collection of airborne eDNA. We used the data collected to assess whether: (1) eDNA can accurately characterize species inventories in a diverse mixed-mammal community, (2) common species overwhelm eDNA inventories, obscuring the presence of rare species in the assemblage, and (3) filter design impacts DNA collection and taxonomic recovery. We hypothesize that eDNA from abundant, common species will not overwhelm eDNA inventories, allowing rare species in the assemblage to still be detected via airborne eDNA despite their low numbers.

Materials and Methods

Air Sampler Design

We constructed three prototype models of air samplers for eDNA collection using computer fans and 3D printed attachments bolted to the fans (Figures 1.1 and 1.2). While some commercial samplers are available, they are generally too large, heavy, and expensive for general use in the field. Our filters are a modified version of Lynggaard et al., (2022) adapted to include an improved support to prevent filters from being drawn into the fan and an adjustable ring, which can secure filters against wind or other environmental disturbance and can permit different filter thicknesses to be used (all 3D printing design files are included as a supplement to this paper – Appendices 1-4). Our filters were designed to meet specific criteria suitable for field ecology, ideally being inexpensive, quiet, easy to construct and repair in the field, operable over long periods of

time on batteries that are readily recharged, light weight for hiking out into the field, and robust to extremes of weather (high heat and humidity).

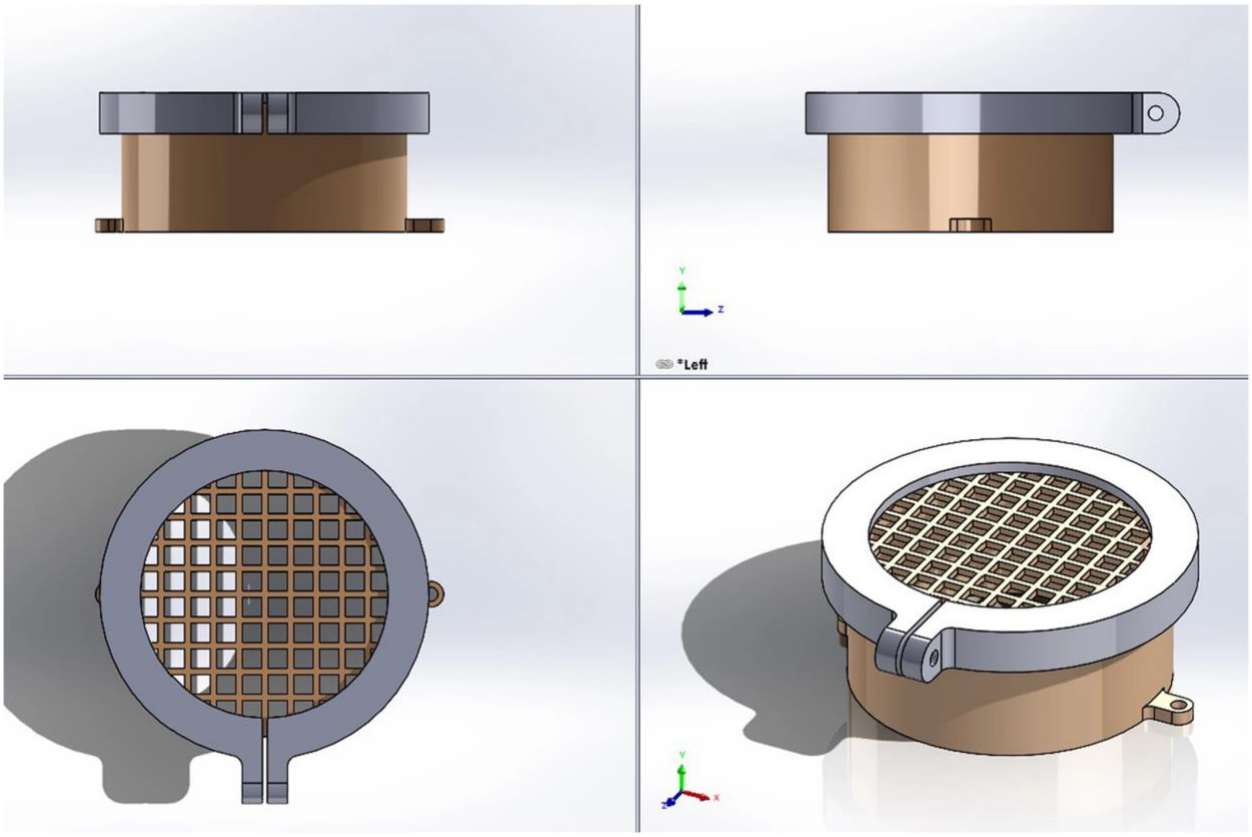


Figure 2.1. Prototype 3D printed filter frames. The base unit (brown) is attached to the blower fan and the ring cinch (gray) can be tightened with a nut and bolt to secure different filter thicknesses. Filter surface area of the small version is 122.5 mm^2 while the large sampler has a surface area of 280 mm^2.

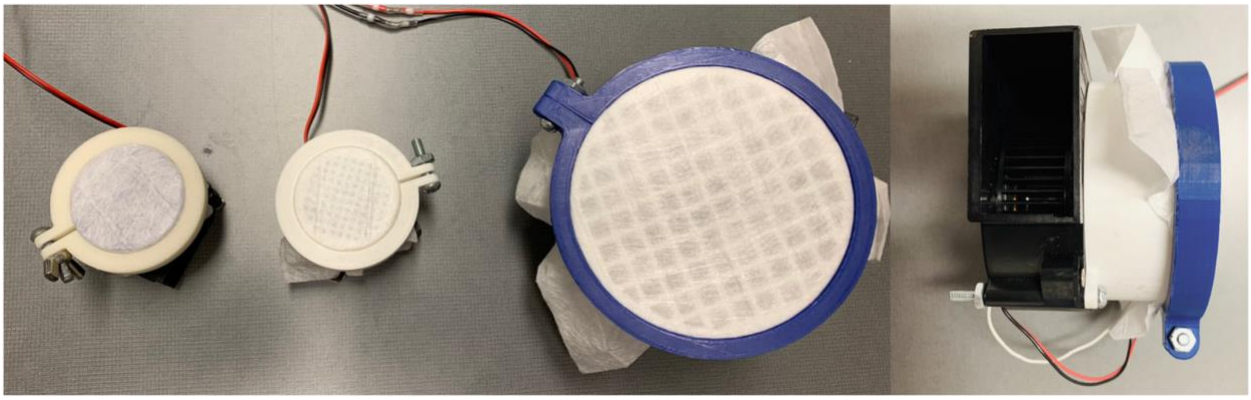


Figure 2.2. Three prototype air filters. A 5v small filter, 12vSmall, and 12vLarge filter (top and side) used for the filtration of air samples and collection of airborne eDNA. The small filters have a surface area of 122.5 mm2 while the large sampler has a surface area of 280 mm2.

Design 1–5v: a 5 V WINSINN 40 mm × 40 mm × 20 mm 2-pin, 1 W, DC blower fan wired to a 5 V USB lead and powered using a standard 5000 mAH power bank. To this we added a custom 3D printed filter frame (see Figures 1.1 and 1.2 and Appendix 1 and 2) attached by two bolts and two nuts. Approximate cost: $20 CAD (including battery), weight (without battery): 83 g, air draw (with filter): 3.2 KmH, filter diameter: 39 mm, surface area: 122.5 mm^2.

Design 2–12vSmall: a 12 V WINSINN 40 mm × 40 mm × 20 mm DC brushless fan, 2-pin, 0.15 A 1.8 W wired to an Enercell 270-052 12VDC Car Power Adapter with a switch for power on/off. To this we added a custom 3D printed filter frame (see Figures 1.1 and 1.2, Appendix 1 and 2) attached by two bolts and two nuts. Approximate cost: $10 CAD, weight (without battery): 117 g, air draw (with filter): 2.5 KmH, filter diameter: 39 mm, surface area: 122.5 mm^2.

Design 3–12vLarge: a 12 V NMB 97 mm × 93.5 mm × 33 mm DC brushless fan, 3-pin, 1.2 A 19.2 W wired to an Enercell 270-052 12VDC Car Power Adapter with a switch for

power on/off. To this we added a custom 3D printed filter frame (see Figures 1.1 and 1.2, Appendix 3 and 4) attached by two bolts and two nuts. Approximate cost: $20 CAD, weight (without battery): 325 g, air draw (with filter): 6.4 KmH, filter diameter: 89 mm, surface area: 280 mm^2.

We powered Design 2 and 3 using RoyPow 30 W PD Power Banks (RoyPow USA), which have a 12V 10A “cigarette” lighter socket compatible with the 12V fans. These power banks can be fully recharged in about 3 h using a 100 W charger. We used a UGREEN 100 W USB C multiport charging station, which could accommodate multiple batteries simultaneously. Net weight for batteries = 821 g each, and these batteries are acceptable in carry-on luggage on most commercial airlines.

For filter material we used Filtrete 1900 Smart Air Filters (3 M) “Merv 13” designed for furnace filtration and which are rated for the capture of pollens, bacteria, and viral sized particles from circulating air. We removed the filter material from the standard wire and cardboard frame and cut squares that would cover our 3D printed filter frames and could be secured using the ring cinch (Figure 1.1, Appendix 1, 2, 3 and 4). We UV sterilized each filter and packaged them for field collections.

Air Sample Collection and Filtration

We collected air samples from 25 April to 7 May 2022 (13 sample days), in Orange Walk District, Belize. Bats were captured in the riverine preserve forest of Lamanai Archeological Reserve, the forests near the Lamanai Outpost Lodge, and the forest fragment of the Ka'kabish Archeological Project using mist nets and harp traps (Permit #

FD/WL/1/21(12&18), York ACC 2021-10). Due to COVID protocols along with the heat and humidity, our first action upon arrival was to open the classroom windows and double doors and turn on ceiling fans. These conditions were maintained throughout the experiment. We deployed samplers in the classroom field lab where captured bats were brought from the field and processed each night. The eDNA was sampled by simultaneously filtering air using the 3 prototypes described. The samplers were positioned next to each other on a bench at chest height at the end of the classroom where the bats are held in bat bags for examination and identification. Samplers were turned on when the first bats were brought into the classroom. They ran for 6–8 h depending on the arrival time of the first bats in the empty classroom each night, and we removed the filters the next morning. We did not sample during the day as the bats captured were released before sunrise and the room was used for other activities during the day. Filters were folded with the exposed side in and frozen in clean sample bags for later analysis. Before each sampling night, we cleaned the 3D printed filter frames with a 50% bleach solution and then water. We collected 37 individual samples (12vLarge n = 13, 12vSmall n = 12, 5v n = 12, one extra 12vLarge filter was collected on a day with minimal bat sampling) over the course of 14 days. KN95 masks and gloves were worn by all researchers when handling filters or when bats were present in the classroom. Over the sample period, 880 individual bats of 35 taxa were present in the room.

DNA Extraction from Filter Material

We performed all laboratory procedures inside a sterilized AirClean 600 PCR workstation while wearing KN95 masks, gloves, and lab coats. We cleaned all instruments using a

10% bleach solution, followed by a 10% ethanol solution, and then rinsed these with sterilized water before each use and between each sample. We cleaned the workstation with a 25% bleach solution and 30 min of UV light before handling any eDNA products. We extracted all filter samples using a Qiagen Blood and Tissue extraction kit as follows. We cut out a 2 cm^2 piece from the center of the filter and incubated it overnight at 56°C in 180 µl ATL buffer and 20 µl proteinase K. After incubation, we removed the filters using clean forceps and spun the filters in QiaShredder spin columns for 3 min at 13,000 rpm. For the rest of the extraction, we followed the manufacturer's guidelines. We processed extraction blanks (an extraction without any filter) along with the samples to act as a negative control during extraction. We quantified the DNA concentration of each sample using a Nanodrop. We froze extracted DNA until use.

PCR and Sequencing

We amplified a region of the mitochondrial 16 S gene for each sample using the mam1 (5′-CGGTTGGGGTGACCTCGGA-3′) and mam2 (5′-GCTGTTACCCTAGGTAACT-3′) primers (≈90 bp + primers) (Calvignac-Spencer, Merkel, et al., 2013; Taylor, 1996) adapted for the Illumina MiSeq sequencing platform. Mitochondrial DNA is commonly used in eDNA work because it correctly diagnoses species level diversity and is found in much higher copy number than nuclear DNA. It has also been shown to work well with airborne eDNA (Clare et al., 2021). Each mix included 7.5 µl of QIAGEN Multiplex PCR Master Mix, 1.25 µl of ddH_2O, 1.75 µl of human blocking primer (5′-GCGACCTCGGAGCAGAACCC-spacerC3-3′) (Calvignac-Spencer, Leendertz, et al., 2013), 0.75 µl of each forward and reverse primer tagged with CS1 and CS2 adaptors and 3 µl of extracted DNA. Because two facilities were used to avoid batch effects (below)

we use different adaptors with slight variation in PCR cycling to achieve the same PCR success. We conducted the first PCR using the following cycle conditions: 95°C for 10 min, 40 cycles of 95°C for 12 s, 59°C for 30 s, 70°C for 25 s, and a final 72°C for 10 min. We performed two additional rounds of PCR under the same conditions but with the number of cycles increased to 45 and the overhang adaptors changed to those specified by Illumina in their 16S metagenomic library preparation guidelines. We included negative (no template) and positive (salmon) controls in each PCR. We used salmon as a positive control because salmon DNA would not be readily available in the tropical environment where the samples were collected, unlike other potential controls such as cow, pig, chicken, or lamb DNA, all of which are animals present on local farms.

We visualized all PCR products, including all controls (positive, negative, and extraction blank) using a 1.5% agarose EtBr gel and run at 110 V for 1 h. Given the unknown nature and efficiency of air sampling for such an ecologically complex community we employed extra sequencing controls. To independently confirm sequencing, avoid batch effects, and detect potential sequencing contamination, the replicates were sent to two separate sequencing facilities for library building. The first CS1/CS2 tagged PCR replicate was sent to Barts and the London Genome Centre, where the products were indexed. The samples were quantified on a TapeStation D100 (Agilent) and normalized and pooled for sequencing using an Illumina MiSeq V3 Micro 2 × 300 cycle run. Two other replicates were sequenced and indexed separately as follows. A sequencing library was prepared from the purified amplicons, indices were added following Illumina's 16S Metagenomic Sequencing Library Preparation protocol but using 1× DreamTaq PCR Master Mix (Thermo Scientific). Indexed PCR products were again purified using Mag-Bind®

TotalPure NGS (Omega Bio-tek) magnetic beads. The purified index products were quantified using a Qubit dsDNA BR Assay Kit, normalized and pooled. The pooled PCRs were sized using a TapeStation D1000 ScreenTape System (Agilent). The libraries were sequenced on an Illumina MiSeq with a V3 MiSeq Reagent kit, 300 cycles. The final library was loaded at 10 pM with a 20% PhiX control spike and sequenced at the NatureMetrics laboratory. Reads were demultiplexed in preparation for bioinformatic analysis and exported as FASTQ files.

Bioinformatics Methods and Statistical Analysis

We processed the demultiplexed sequences using the DADA2 pipeline (Callahan et al., 2016) in RStudio (RStudio Team, 2021). The forward and reverse reads were filtered, trimmed to 90 bp, and errors in the sequence data were removed based on the learned error rates generated by the DADA2 *learnErrors* function. We removed primer sequences using cutadapt 3.7 in paired-end mode (Martin, 2011). We merged paired reads and generated amplicon sequence variants (ASVs). Any chimaeras detected at this stage were removed and the ASVs were exported as a FASTA file. We used BLAST to compare individual ASVs to the full nucleotide collection in NCBI to evaluate likely taxon of origin and use updated taxonomic designations following BatNames (https://batnames.org), Mammal Species of the World (http://www.departments.bucknell.edu/biology/resources/msw3/browse.asp), and Catalogue of Life (https://www.catalogueoflife.org/). We removed ASVs matched to human DNA, which was ubiquitous in the sampled environment. Next, we removed ASVs that matched Black-tailed Jackrabbit (*Lepus californicus*) as these are likely from a previous sample processed in our lab. A single ASV matched to *Cervus canadensis*

nannodes was also removed as it does not occur in our study area and was present in samples that had previously been processed in the lab. All matches to fish species and one match to a Harbor porpoise (*Phocoena phocoena*) were considered contaminates during the sequencing process as the sequencing facility primarily processes aquatic samples. We then disregarded the positive control, *Salmo salar*. The ASVs that had a match >95% identity (100% overlap) to either bats or other taxa known to be in the area were kept for further scrutiny. Four fell below this but were retained based on known room occupancy and matches to sister taxa where the local species was not represented in the database. We retained all ASVs matched to a bat species known to be in the classroom regardless of read count. For matches to other taxa known to be in the area, ASVs with read counts below 20 were discarded, which is the threshold that captures all true positives (bats we know were in the classroom and non-target species seen in the area).

We divided the ASVs into high-quality detections, low-quality detections, and very low-quality detections. High-quality detections are those that would be retained using a highly conservative filtering approach of excluding any ASV detection with a read count below the highest read count found for any taxa detected in the negative controls. Low-quality detections were those which fell below the read count of the highest negative control contaminant but were matched with bat taxa known to be present in the classroom. Similar to low-quality detections, very low-quality bat detections would normally be excluded based on very low read count, below any contaminant, but they matched bat species known to be in the classroom or area and had no other potential source in our lab. For other mammals, very-low quality detections represent species that would be excluded based on negative controls but were seen in the area. Any identifications that

did not fall into the above categories, such as non-Neotropical bat species were fully explored to try and determine the source and are discussed further below.

As the data was not normally distributed, we performed a Kruskal-Wallis anova to compare both the total read counts (library size) and DNA concentration per prototype sampler design to test whether read count or DNA concentration differed by sampler type (5v, 12vSmall 12vLarge). We used Pearson's correlation to assess the relationship between cumulative species abundance over the 2-week sampling period and recovered cumulative read count per species across all three replicates.

To further independently verify the collection of bat eDNA from air, a subset of five samples were sent to a collaborating lab at Northern Arizona University. This analysis used COI and 12 S markers instead of the 16 S region used on the full sample set. Full methods for this verification step are presented in Appendix S5.

Results

Species Detections

For the main 16s data generated at NatureMetrics we processed approximately 7.16 million reads. Following filtering, denoising, merging and chimera detection this was reduced to approximately 4.61 million reads for further processing including all PCRs, two extraction blanks, three PCR blanks, two reagent blanks (without water as a template), and three positive controls ($\bar{x}$ /sample replicate of 55,757 SE ± 948). These were reduced to 2305 ASVs ($\bar{x}$ = 27.4/replicate). For the subset data run at the QMUL Genome Center we processed approximately 1.4 million reads, 0.98 million post filtering ($\bar{x}$/sample replicate of 21,259 SE ± 840). After the exclusion of known contaminants (e.g., human)

247 ASVs successfully met the match criteria and read count criteria for retention. These ASVs were identified as belonging to 41 taxa, including 27 bat taxa (three taxa could not be identified to species and we retained only taxonomy at genus level) (Figures 1.3 and 1.4), a species of amphibian, five species of non-bat native mammals, and eight species of domesticated animals (Figures 1.3 and 1.4). Of the bat taxa identified, 11 were considered high-quality detections and 11 were considered low-quality. An additional five bat taxa were identified as very low-quality detections (very low read counts) but did match species known to be present during the sampling period (Figure 1.3). One of these bats, *Rhynchonycteris naso* was caught but not brought back to the classroom. *R. naso* typically forage over water and were caught using a boat. Thus, these individuals were processed in the field at the site of capture and released. However, cloth bags used to contain these bats in the field were shaken out in the classroom on multiple occasions. The other four (*Bauerus dubiaquercus*, *Lasiurus ega*, *Myotis* spp. and *Vampyressa thyone*) were present in classroom. Daily captures compared to detections can been seen in Figure S1. Four detections in the classroom occurred prior to our bat captures and include species of common local bats whose DNA could have been drawn in from the outside by fans.

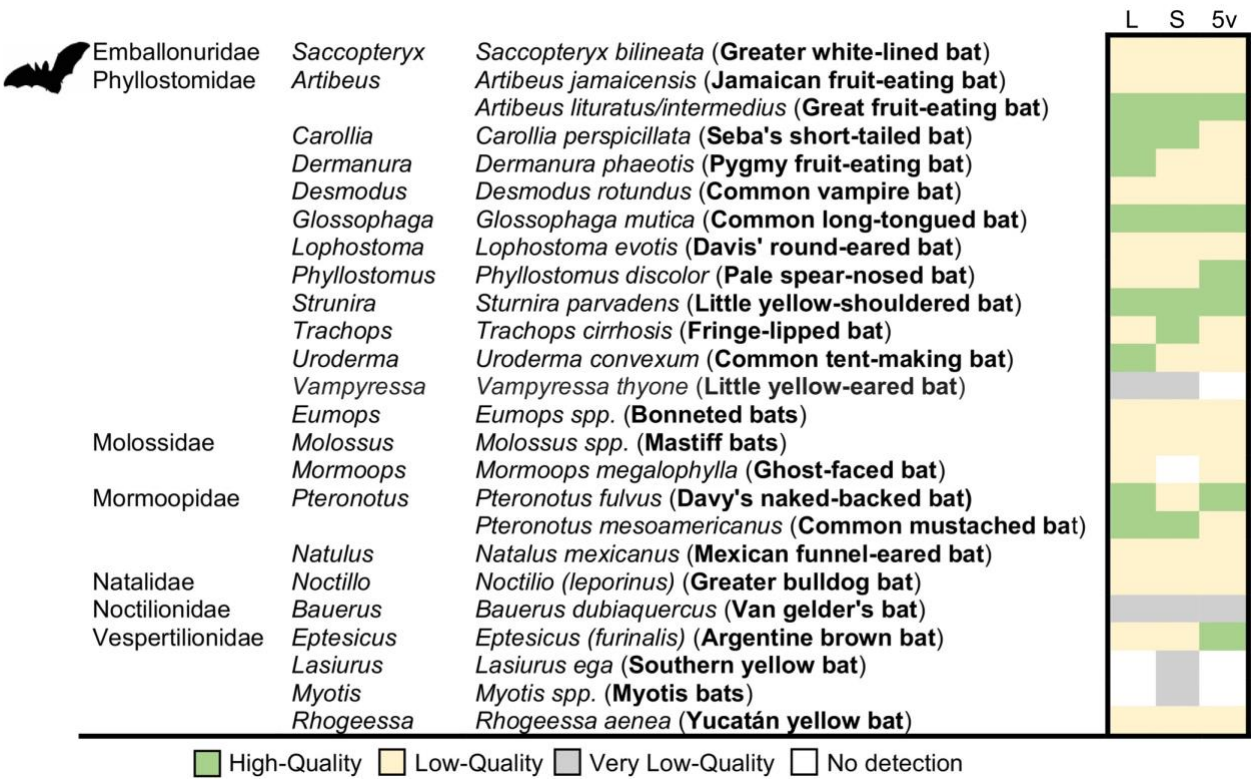


Figure 2.3. Bat species present in the classroom and detected using active airborne eDNA sampling by three prototype samplers: 12vLarge (n = 13), 12vSmall (n = 12), and 5v small (n = 12). High-quality detections are indicated in green, low-quality detections in yellow, and very low-quality detections in gray. White indicates that the taxon was not detected. Noctilio (leporinus) and Eptesicus (furinalis) could not be identified beyond genus; however, there are only one Noctilio and Eptesicus species known in the area thus species is indicated by ().

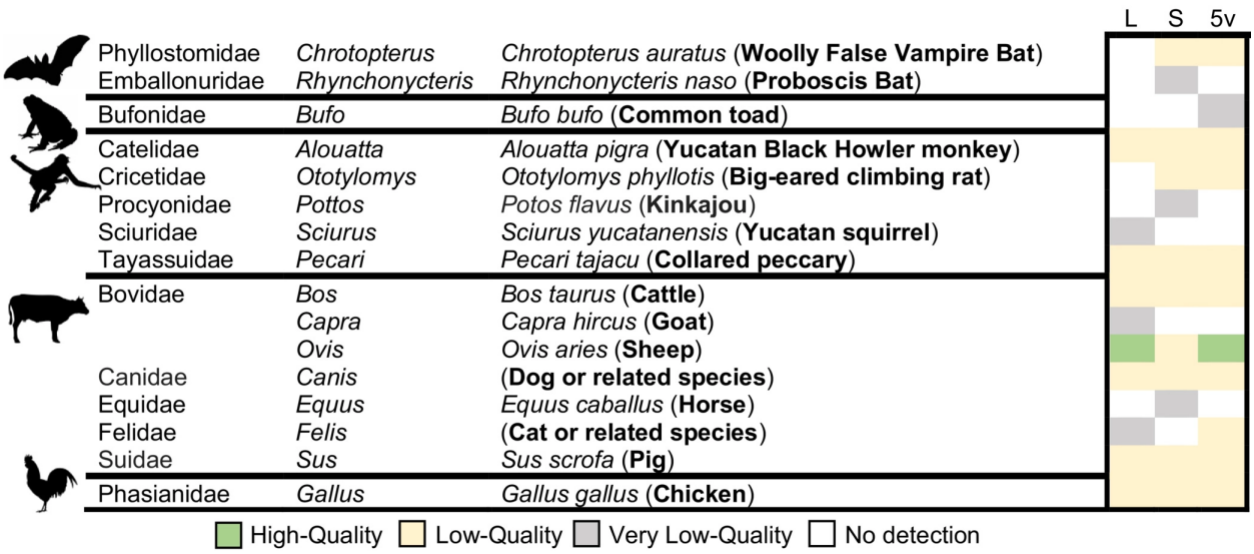


Figure 2.4. Bat species not brought to the classroom and non-target vertebrate species detected using active airborne eDNA sampling by three prototype samplers: 12vLarge (n = 13), 12vSmall (n = 12) and 5v small (n = 12). High-quality detections are indicated in green, low-quality detections in yellow, and very low-quality detections in gray. White indicates that the taxon was not detected.

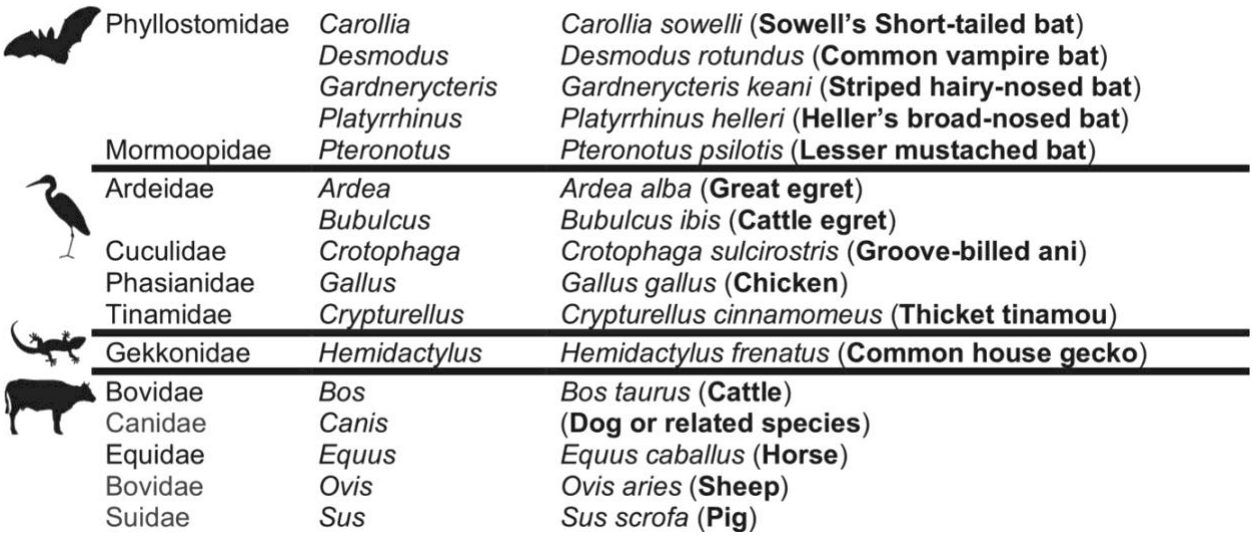
Chrotoperus auritus was a low-quality detection. It is common in the area and frequently caught but was not captured during the sampling period, though it is possible that one or more of these bats was briefly trapped in a mist net or harp trap that was later brought back to the classroom, and DNA may be on equipment from previous years.

In addition to the taxa described above, we detected four bat taxa not found in the Neotropics. These were detected with extremely low read counts and were subsequently traced to likely secondary transfer of forensic trace material on equipment used elsewhere in the world by various team members in the months just prior to this field trip.

Of the non-bat native mammals detected, three were low-quality detections, while the remaining two, the Kinkajou (*Potos flavus*) and the Yucatan squirrel (*Sciurus*

yucatanensis), were very low-quality detections but are known in the area. Kinkajous were seen around the classroom and one night played in the trees over our nets and team. Their DNA is not unexpected in the area, on our equipment, and on our clothing. The amphibian detection was a very low-quality detection as was the horse (*Equus caballus*) and goat (*Capra hircus*). Except for the high-quality sheep (*Ovis aries*) detections, the remaining domestic mammal and bird detections were considered low-quality (Figure 1.4).

Five samples sent for independent verification led to the identification of 16 species including five bat species, five bird species, one reptile and five non-native mammals. Three of the five bat species, four of the bird species and the reptile species detected in this subset were not detected in our analysis (Figure 1.5) bringing the total number of taxa detected to 49 with 30 bat taxa detected. This verification used a different target amplicon (see Appendix S5) and identification approach. These novel detections included two species for which the 16S region is not present in the reference database, and thus they were not detected until a complimentary approach was used. After accounting for species that we could not differentiate beyond genus, we identified all but 3 rare bats known to be present in the room during the sampling period.



Phyllostomidae	*Carollia*	*Carollia sowelli* (**Sowell's Short-tailed bat**)
	Desmodus	*Desmodus rotundus* (**Common vampire bat**)
	Gardnerycteris	*Gardnerycteris keani* (**Striped hairy-nosed bat**)
	Platyrrhinus	*Platyrrhinus helleri* (**Heller's broad-nosed bat**)
Mormoopidae	*Pteronotus*	*Pteronotus psilotis* (**Lesser mustached bat**)
Ardeidae	*Ardea*	*Ardea alba* (**Great egret**)
	Bubulcus	*Bubulcus ibis* (**Cattle egret**)
Cuculidae	*Crotophaga*	*Crotophaga sulcirostris* (**Groove-billed ani**)
Phasianidae	*Gallus*	*Gallus gallus* (**Chicken**)
Tinamidae	*Crypturellus*	*Crypturellus cinnamomeus* (**Thicket tinamou**)
Gekkonidae	*Hemidactylus*	*Hemidactylus frenatus* (**Common house gecko**)
Bovidae	*Bos*	*Bos taurus* (**Cattle**)
Canidae	*Canis*	(**Dog or related species**)
Equidae	*Equus*	*Equus caballus* (**Horse**)
Bovidae	*Ovis*	*Ovis aries* (**Sheep**)
Suidae	*Sus*	*Sus scrofa* (**Pig**)

Figure 2.5.Species detected from a subset of samples (n = 5) using COI and 12S markers, and alternative lab protocols at the Northern Arizona University for independent confirmation of detections.

Sampler Comparison and Read Count-Abundance Correlation

Neither read count ($\chi^2_2 = 1.41$, $p = 0.49$) nor DNA concentration ($\chi^2_2 = 1.93$, $p = 0.38$) differed significantly between the three sampler types that we employed. The 12vLarge samplers detected nine taxa with high read counts, 19 with low read counts, and five with very low read counts (Figure 1.3). The 12vSmall samplers detected six taxa with high read counts, 23 with low read counts, and seven with very low read counts (Figures 1.3 and 1.4). The 5v samplers detected seven taxa with high read counts, 24 with low read counts and two with very low read counts (Figures 1.3 and 1.4). Read count and cumulative species abundance in the classroom had a positive correlation (Pearson's $r = 0.71$, $t_{24} = 4.95$, $p < 0.001$) (Figure 1.6).

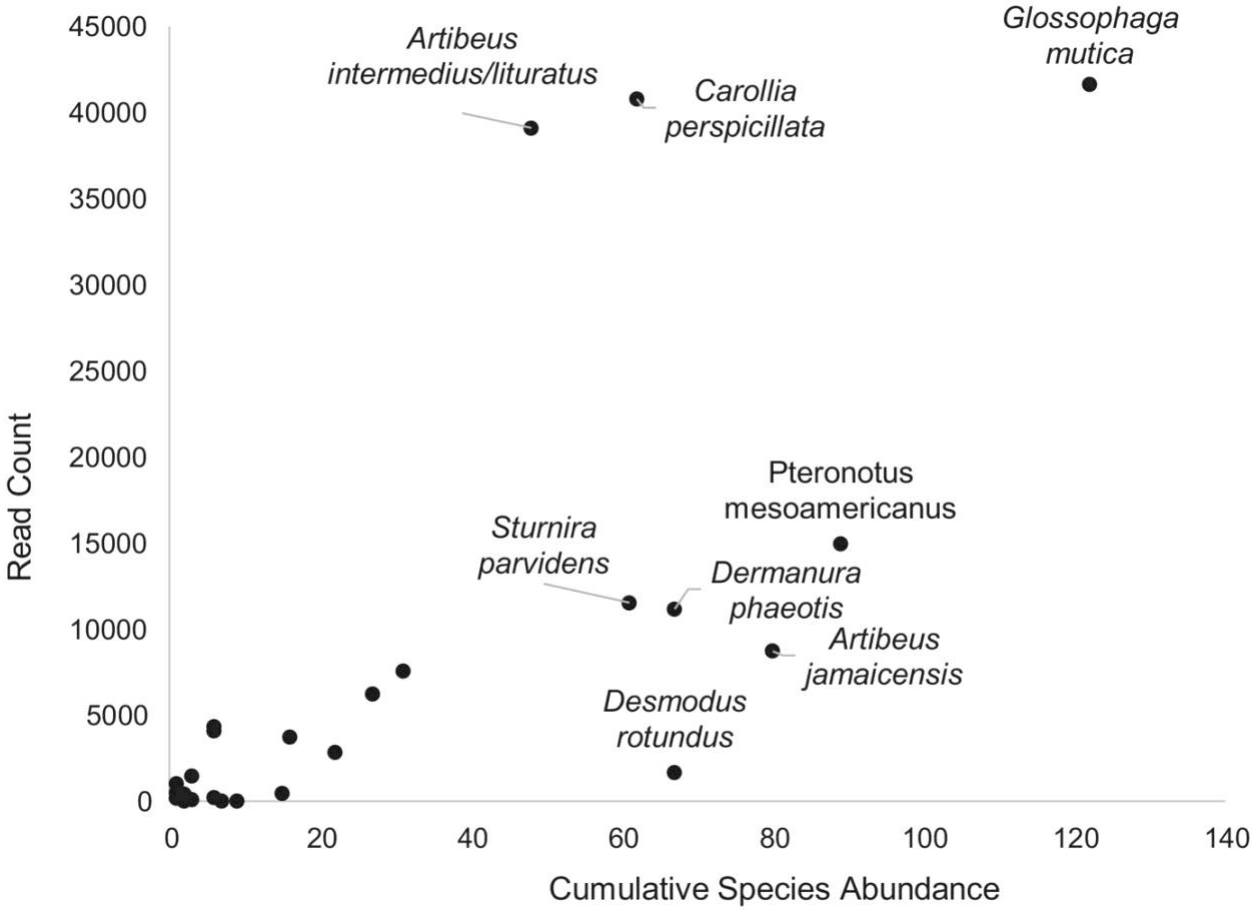


Figure 2.6.Total summed read counts across PCR replicates and their corresponding total bat species abundance over the 2-week sampling period. The eight most abundant species are labeled. (Pearson's r = 0.71, t24 = 4.95, p < 0.001).

Discussion

Our objective was to assess the efficiency of airborne eDNA in documenting a diverse and ecologically complex community, and to test three new prototype samplers and a novel filter material for the collection of airborne eDNA. We used an artificial classroom “roost” where a known community of bats was brought for identification each day. Our data demonstrated the success of all three sampler designs for accurately cataloging species inventories. With a high degree of accuracy, airborne eDNA was able to

document an ecologically diverse (35 taxa) community with highly skewed (taxon counts varied from 1 to 122 individuals) relative species abundances. These results highlight airborne eDNA's potential for larger biodiversity surveys, particularly in caves and roosts, which can be hard to access and monitor efficiently.

Three prototype samplers

We found no significant difference in DNA concentration or read count between our 3 different types of samplers, which suggests that flow rate does not have a significant impact on the amount of eDNA collected. It is likely that other factors such as surface area of the collector, sampling time, and environmental conditions may have a greater influence on the amount of eDNA collected. Serrao et al., 2021 collected low concentrations of airborne eDNA despite actively sampling bats in an enclosed space. Johnson et al. (2021) and Klepke et al. (2022) seemed to have greater success using dust traps and tubs of water, respectively. Neither of the latter passive sampling methods could control flow rate, but relied on wind and air movement over a larger surface collecting area than the filters used by Serrao et al., 2021. The surface area of the filter should be considered when refining airborne eDNA collection methods, as larger surface areas may collect more material but be harder to process in a lab. Other factors which may influence sampling include sampling duration (Klepke et al. 2022) and external environmental factors (Johnson et al. 2021) such as heat, UV exposure, rain, and humidity, but none could be measured in our environmentally stable situation. Our data captured a very good representation of the total species richness present over the 2-week sampling period, but for any given night we only captured a small portion of the room's diversity. Using more samplers to generate more biological replicates can be expected to

improve the detection of diversity per night, though an optimal sampling design is not yet clear. A number of commercial air samplers are available but all models we are familiar with suffer from a number of key limitations. First, most are extremely expensive (one model was priced at approximately $13,000 USD/unit) making it impossible to deploy them in large scale sampling arrays and risky to leave unprotected. Second, they are often extremely heavy or large making them hard to take to remote areas (one model is the size of a suitcase). Finally, many are designed for medical settings where there is access to power and cannot easily be run by batteries, while those that do use batteries often violate airport transport laws on battery size. For field practicality, our goal was to test something that could be made at low cost, easily repaired and small and light enough for transport. For reference, we carried 40 samplers and their batteries in carry-on suitcases to our field location and they could be transported in regular backpacks for field deployment.

Characterization of a complex tropical bat community

When we analyzed data produced at all facilities, we identified all but 3 bat taxa present in the classroom. *G. commissarisi* is not in the reference collection thus detection was impossible. The other two missing species are considered rare and only a small number of individuals were caught over the entire sampling period (*Micronycteris microtis* *n* = 1, *Micronycteris schmidtorum* *n* = 3). When we consider the main collection separately from the subset sent for independent verification, *Gardnerycteris keenani* (*n* = 5), *Carollia sowellii*(*n* = 53), and *Platyrrhinus helleri* (*n* = 13) were detected only in the material processed at our secondary lab site at the Northern Arizona University.

Both *G. keenani* and *P. helleri* are not common so their detections only once in the subsample may not be surprising. At the time of analysis, *P. helleri* and *C. sowellii* did not have an available reference sequence for the 16s amplicon, and so were only detected in the alternative COI and 12s markers used in our confirmatory test samples at the Northern Arizona University.

While our results suggest that there is a moderate correlation between read count and total species abundance, it is not clear what makes a species “detectable” using air samples. In a previous study, Clare et al., (2022) failed to detect some target species in their enclosures such as the Maned wolves (*Chrysocyon brachyurus*) and Ring-tailed lemurs (*Lemur catta*). Similarly, Lynggaard et al., (2022) failed to detect four zoo mammals during their open-air sampling. Neither saw a relationship with body size to explain false negatives. In our correlation, four species stand out as outliers in abundance vs. read count but this may reflect handling practices. Three pairs of species (*Artibeus lituratus* & *A. intermedius*, *Carollia perspicillata* & *C. sowelli* and *Glossophaga mutica* & *G. commissarisi*) are difficult to differentiate without a hand lens, calipers, and considerable experience with local characters such as the angle of teeth or length of tibia required for correct identification. Consequently, every single individual is examined carefully. In contrast, *Desmodus rotundus* is not hard to identify, and often simply touching the bag holding the bat will elicit a characteristic vocalization and no further examination is required. We thus speculate that the disproportionally high read counts from the first three taxa (*Artibeus lituratus* & *A. intermedius*, *Carollia perspicillata* & *C. sowelli* and *Glossophaga mutica* & *G. commissarisi*) and low counts from the latter (*D. rotundus*) could simply be a result of handling routines.

While we failed to detect some species, which are uncommon to the area, we did detect others including *Mormoops megalophylla*, *Natalus mexicanus*, and *Saccopteryx bilineata*. The first two detections picked up a single individual caught over the entire 2 weeks; and the latter detection found a taxon that is rarely brought back to the classroom because they are considered fragile and quickly recorded and released when caught. As such, our results suggest that rare species can be detected even when there is a high abundance of common species present. It remains unclear why some species are not detected when present; however, it is thought that certain species shed less DNA than others based on their physiology, behavior and metabolism making them harder to detect (Klymus et al., 2015; Sassoubre et al., 2016; Thalinger et al., 2021). Primer mismatches can also make it less likely for amplification to occur and thus a multimarker approach is recommended. A careful review of references showed no forward primer mismatches in the taxa we failed to detect and almost no mismatches across the entire bat reference collection, making mismatch amplification errors unlikely in this case. We, therefore, suspect additional sampling effort is required to capture these undetected species.

The detection of rare species is one of the most highly-cited advantages of using eDNA approaches to monitor ecosystems. Johnson et al. (2023) detected the Texas toad (*Anaxyrus speciosus*), which opportunistically breeds in temporary water pools, in a single airborne eDNA sampling period after a large rainstorm. Similarly, Clare et al. (2022) detected the European hedgehog (*Erinaceus europaeus*), a listed species in the UK, during the winter when they are less active and occur in lower abundances. In this context, our results are promising in that they suggest that eDNA air sampling can be effective at

detecting rare taxa even in highly diverse communities dominated by large numbers of some species.

Non-target detections

In addition to bats, we detected a variety of non-target species including five local and well-known mammals such as the Yucatan black howler monkey (*Alouatta pigra*), Kinkajous and the Big-eared climbing rat (*Ototylomys phyllotis*). The other two species, the Hispid cotton rat (*Sigmodon hispidus*) and the Eastern cottontail (*Sylvilagus floridanus*), were detected but were treated with caution as DNA from both species had been present in our lab recently. Samples taken on the first day of our trip (i.e., prior to the processing of any bats) were nearly empty and only contained very low read counts e.g. howler monkey. It is likely that opening the windows and turning on the ceiling fan immediately mixed the air in the room and pulled eDNA in through the windows, thus resulting in the detection of non-target species (e.g., the howler monkeys, which routinely visit trees near the classroom). In addition to possibly sucking in eDNA from outside the room, open windows, doors, and the use of fans in the classroom may have created a more homogeneous mix of eDNA from the bats in the room over subsequent days and DNA on the fans could have been tossed into the air.

Relative air movement is an important consideration in designing future sampling strategies where such air mixing is not present. Sampling more locations within a space that is not being “mixed” may be required to achieve high species recovery when fans are not in use. Or, in closed areas without wind, fans could be introduced to try and assist with detections. During their studies, Clare et al. (2022) and Lynggaard et al. (2022)

similarly detected non-target species in enclosures, with this eDNA thought to have traveled hundreds of meters from the most likely source. Cow (*Bos taurus*), sheep (*Ovis aries*), pig (*Sus scrofa*), chicken (*Gallus gallus*), *Canis* (dog) and *Felis* (cat) detected in our samples are common lab contaminants (Klepke et al., 2022), but these taxa are also common in our study area (e.g., chickens, dogs, and cats roam free on the site) making it likely that the domestic species detected are true positives.

Forensic detection of secondary transfer material

We were particularly interested in our detection of several bats not found in the Neotropics. Each was identified from only a handful of sequences, and we put special effort into tracking every possible source without simply dismissing low read count IDs during standard bioinformatic filtering. We strongly suspect these detections were a result of eDNA brought in on individual gear and in several cases were able to trace the source. While we wash clothes and use dedicated local equipment, some items cannot be washed (e.g., head lamps, computers, cameras) making secondary transfer possible. In the original proof-of-concept paper by Clare et al., (2021), non-target dog DNA was detected and subsequently hypothesized to have been brought in on the clothes of one of the individuals caring for the animals. These non-target detections suggest that airborne eDNA may be a very sensitive detection method, and that extreme care needs to be taken evaluating data. It also raises the same issue for all eDNA work. As we learn more about the pervasiveness of eDNA and our detection methods become more sensitive it becomes impossible to eliminate all sources of potential secondary transfer. In a hypothetical situation, a pump which helps syphon water through a filter will carry

eDNA, and can toss this into the air, which settles in the water and is then detected in the same way. Some equipment is simply impossible to sterilize. The difference here is that because of the controlled situation of our design, we could detect these events and then went to extremes to trace the source of even the rarest ASVs. It is customary to dismiss low read count detections as noise during filtering, but exploring the potential sources of such DNA may yield information about methods and how to control contamination in eDNA work.

Considerations in bioinformatic handling of eDNA data

Commonly used approaches to filter metabarcoding data based on read counts include: singleton removal, negative control thresholds, and rarefaction (Alberdi et al., 2018; McMurdie & Holmes, 2014). Singleton removal may dismiss sequences that appear only once or detections found only in a minority of PCR replicates. This is a conservative method, and it may be appropriate when underlying data sources are entirely unknown. However, these methods would remove some rare species present in the classroom but only detected by a low read coverage. Detection of rare species may require some flexibility; thus, we used a classification of detection reliability rather than strict filtering. We had the advantage of knowing a priori what species were present, making this a low-risk decision for this study, but our results suggest that it may be worth reporting data with quality rankings or under different filter methods to give a better representation of the data. This approach also led to our discovery of potentially secondarily transferred material. Similarly, there are a variety of approaches to negative control thresholds such as removing all ASVs found in the negative controls (Klepke et al. 2022), or

removing/highlighting read counts lower than those in the negative controls (Clare et al., 2022; Evans et al., 2017). Had we used this method, the only detections reported would have been the 12 high-quality detections, with the 21 low-quality detections and nine very low-quality detections discarded (Figures 1.3 and 1.4) though they are known true positives. Normalizing data is also an approach sometimes used in metabarcoding, but this is controversial. Rarefaction can result in the unnecessary loss of data (McMurdie & Holmes, 2014) and are problematic when you expect samples to be empty. For example, subsampling to a common read count with the extremely low concentration eDNA in air would result in an almost total loss of data. It also makes little sense procedurally given our objectives (i.e., looking at total diversity over the 2-week period, not nightly diversity).

The primary problem is that human contamination is impossible to avoid when sampling airborne eDNA. The large amount of human DNA, relative to our target species, makes threshold filtering hard to employ (Clare et al., 2022). Human DNA overwhelms our amplifications and reduces the relative recovery of bat DNA despite human blocking probes (Calvignac-Spencer, Leendertz, et al., 2013; Calvignac-Spencer, Merkel, et al., 2013). Blocking probes also led to a problem with our positive controls being misleading in their apparent amplification (we amplified very little salmon DNA; the sequences we recovered were mostly human). Taxon-specific primers might correct this making more stringent quality filtering possible, but with the risk of losing non-target detections (e.g., Howler monkeys and Kinkajous). Other studies have also highlighted the need for taxon-specific assays to avoid amplifying contaminants and primer bias towards non-target species (Klepke et al. 2022; Roger et al. 2022).

Future applications

Our results showcase airborne eDNA's potential in detecting elusive species such as bats. As they are nocturnal and can roost in hard-to-reach places, bats can be difficult to monitor. Some species also do not produce species specific echolocation calls, making acoustics identification challenging particularly in the diverse tropical regions (Meyer, 2015). These detection and monitoring challenges can be especially prominent during roost surveys. Such surveys are vital to bat conservation (Kelm et al., 2021; Villalobos-Chaves et al., 2016; Voss et al., 2016), but can be difficult and resource intensive to conduct using existing methods. Such methods may not be possible for some roosts (i.e., physically inaccessible, or too dangerous for humans) and the risk of disrupting roosting bats is a particular fear during hibernation. As airborne eDNA does not require physical access to the bats themselves, it provides a potentially transformative roost survey tool (Clare et al., 2021). Given that the classroom is an enclosed space much like a roost, and that multiple bat species are known to roost together, our results provide strong evidence that using airborne eDNA to detect bats could accurately characterize the species composition of a roost or cave. Using airborne eDNA could improve accessibility and survey efficiency while minimizing disturbance, making roost surveys a logical future application of this detection method.

Conclusions

Our data suggest that sampling airborne eDNA can be done using simple and inexpensive equipment. However, more research is needed to determine the most appropriate sampling strategies with a focus on the optimization of biological replicates,

run times, and environmental conditions to maximize the amount of eDNA collected as well as the efficacy of taxon-specific primers and/or more efficient methods to block the amplification of human DNA. We demonstrated that airborne eDNA can efficiently inventory species in diverse mixed-mammal communities and even rare species can be detected. Airborne eDNA studies could be especially useful for bat roost surveys, early detection of invasive species, and monitoring rare and endangered species whose abundances are low.

Acknowledgements

We wish to thank the staff at Lamanai Field Research Center for all their assistance with sampling logistics and research permits. We also thank colleagues who helped with field work and bat captures during the 2022 field season, and NatureMetrics of London, UK for sequencing data. Particular thanks to Will Clare, Matt Clare, Kaya Courie, Annie Floyd and Owen Floyd who built and tested dozens of prototype filters and Jerry J Grech for the design of our 3D printing. This work was funded by the Natural Sciences and Engineering Research Council of Canada through the Discovery Grants Program to ELC and an NSERC of Canada CGS-M, Academic Excellence Fund Award, York Graduate Scholarship and the Vernon Oliver Stong Graduate Scholarship in Science to NRG.

Chapter 3:

Published in PeerJ as follows:

Garrett NR, Watkins J, Francis CM, Simmons NB, Ivanova N, Naaum A, Briscoe A, Drinkwater R, Clare EL. 2023. Out of thin air: surveying tropical bat roosts through air sampling of eDNA. *PeerJ* 11:e14772 https://doi.org/10.7717/peerj.14772

Chapter Summary

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.

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 (Kelm et al., 2021; Villalobos-Chaves et al., 2016). 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 et al., 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 (Aguirre et al., 2003; Humphrey, 1975; 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 (Kelm et al., 2021; Voss et al., 2016). Annual roost surveys using visual counts, mist nets, acoustic monitoring, and PIT tagging are conducted in many regions to estimate population health (Bat Conservation Trust, 2021; Kaarakka, 2020). 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 et al., 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 et al., 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 et al., 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., 2022 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 accumulates in sufficient quantities in natural roosts to document the roosting ecology of cavity-roosting neotropical bat species.

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 (Figure 2.1A–C); 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 uncertain size in secondary forest (Indian Creek Cave) and one relatively small natural cave in a small, cleared hill in a pasture (Schoolhouse Cave; Figure 2.1E), both in the vicinity of Indian Creek (Table 2.1). At the Schoolhouse Cave, we placed our samplers 3

to 5 m inside and spanning the width of the cave (3 to 4 m) (Figure 2.1E). 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 (Figure 2.1A) was approximately 4 to 5 m in height and 2 to 3 m in width. The White Room roost (Figure 2.1B) 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 (Figure 2.1C) and 1.5 to 2 m tall. Three of the four hollow trees that we sampled were large Guanacaste trees around LAM (Figures 2.1D and F), 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.

Figure 3.1. 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)).

Table 3.1 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	12V Large	Run Time (hrs)
Natural Caves	Vampire Cave (virgin cave)	2	8
	School Room Cave	6 (2)	~24
Artificial Tunnels	High Tunnel	1	~24
	Natalus Tunnel	1 (2)	~24
	Plaza Tunnel	1 (2)	~24
	Tin Roof	1	~24
Man-made	Helen's House	1	~24
	Cistern	2	6
Tree Roosts	High Temple Hollow Tree	1 (2)	6
	Museum Tree	1	6
	Sugar Mill High Tree	1 (3)	6
	Sugar Mill Low Tree	1 (2)	6

We filtered roost air by deploying 12V Large samplers as described by Garrett et al. (2023) (Chapter 2) in each roost (Table 2.1 and Figure 2.1). 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 2.1). 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) (Chapter 2). 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 (Calvignac-Spencer, Merkel, et al., 2013; Taylor, 1996) 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 (Figure 2.1E) 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 et al., 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 2.2). 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* (Figure 2.2H; see discussion)).

*Table 3.2. Summary of roost survey detections grouped by roost type. Taxa with a % match lower than 95% are denoted with a *.*

Type	Roost	Bats	Other Vertebrates
Natural Caves	Vampire Cave (virgin cave)	*Carollia perspicillata* *Desmodus rotundus* *Glossophaga mutica* *Natalus mexicanus*	*Bos taurus*
	School Room Cave	*Carollia perspicillata* *Glossophaga mutica* *Natalus mexicanus* *Saccopteryx bilineata* *Trachops cirrhosus*	*Alouatta spp.** *Bos taurus* *Canis spp.* *Equus caballus* *Ototylomys phyllotis* *Leptodactylus fragilis* *Ovis aries* *Scinax staufferi* *Sus scrofa* *Sylvilagus floridanus* *Trachycephalus typhonius*
Artificial tunnels	High Tunnel (Zinc Room)		*Canis spp.*
	Natalus Tunnel (Red Room)	*Carollia perspicillata* *Saccopteryx bilineata*	*Ototylomys phyllotis* *Sus scrofa* *Trachycephalus typhonius*
	Plaza Tunnel (Palace Structure)	*Carollia perspicillata* *Glossophaga mutica* *Natalus mexicanus* *Sturnira parvadens* *Trachops cirrhosus*	*Bos taurus* *Canis spp.* *Dendropsophus microcephalus* *Ototylomys phyllotis* *Ovis aries* *Leptodactylus fragilis* *Sus scrofa*
	Tin Roof (Zinc Room)	*Chrotopterus auritus**	*Bos taurus* *Ototylomys phyllotis*
Man-made	Helen's House	*Glossophaga mutica*	*Bos taurus* *Canis spp.*
	Cistern		*Bos taurus* *Canis spp.*
Tree Roosts	High Temple Hollow Tree		
	Museum Tree	*Molossus spp.*	*Alouatta spp.**
	Sugar Mill High Tree	*Desmodus rotundus* *Diaemus youngi* *Saccopteryx bilineata*	*Bos taurus* *Ototylomys phyllotis* *Ovis aries*
	Sugar Mill Low Tree	*Pteronotus fulvus* *Sturnira parvidens*	

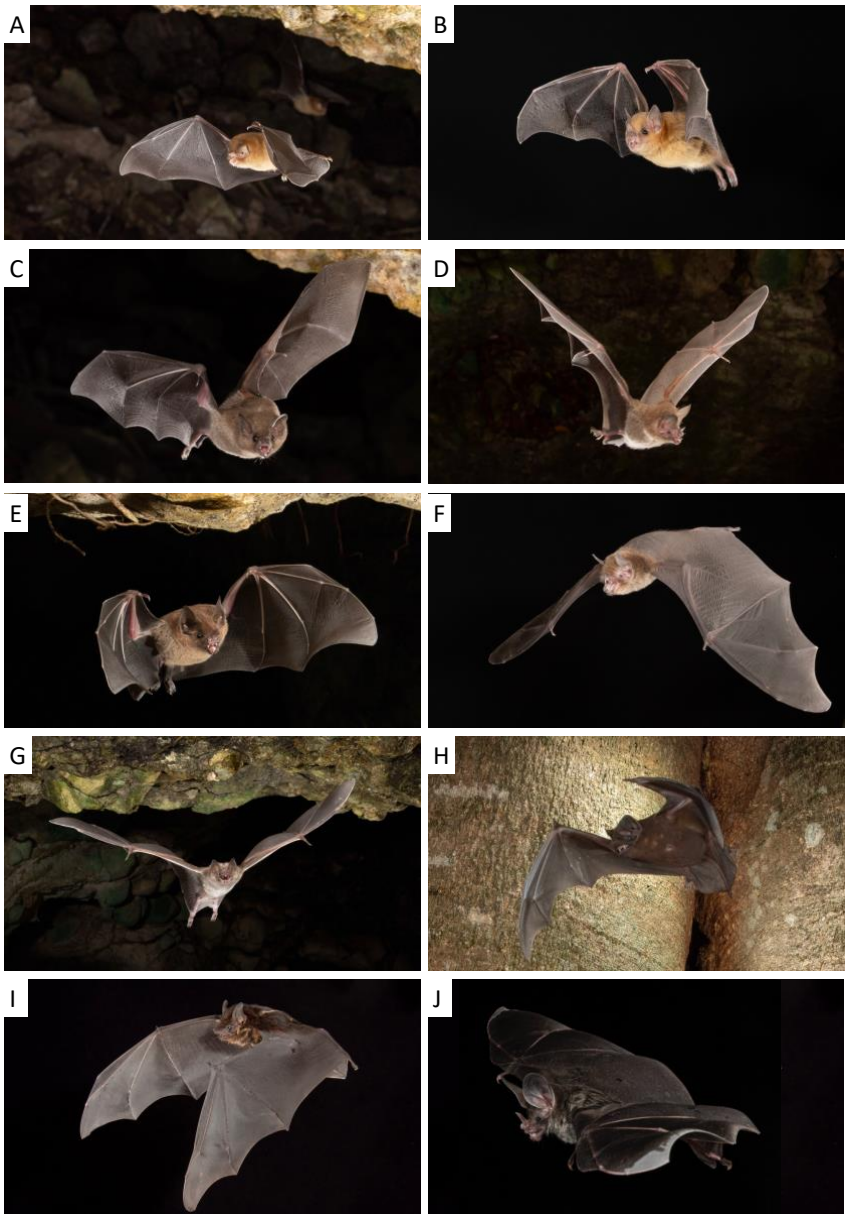


Figure 3.2. With the exception of Diaemus youngi, all bats detected using airborne eDNA have also been documented in the study area from camera traps at roost exits, from mist net captures and/or from being captured in roosts. Natalus mexicanus (A), Glossophaga mutica (C), Carollia sp. (E), Desmodus 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).

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 2.2). 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 2.2). 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 2.2). 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 (Figure 2.3).

Figure 3.3. The big eared climbing rat Ototylomys phyllotis has been seen and photographed sharing roosting areas with bats in the Ka'kabish site before (A, B) and in the area around the tree roosts of the preserve forests (C) and was both seen and recorded on acoustic equipment during the filed season. The DNA of Ototylomys was detected in both natural and man-made bat roosts and a hollow tree. This suggests a widespread roost sharing behaviour.

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 (Figure 4.2A). In contrast, for total diversity (bats and other vertebrate taxa) species richness (q = 0) does not reach an asymptote (Figure 2.4B), indicating increased sampling may add more species. However, for the other orders of diversity (q = 1, 2) curves do reach an asymptote (Figure 2.4B). 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 et al., 2022). The mismatch between q = 0 for total diversity, and the bat-only profile could indicate that the current sampling was insufficient for rarer non-bat vertebrates (Figure 2.4). 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 (Figure 2.5). Both the normality and homogeneity assumptions were met. See appendix 1 and 2 (Figure S1 and S2) for detections by sampler.

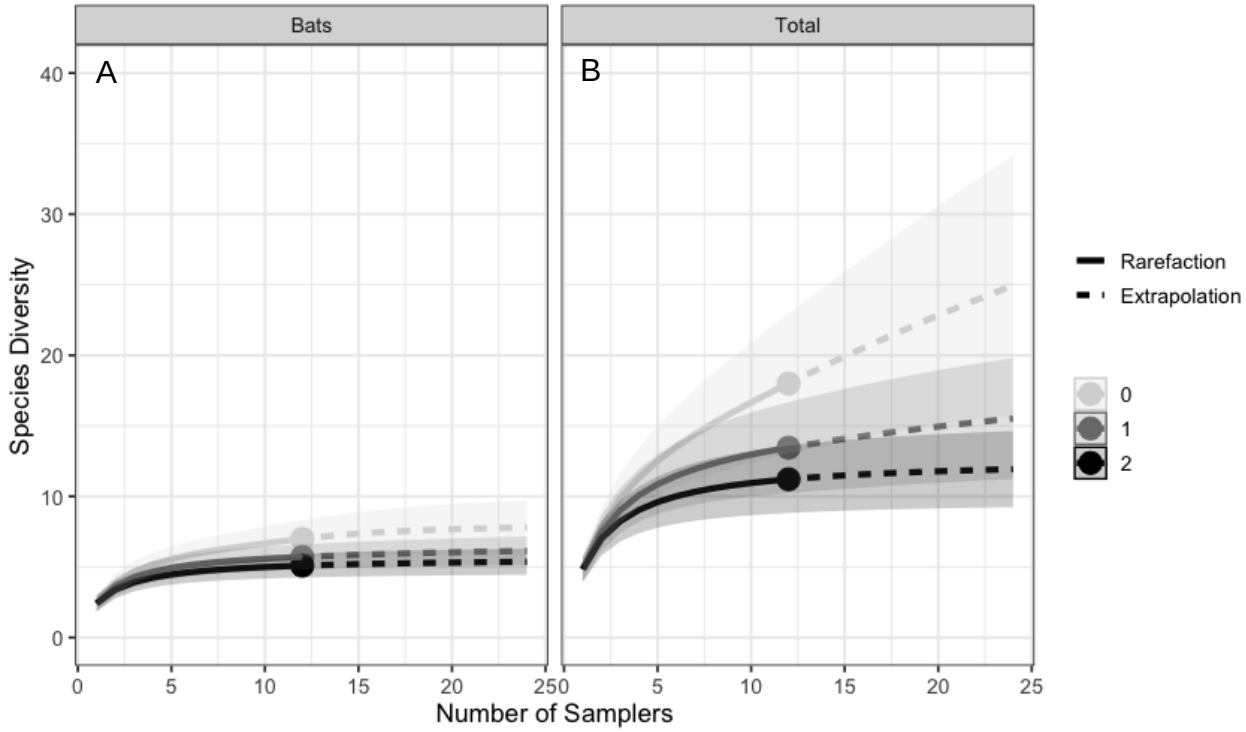


Figure 3.4. Accumulation curves for bat diversity and total diversity for three orders equivalent to species richness (q = 0), the Shannon index (q = 1) and the Simpson index (q = 2). Including 95% confidence intervals and extrapolated to double the observed value (solid circle).

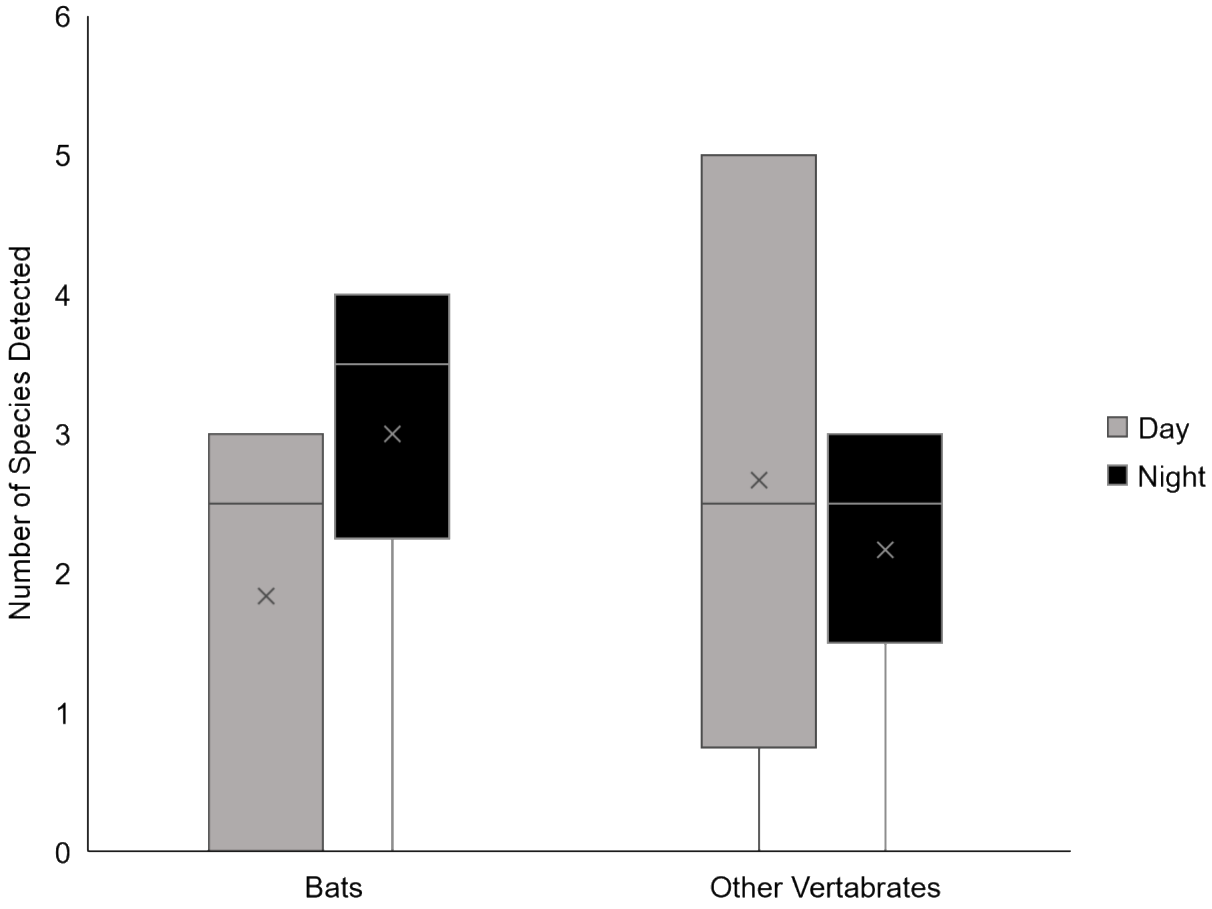


Figure 3.5. The mean (x) number of species detected during the day (grey, n = 6) and night (black, n = 6) did not differ significantly for bat species (t5 = −1.19, p = 0.14) and other vertebrates (t5 = 0.44, p = 0.34) at the Schoolhouse Cave. Graph shows the range (line), 5th, 50th (median) and 95th percentiles in the boxes.

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 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 et al., 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 et al., 2020). Based on camera trap images (*e.g*., Figure 2.2H) 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 (Figures 2.1D 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 (Figure 2.1A) 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 (Nester et al., 2022; Weltz et al., 2017), amphibians (Plante et al., 2021), birds (Neice & McRae, 2021) and marine mammals (Juhel et al., 2021; Ma et al., 2016). 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 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.

Acknowledgments

We wish to thank the staff at Lamanai Field Research Center for all their assistance with sampling logistics and research permits. We also thank colleagues who helped with field work and bat captures during the 2022 field season. Thanks to Will Clare, Matt Clare, Kaya Courie, Annie Floyd and Owen Floyd who built and tested the three prototype filters, and Jerry J. Grech for the design of our 3D printing. Thanks to Brock and Sherri Fenton for photographs and field logistics, Helen Haines for access to her attic, and Alejandro Maeda-Obregon for help with the DADA2 pipeline. Helpful comments were provided by two reviewers which greatly enhanced the content of the manuscript.

Chapter 4: Conclusions

The use of eDNA an ecological analysis and monitoring tool is a rapidly expanding field. Because eDNA exists as fragments of DNA in the environment it can be used to detect species without needing to access the target organism physically or visually. eDNA often presented as a relatively non-invasive technique (Garrett et al., 2023) which uses less time and resources than traditional methods (Johnson et al., 2021). With the recent discovery of terrestrial vertebrate DNA in the air (Clare et al., 2021, 2022; Lynggaard et al., 2022), airborne eDNA offers another potential tool in the biodiversity monitoring toolbox. As the use of airborne eDNA in a terrestrial vertebrate context is still in its infancy, my thesis sought to determine if it could be used in a diverse mixed-mammal community setting and if it was successful at detecting target taxa in the field.

Major Findings from Chapter 2

In Chapter 1 my objectives were to use eDNA to accurately characterize species inventories in a diverse mixed-mammal community, determine if common species overwhelm eDNA inventories, obscuring the presence of rare species in the assemblage, and determine how filter design impacts DNA collection and taxonomic recovery. Based on the results of this experiment, I can conclude that the prototypes were successful in capturing airborne eDNA. While flow rate does not appear to have in impact on the concentration of eDNA collected, other factors such as surface area might. The data shows that airborne eDNA can successfully characterize species inventories of high diversity, mixed mammal communities. Within these communities, it can detect rare species at low abundances at the same times as common species at high abundances in

high density scenarios. As such, it could be a useful tool for early detection of invasive species and monitoring critically endangered species whose abundance is very low (Loeza-Quintana et al., 2020; Vörös et al., 2017). It appears to be very sensitive and can detect non-target species as well as traces of eDNA brought in via secondary transfer (i.e., on gear), indicating a need for careful consideration when designing and implementing studies as it is vulnerable to contamination as well as at the bioinformatic stage. Current bioinformatic criteria results in loss of potentially valid signals.

Major Findings from Chapter 3

In Chapter 2 my objective was survey known and suspected bat roosts using airborne eDNA under natural field conditions. Airborne eDNA can successfully detect vertebrates under natural field conditions and survey several roost types. It can detect vertebrates both inside the roosts and it the surrounding areas. Airborne eDNA could provide a non-invasive survey method not only for bat roosts, but other cavity dwelling species. I found evidence of eDNA “seeping” into the roosts. The cow detected in the attic and the pigs in the schoolhouse cave are not entirely false positives as these animals are common in the surrounding area, but rather and an indication that eDNA from the surrounding area may be detected in the roosts. This provides evidence that it is not an isolated system. As such, critical thinking is needed at the bioinformatic stage to ensure that accurate depiction of roost community composition is presented.

Final Conclusions and Future Considerations

While it is clear from the results of both projects that airborne eDNA can detect and characterize complex communities and detect rare and elusive species like its aquatic

counterpart, there are still several questions that need to be addressed. It is unclear how many samplers should be taken at a time to arcuately capture the total diversity of a given airspace. Increased biological replication will likely produce better results. Running samples for longer will also increase the amount of eDNA collected (Klepke et al. 2022), thus likely impact the complexity of the detections. Given the early evidence with a small sample size that more bat species are detected when they are most active, when you choose to sample is likely to impact your results especially if you are targeting a specific taxa or group of organisms. Using taxa or species specific primers will reduce human contamination and also help in targeted surveys. More research into how long to sample and when to sample will refine this method and better the chances of capturing total diversity. It is still unclear how environmental conditions impact the active sampling of airborne eDNA or how localized the signal is. For example, it is unclear exactly how long signals persist in the environment and how far they can drift. Answering such questions will help develop a more refined sampling protocol that could potentially be applied to more complex ecological questions such as modeling roost occupancy or predator-prey interactions in dens. Ultimately once these gaps are addressed, airborne eDNA could offer semi-automated sampling for the terrestrial ecosystem much like what we see in aquatic systems and add another approach to existing biodiversity monitoring methods.

References

Abrego, N., Norros, V., Halme, P., Somervuo, P., Ali-Kovero, H., & Ovaskainen, O. (2018). Give me a sample of air and I will tell which species are found from your region: Molecular identification of fungi from airborne spore samples. *Molecular Ecology Resources*, *18*(3), 511–524. https://doi.org/10.1111/1755-0998.12755 PMID: 29330936

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

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2018). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, *9*(1), 134–147. https://doi.org/10.1111/2041-210X.12849

Altringham, J. D. (2011). Roosting and Feeding Ecology. In *Bats: From Evolution to Conservation* (pp. 138–150). Oxford University Press.

Andersen, K., Bird, K. L., Rasmussen, M., Haile, J., Breuning-Madsen, H., Kurt H. Kjær, Ludovic Orlando, M. Thomas P. Gilbert, & Eske Willerslev. (2012). Meta-barcoding of 'dirt' DNA from soil reflects vertebrate biodiversity. *Molecular Ecology*, *21*(8), 1966–1979. https://doi.org/10.1111/j.1365-294X.2011.05261.x

Armstrong, A. J., Walker, F. M., Sanville, C., Martin, S., & Szewczak, J. M. (2022). Bat Use of Hollows in California's Old-Growth Redwood Forests: From DNA to Ecology. *Animals*, *12*(21), 2950. https://doi.org/10.3390/ani12212950

Barnes, M. A., & Turner, C. R. (2015). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*(1), 1–17. https://doi.org/10.1007/s10592-015-0775-4

Bat Conservation Trust. (2021). *National Bat Monitoring Programme Annual Report 2021*. https://www.bats.org.uk/our-work/national-bat-monitoring-programme/reports/nbmp-annual-report

Behrens, P., Kiefte-de Jong, J. C., Bosker, T., Rodrigues, J. F. D., 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. https://doi.org/10.1073/pnas.1711889114

Bohmann, K., Evans, A., Gilbert, M. T. P., Carvalho, G. R., Creer, S., Knapp, M., Yu, D. W., & Bruyn, M. de. (2014). Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*, *29*(6), 358–367. https://doi.org/10.1016/j.tree.2014.04.003

Brigham, R. M., Broders, H. G., Toth, C. A., Reimer, J. P., & Barclay, R. M. R. (2018). Observations on the Roosting and Foraging Behavior of Woolly False Vampire Bats, Chrotopterus auritus, in Belize. *Caribbean Naturalist*, *47*, 1–7.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). dada2: High-resolution sample inference from illumina amplicon data. *Nature Methods, 13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Calvignac-Spencer, S., Leendertz, F. H., Gilbert, M. T. P., & Schubert, G. (2013). An invertebrate stomach's view on vertebrate ecology. *BioEssays*, *35*(11), 1004–1013. https://doi.org/0.1002/bies.201300060

Calvignac-Spencer, S., Merkel, K., Kutzner, N., Kühl, H., Boesch, C., Kappeler, P. M., Metzger, S., Schubert, G., & Leendertz, F. H. (2013). Carrion fly-derived DNA as a tool for comprehensive and cost-effective assessment of mammalian biodiversity. *Molecular Ecology*, *22*(4), 915–924. https://doi.org/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.

Clare, E. L., Economou, C. K., Bennett, F. J., Dyer, C. E., Adams, K., McRobie, B., Drinkwater, R., & Littlefair, J. E. (2022). Measuring biodiversity from DNA in the air. *Current Biology*. https://doi.org/10.1016/j.cub.2021.11.064

Clare, E. L., Economou, C. K., Faulke, C. G., Gilbert, J. D., Bennett, F., Drinkwater, R., & Littlefair, J. E. (2021). eDNAir: Proof of concept that animal DNA can be collected from air sampling. *PeerJ (San Francisco, CA)*, *9*, e11030–e11030. https://doi.org/10.7717/peerj.11030

Clare, E. L., & Simmons, N. B. (2021). *A Field Key to Bats of Lamani, Belize V.5*.

Clare, E., Lim, B., Fenton, M., & Herbert, P. (2011). *Neotropical Bats: Estimating Species Diversity with DNA Barcodes. 6*(7), e22648. https://doi.org/doi:10.1371/journal.pone.0022648

Cristescu, M. E., & Hebert, P. D. N. (2018). Uses and Misuses of Environmental DNA in Biodiversity Science and Conservation. *Science and Conservation*, *49*(1), 209–230. https://doi.org/10.1146/annurev-ecolsys-110617-062306

Deiner, K., Bik, H. M., Mächler, E., Seymour, M., Anaïs, A., Florian Altermatt, Simon Creer, Iliana Bista, David M. Lodge, Natasha de Vere, Michael E. Pfrender, & Louis Bernatchez. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. *Molecular Ecology*, *26*(21), 5872–5895. https://doi.org/10.1111/mec.14350

Deiner, K., Fronhofer, E. A., Mächler, E., Walser, J.-C., & Altermatt, F. (2016). Environmental DNA reveals that rivers are conveyer belts of biodiversity information. *Nature Communications*, *7*(1), 12544–12544. https://doi.org/10.1038/ncomms12544

Díaz-Ferguson, E. E., & Moyer, G. R. (2014). History, applications, methodological issues and perspectives for the use of environmental DNA (eDNA) in marine and freshwater environments. *Revista de Biología Tropical*, *62*(4), 1273–1284. https://doi.org/10.15517/rbt.v62i4.13231

Doyle, E. D., Prates, I., Sampaio, I., Koiffmann, C., Jr., W. A. S., Carnaval, A. C., & Harris, E. E. (2021). Molecular phylogenetic inference of the howler monkey radiation (Primates: Alouatta). *Primates*, *62*(1), 177–188. https://doi.org/10.1007/s10329-020-00854-x

Drinkwater, R., Jucker, T., Potter, J. H. T., Swinfield, T., Coomes, D. A., Salde, E. M., Glibert, M. T. P., Lewis, O. T., Bernard, H., Struebig, M. J., Clare, E., & Rossiter, S. J. (2021). Leech blood-meal invertebrate-derived DNA reveals differences in

Bornean mammal diversity across habitats. *Molecular Ecology*, *30*(13), 3299–3312. https://doi.org/10.1111/mec.15724

Drinkwater, R., Schnell, I. B., Bohmann, K., Bernard, H., Veron, G., Clare, E., Gilbert, M. T. P., & Rossiter, S. J. (2019). Using metabarcoding to compare the suitability of two blood-feeding leech species for sampling mammalian diversity in North Borneo. *Molecular Ecology Resources*, *19*(1), 105–117. https://doi.org/10.1111/1755-0998.12943

Drinkwater, R., Williamson, J., Clare, E. L., Chung, A. Y. C., Rossiter, S. J., & Slade, E. (2021). Dung beetles as samplers of mammals in Malaysian Borneo—A test of high throughput metabarcoding of iDNA. *PeerJ (San Francisco, CA)*, *9*, e11897–e11897. https://doi.org/10.7717/peerj.11897

Esquivel, D. A., Peña, S., Aya-Cuero, C., & Tavares, V. da C. (2020). Bats and Termite Nests: Roosting Ecology of Lophostoma brasiliense (Chiroptera: Phyllostomidae) in Colombia. *Mastozoologia Neotropical*, *27*(1), 72–80. https://doi.org/10.31687/saremMN.20.27.1.0.11

Evans, N. T., Li, Y., Li, Y., Renshaw, M. A., Olds, B. P., Deiner, K., Turner, C. R., Jerde, C. L., Lodge, D. M., Lamberti, G. A., & Pfrender, M. E. (2017). Fish community assessment with eDNA metabarcoding: Effects of sampling design and bioinformatic filtering. *Canadian Journal of Fisheries and Aquatic Sciences*, *74*(9), 1362–1374. https://doi.org/10.1139/cjfas-2016-0306

Fantinato, C., Gill, P., & Fonneløp, A. E. (2022). Detection of human DNA in the air. *Forensic Science International: Genetics Supplement Series*, *8*, 282–284. https://doi.org/10.1016/j.fsigss.2022.10.063

Fenton, M. B., Bouchard, S., Hollis, L., Johnston, D. S., Lausen, C. L., Ratcliffe, J. M., Riskin, D. K., Taylor, J. R., & Zigouris, J. (2001). The bat fauna of Lamanai, Belize: Roosts and trophic roles. *Journal of Tropical Ecology*, *17*(4), 511–424. https://doi.org/10.1017/S0266467401001389

Fenton, M. B., & Ratcliffe, J. M. (2010). Bats. *Current Biology*, *20*(24), R1060–R1062. https://doi.org/10.1016/j.cub.2010.10.037

Fenton, M. B., Vonhof, M. J., Bouchard, S., Gill, S. A., Johnston, D. S., Reid, F. A., Riskin, D. K., Standing, K. L., Taylor, J. R., & Wagner, R. (2000). Roosts Used by Sturnira lilium (Chiroptera/ Phyllostomidae) in Belize. *Association for Tropical Biology and Conservation*, *32*(4a), 729–733. https://doi.org/10.1646/0006-3606(2000)032[0729:RUBSLC]2.0.CO 2

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*(4), 423–425. https://doi.org/10.1098/rsbl.2008.0118

Franklin, T. W., McKelvey, K. S., Golding, J. D., Mason, D. H., Dysthe, J. C., Pilgrim, K. L., Squires, J. R., Aubry, K. B., Long, R. A., Greaves, S. E., Raley, C. M., Jackson, S., MacKay, P., Lisbon, J., Sauder, J. D., Pruss, M. T., Heffington, D., & Schwartz, M. K. (2019). Using environmental DNA methods to improve winter surveys for rare carnivores: DNA from snow and improved noninvasive techniques. *Biological Conservation*, *229*, 50–58. https://doi.org/10.1016/j.biocon.2018.11.006

Garrett, N. R., Watkins, J., Francis, C., Simmons, N. B., Ivanova, N. V., Naaum, A., Briscoe, A., Drinkwater, R., & Clare, E. L. (2023). Out of thin air: Surveying

tropical bat roosts through air sampling of eDNA. *PeerJ*. https://doi.org/10.7717/peerj.14772

Garrett, N. R., Watkins, J., Simmons, N. B., Fenton, M. B., Maeda-Obregon, A., Sanchez, D. E., Froehlich, E. M., Walker, F. M., Littlefair, J. E., & Clare, E. L. (2022). Airborne eDNA documents a diverse and ecologically complex tropical bat and other mammal community. *Environmental DNA*.

Grafe, T. U., Schöner, C. R., Kerth, G., Junaidi, A., & Schöner, M. G. (2011). A novel resource–service mutualism between bats and pitcher plants. *Biology Letters*, *7*(3), 436–439. https://doi.org/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*. https://doi.org/10.1111/1755-0998.13629

Haile, J., Froese, D. G., MacPhee, R. D. E., Roberts, R. G., Arnold, L. J., Reyes, A. V., Rasmussen, M., Nielsen, R., Brook, B. W., Robinson, S., Demuro, M., Gilbert, M. T. P., Munch, K., Austin, J., Cooper, A., Barnes, I., Möller, P., & Willerslev, E. (2009). Ancient DNA reveals late survival of mammoth and horse in interior Alaska. *Proceedings of the National Academy of Sciences*, *106*(52), 22352–22347. https://doi.org/10.1073/pnas.0912510106

Hall, T. A. (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

https://www.academia.edu/2034992/BioEdit_a_user_friendly_biological_sequence_alignment_editor_and_analysis_program_for_Windows_95_98_NT

Hallam, J., Clare, E., Jones, J. I., & Day, J. J. (2021). Biodiversity assessment across a dynamic riverine system: A comparison of eDNA metabarcoding versus traditional fish surveying method. *Environmental DNA*. https://doi.org/10.1002/edn3.241

Hemprich-Bennett, D. R., Kemp, V. A., Blackman, J., Struebig, M. J., Lewis, O. T., Rossiter, S. J., & Clare, E. L. (2021). Altered structure of bat–prey interaction networks in logged tropical forests revealed by metabarcoding. *Molecular Ecology*. https://doi.org/10.1111/mec.16153

Herrera, J. P., Duncan, N., Clare, E., Fenton, M. B., & Simmons, N. (2018). Disassembly of fragmented bat communities in Orange Walk District, Belize. *Acta Chiropterologica*, *20*(1), 147–159. https://doi.org/10.3161/15081109ACC2018.20.1.011

Hofreiter, M., Mead, J. I., Martin, P., & Poinar, H. N. (2003). Molecular Caving. *Current Biology*, *13*(18), R693–R695. https://doi.org/10.1016/j.cub.2003.08.039

Hsieh, T., Ma, K., & Chao, A. (2022). *INEXT: Interpolation and Extrapolation for Species Diversity* (R package version 3.0.0). http://chao.stat.nthu.edu.tw/wordpress/software_download/

Huerlimann, R., Cooper, M. K., Edmunds, R. C., Villacorta-Rath, C., Port, A. L., Robson, H. L. A., Strugnell, J. M., Burrows, D., & Jerry, D. R. (2020). Enhancing tropical conservation and ecology research with aquatic environmental DNA methods: An

introduction for non-environmental DNA specialists. *Animal Conservation*, *23*(3), 632–645. https://doi.org/10.1111/acv.12583

Humphrey, S. R. (1975). Nursery Roosts and Community Diversity of Nearctic Bats. *Journal of Mammalogy*, *56*(2), 321–346. https://doi.org/10.2307/1379364

Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., Letcher, B. H., & Whiteley, A. R. (2015). Distance, flow and PCR inhibition: EDNA dynamics in two headwater streams. *Molecular Ecology Resources*, *15*(1), 216–227. https://doi.org/10.1111/1755-0998.12285

Janicki, A. F., Frick, W. F., Kilpatrick, A. M., Parise, K. L., Foster, J. T., & McCracken, G. F. (2015). Efficacy of Visual Surveys for White-Nose Syndrome at Bat Hibernacula. *PLoS ONE*, *10*(1). https://doi.org/10.1371/journal.pone.0133390

Ji, Y., Baker, C. C. M., Popescu, V. D., Wang, J., Wu, C., Wang, Z., Li, Y., Wang, L., Hua, C., Yang, Z., Yang, C., Xu, C. C. Y., Diana, A., Wen, Q., Pierce, N. E., & Yu, D. W. (2022). Measuring protected-area effectiveness using vertebrate distributions from leech iDNA. *Nature Communications*, *13*(1), 1555–1555. https://doi.org/10.1038/s41467-022-28778-8

Johnson, M., Barnes, M. A., 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*. https://doi.org/10.1002/edn3.388

Johnson, M. D., Cox, R. D., & Barnes, M. A. (2019). Analyzing airborne environmental DNA: A comparison of extraction methods, primer type, and trap type on the

ability to detect airborne eDNA from terrestrial plant communities. *Environmental DNA (Hoboken, N.J.)*, *1*(2), 176–185. https://doi.org/10.1002/edn3.19

Johnson, M. D., Fokar, M., Cox, R. D., & Barnes, M. A. (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. https://doi.org/10.1186/s12862-021-01947-x

Juhel, J.-B., Marques, V., Fernández, A. P., Borrero-Pérez, G. H., Martinezguerra, M. M., Valentini, A., Dejean, T., Manel, S., Loiseau, N., Velez, L., Hocdé, R., Letessier, T. B., 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. https://doi.org/10.1002/ece3.7057

Kaarakka, H. (2020). *Roost Monitoring Report*. Wisconsin Department of Natural Resources. https://wiatri.net/inventory/bats/volunteer/roosts/pdf/RoostReport2020.pdf

Kelm, D. H., Toelch, U., & Jones, M. M. (2021). Mixed-species groups in bats/ non-random roost associations and roost selection in neotropical understory bats. *Frontiers in Zoology*, *18*(53). https://doi.org/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. https://doi.org/10.2108/zs180172

Klepke, M. J., Sigsgaard, E. E., Jensen, M. R., Olsen, K., & Thomsen, P. F. (2022). Accumulation and diversity of airborne, eukaryotic environmental DNA. *Environmental DNA*, 1–17. https://doi.org/10.1002/edn3.340

Klüg-Baerwald, B. J., Lausen, C. l., Willis, C. K. R., & Brigham, R. M. (2017). Home is where you hang your bat: Winter roost selection by prairie-living big brown bats. *Journal of Mammalogy, 98*(3), 752–760. https://doi.org/10.1093/jmammal/gyx039

Klymus, K. E., Richter, C. A., Chapman, D. C., & Paukert, C. (2015). Quantification of eDNA shedding rates from invasive bighead carp Hypophthalmichthys nobilis and silver carp Hypophthalmichthys molitrix. *Biological Conservation*, *183*, 77–84. https://doi.org/10.1016/j.biocon.2014.11.020

Korpelainen, H., & Pietiläinen, M. (2017). Biodiversity of pollen in indoor air samples as revealed by DNA metabarcoding. *Nordic Journal of Botany*, *35*(5), 602–608. https://doi.org/10.1111/njb.01623

Kraaijeveld, K., de Weger, L. A., Ventayol García, M., Buermans, H., Frank, J., Hiemstra, P. S., & den Dunnen, J. T. (2015). Efficient and sensitive identification and quantification of airborne pollen using next-generation DNA sequencing. *Molecular Ecology Resources*, *15*(1), 8–16. https://doi.org/10.1111/1755-0998.12288

Kunz, T. H. (1982). Roosting ecology of bats. In *Ecology of bats* (pp. 1–56). Plenum Press.

Kunz, T. H. (2003). *Censusing Bats: Challenges, Solutions, and Sampling Biases* (Biological Resources Discipline, Information and Technology Report, pp. 9–17)

[Government]. U.S. Geological Survey. https://pubs.usgs.gov/itr/2003/0003/report.pdf

Littlefair, J. E., Allerton, J. J., Brown, A. S., Butterfield, D. M., Robins, C., Economou, C. K., Garrett, N. R., & Clare, E. L. (In Press). A vault of biodiversity data is concealed in plain sight. *Current Biology*.

Littlefair, J. E., Hrenchuk, L. E., Rennie, M. D., & Cristescu, M. E. (2020). Thermal stratification and fish thermal preference explain vertical eDNA distributions in lakes. *Molecular Ecology*, *30*(13), 3083–3096. https://doi.org/10.1111/mec.15623

Loeza-Quintana, T., Crookes, S., Li, P. Y., Reid, D. P., Smith, M., & Hanner, R. H. (2020). Environmental DNA detection of endangered and invasive species in Kejimkujik National Park and Historic Site. *Genome*, *64*(3), 172–180. https://doi.org/10.1139/gen-2020-0042

Longhi, S., Cristofori, A., Gatto, P., Cristofolini, F., Grando, M. S., & Gottardini, E. (2009). Biomolecular identification of allergenic pollen: A new perspective for aerobiological monitoring? *Annals of Allergy, Asthma, & Immunology, 103*(6), 508–514. https://doi.org/10.1016/S1081-1206(10)60268-2

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

Loureiro, L. D., Engstrom, M. D., & Lim, B. K. (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*, 106690. https://doi.org/10.1016/j.ympev.2019.106690

Lyman, J. A., Sanchez, D. E., Hershauer, S. N., Sobek, C. J., Chambers, C. L., Zahratka, J., & Walker, F. M. (2022). Mammalian eDNA on herbaceous vegetation? Validating a qPCR assay for detection of an endangered rodent. *Environmental DNA*. https://doi.org/10.1002/edn3.331

Lynggaard, C., Bertelsen, M. F., Jensen, C. V., Johnson, M. S., Frøslev, T. G., Olsen, M. T., & Bohmann, K. (2022). Airborne environmental DNA for terrestrial vertebrate community monitoring. *Current Biology*. https://doi.org/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. https://doi.org/10.1007/s12686-016-0597-9

Martin, M. (2011). Cutadapt Removes Adapter Sequences from High-Throughput Sequencing Reads. *EMBnet Journal*, *17*(1). https://doi.org/DOI:10.14806/ej.17.1.200

McMurdie, P. J., & Holmes, S. (2014). Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible. *PLoS Computational Biology*, *10*(4), p.e1003531-e1003531. https://doi.org/10.1371/journal.pcbi.1003531

Mena, J. L., Yagui, H., Tejeda, V., Bonifaz, E., Bellemain, E., Valentini, A., Tobler, M. W., 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-n/a. https://doi.org/10.1002/eap.2335

Mercer, C., Taylor, D., Henry, J., & Linacre, A. (2023). DNA accumulation and transfer within an operational forensic exhibit storeroom. *Forensic Science International: Genetics*, *62*, 102799. https://doi.org/10.1016/j.fsigen.2022.102799

Meyer, C. F. J. (2015). Methodological challenges in monitoring bat population- and assemblage-level changes for anthropogenic impact assessment. *Mammalian Biology*, *80*(3), 159–169. https://doi.org/10.1016/j.mambio.2014.11.002

Mohanty, R. P., Buchheim, M. A., & Levetin, E. (2017). Molecular approaches for the analysis of airborne pollen A case study of Juniperus pollen. *Annals of Allergy, Asthma, & Immunology, 118*(2), 204-211.e2. https://doi.org/10.1016/j.anai.2016.11.015

Monuki, K., Barber, P. H., & Gold, Z. (2021). EDNA captures depth partitioning in a kelp forest ecosystem. *PLoS ONE*, *16*(11), p.e0253104-e0253104. https://doi.org/10.1371/journal.pone.0253104

Mormann, B. M., & Robbins, L. W. (2007). Winter roosting ecology of eastern red bats in southwest Missouri. *The Journal of Wildlife Management*, *71*(1), 213–217. https://doi.org/10.2193/2005-622

Neice, A. A., & McRae, S. B. (2021). An eDNA diagnostic test to detect a rare, secretive marsh bird. *Global Ecology and Conservation*, *27*, e01529. https://doi.org/10.1016/j.gecco.2021.e01529

Nester, G. M., Heydenrych, M. J., Berry, T. E., Richards, Z., Wasserman, J., White, N. E., Brauwer, M. D., 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*. https://doi.org/10.1002/edn3.365

Núñez, A., Paz, G. A. de, Ferencova, Z., Rastrojo, A., Guantes, R., García, A. M., Alcamí, A., Gutiérrez-Bustillo, A. M., & Moreno, D. A. (2017). Validation of the Hirst-Type Spore Trap for Simultaneous Monitoring of Prokaryotic and Eukaryotic Biodiversities in Urban Air Samples by Next-Generation Sequencing. *Applied and Environmental Microbiology, 83*(13). https://doi.org/10.1128/AEM.00472-17

Patriquin, K. J., & Ratcliffe, J. M. (2016). Should I Stay or Should I Go? Fission–Fusion Dynamics in Bats. In *Sociality in Bats* (1st ed., pp. 65–103). Springer Cham. https://doi.org/10.1007/978-3-319-38953-0_4

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*. https://doi.org/10.1002/edn3.233

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (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. https://doi.org/10.1111/1365-2664.12306

Reid, F. A. (2009). Chiroptera. In *A Field Guide to the Mammals of Central America and Southeast Mexico* (Second, pp. 73–177). Oxford University Press.

Roger, F., Ghanavi, H. R., Danielsson, N., Wahlberg, N., Löndahl, J., Pettersson, L. B., Andersson, G. K. S., Olén, N. B., & Clough, Y. (2022). Airborne environmental

DNA metabarcoding for the monitoring of terrestrial insects—A proof of concept from the field. *Environmental DNA*, *4*(4), 790–807. https://doi.org/10.1002/edn3.290

RStudio Team. (2021). *RStudio: Integrated Development Environment for R.* URL http://www.rstudio.com/.

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (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. https://doi.org/10.1016/j.gecco.2019.e00547

Ryan, E., Bateman, P., Fernandes, K., Heyde, M. van der, & Nevill, P. (2022). EDNA metabarcoding of log hollow sediments and soils highlights the importance of substrate type, frequency of sampling and animal size, for vertebrate species detection. *Environmental DNA*, *4*(4), p.940-953. https://doi.org/10.1002/edn3.306

Rydell, J., Russo, D., Sewell, P., Seamark, E. C. J., Francis, C. M., Fenton, S. L., & Fenton, M. B. (2022). Bat selfies: Photographic surveys of flying bats. *Mammalian Biology*. https://doi.org/10.1007/s42991-022-00233-7

Sassoubre, L. M., Yamahara, K. M., Gardner, L. D., & Boehm, A. B. (2016). Quantification of Environmental DNA (eDNA) Shedding and Decay Rates for Three Marine Fish. *Environmental Science & Technology*, *50*(19), 10456–10464. https://doi.org/10.1021/acs.est.6b03114

Schmelzle, M. C., & Kinziger, A. P. (2016). Using occupancy modelling to compare environmental DNA to traditional field methods for regional-scale monitoring of

an endangered aquatic species. *Molecular Ecology Resources*, *16*, 895–908. https://doi.org/10.1111/1755-0998.12501

Schnell, I. B., Thomsen, P. F., Wilkinson, N., Rasmussen, M., Jensen, L. R. D., Willerslev, E., Bertelsen, M. F., & Gilbert, M. T. P. (2012). Screening mammal biodiversity using DNA from leeches. *Current Biology*, *22*(8), R262-263. https://doi.org/10.1016/j.cub.2012.02.058

Serrao, N. R., Weckworth, J. K., McKelvey, K. S., Dysthe, J. C., & Schwartz, M. K. (2021). Molecular genetic analysis of air, water, and soil to detect big brown bats in North America. *Biological Conservation*, *261*, 109252. https://doi.org/10.1016/j.biocon.2021.109252

Shadi, S., Spall, J. L., Gibson, J. F., & Hjibabeai, M. (2012). Next-generation Sequencing Technologies for Environmental DNA Research. *Molecular Ecology*, *21*(8), 1794–1805. https://doi.org/10.1111/j.1365-294X.2012.05538.x

Sønstebø, J. H., Gielly, L., Brysting, A. K., Elven, R., Edwards, M., Haile, J., Willerslev, E., Coissac, E., Rioux, D., Sannier, J., Taberlet, P., & Brochmann, C. (2010). Using next-generation sequencing for molecular reconstruction of past Arctic vegetation and climate. *Molecular Ecology Resources*, *10*(6), 1009–1018. https://doi.org/10.1111/j.1755-0998.2010.02855.x

Speakman, J. R., Webb, P. I., & Racey, P. A. (1991). Effects of Disturbance on the Energy Expenditure of Hibernating Bats. *Journal of Applied Ecology*, *28*(3), 1087–1104. https://doi.org/10.2307/2404227

Taberlet, P., Coissac, E., Hjibabeai, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular Ecology*, *21*(8), 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Taylor, P. G. (1996). Reproducibility of Ancient DNA Sequences from Extinct Pleistocene Fauna. *Molecular Biology and Evolution*, *13*(1), 283–285. https://doi.org/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*. https://doi.org/10.3389/fevo.2021.623718

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., & Orlando, L. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*(11), 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, *183*, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Venter, J. C., Remington, K., Heidelberg, J. F., Rusch, D., Eisen, J. A., Wu, D., Paulsen, I., Nelson, K. E., Nelson, W., Fouts, D. E., Levy, S., Knap, A. H., Lomas, M. W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., … Smith, H. O. (2004). Environmental Genome Shotgun Sequencing of the Sargasso Sea. *Science*, *304*(5667), 66–74. https://doi.org/10.1126/science.1093857

Villalobos-Chaves, D., Murillo, J. V., Valerio, E. R., Keeley, B. W., & 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. https://doi.org/10.15517/rbt.v64i3.21093.

Vörös, J., Márton, O., Schmidt, B. R., Gál, J. T., & Jelić, D. (2017). Surveying Europe's Only Cave-Dwelling Chordate Species (Proteus anguinus) Using Environmental DNA. *PLoS ONE*, *12*(1), e0170945–e0170945. https://doi.org/10.1371/journal.pone.0170945

Voss, R. S., Fleck, D. W., Strauss, R. E., Velazco, P. M., & Simmons, N. B. (2016). Roosting ecology of Amazonian bats: Evidence for guild structure in hyperdiverse mammalian communities. *American Museum Novitates*, *3870*, 1–43. https://doi.org/10.1206/3870.1

Walker, F. M., Sanchez, D. E., Froehlich, E. M., Lyman, J. A., Owens, M. D., & Lear, K. (2022). Endangered nectar-feeding bat detected by environmental DNA on flowers. *Animals*, *12*(22), 3075. https://doi.org/10.3390/ani12223075

Weltz, K., Lyle, J. M., Ovenden, J., Morgan, J. A. T., Moreno, D. A., & Semmens, J. M. (2017). Application of environmental DNA to detect an endangered marine skate species in the wild. *PLoS ONE*, *12*(6), e0178124–e0178124. https://doi.org/10.1371/journal.pone.0178124

Willerslev, E., Hansen, A. J., Binladen, J., Brand, T. B., Gilbert, M. T. P., Shapiro, B., Bunce, M., Wiuf, C., & Gilichinsky, D. A. (2003). Diverse Plant and Animal Genetic Records from Holocene and Pleistocene Sediments. *Science (American*

Association for the Advancement of Science), *300*(5620), 791–795. https://doi.org/10.1126/science.1084114

Willerslev, E., Hansen, A. J., Christensen, B., Steffensen, J. P., & Arctande, P. (1999). Diversity of Holocene life forms in fossil glacier ice. *Proceedings of the National Academy of Sciences - PNAS*, *96*(14), 8017–8021. https://doi.org/10.1073/pnas.96.14.8017

Willson, D. E., & Mittermeier, R. A. (2019). Pteronotus fulvus. In *Handbook of the Mammals of the World: Mormoopidae* (Vol. 9, pp. 424-443:438). Lynx Edicions. https://doi.org/10.5281/zenodo.6606810

Appendix A: Small Ring

edn3385-sup-0001-AppendixS1.STL 43.8 KB

Appendix B: Small Housing

edn3385-sup-0002-AppendixS2.STL 158.1 KB

Appendix C: Large Ring

edn3385-sup-0003-AppendixS3.STL 47.5 KB

Appendix D: Large Housing

edn3385-sup-0004-AppendixS4.STL 156.2 KB

Appendix E: Figure S1

A)

Species	2022-04-24	2022-04-25	2022-04-26	2022-04-27	2022-04-28	2022-04-29	2022-04-30
Saccopteryx bilineata		1	5				
Artibeus jamaicensis	1	7	10	3	8	2	2
Artibeus lituratus/intermedius		6	1	8	9		5
Carollia perspicillata		3	22	10			7
Dermanura phaeotis	4	7	10	4	11	2	11
Desmodus rotundus		18	4	6			1
Glossophaga mutica	6	22	7	33	3	3	8
Lophostoma evotis					2		
Phyllostomus discolor					3		
Sturnira parvadens		15	2	13	3		3
Trachops cirrhosis			5				1
Uroderma convexum	1		1		4		2
Vampyressa thyone							
Eumops spp.						1	
Molossus spp.	10			3		3	
Mormoops megalophylla					1		
Pteronotus fulvus		1			6		
Pteronotus mesoamericanus	1	6	12	2	10	2	
Natalus mexicanus						1	
Noctilio (leporinus)					2		
Bauerus dubiaquercus		1			2		
Eptesicus (furinalis)	4	1		1	4		
Lasiurus ega	5		1				
Myotis spp.		1		1	5		
Rhogeessa aenea	1	2	2	2	2	3	

High Quality Low Quality No detection

B)

Species	2022-05-01	2022-05-02	2022-05-03	2022-05-04	2022-05-05	2022-05-06	2022-05-07
Saccopteryx bilineata							
Artibeus jamaicensis	1	9	9	28			
Artibeus lituratus/intermedius		8	8	3			
Carollia perspicillata		5	14	1			
Dermanura phaeotis	1	5	6	6			
Desmodus rotundus		14	6	18			
Glossophaga mutica	4	8	6	22			
Lophostoma evotis							
Phyllostomus discolor			3				
Sturnira parvadens	1	3	5	16			
Trachops cirrhosis							
Uroderma convexum		1	7	6			
Vampyressa thyone				2			
Eumops spp.							
Molossus spp.	11						
Mormoops megalophylla							
Pteronotus fulvus		19	4	1			
Pteronotus mesoamericanus	8	26	21	1			
Natalus mexicanus							
Noctilio (leporinus)	1						
Bauerus dubiaquercus							
Eptesicus (furinalis)	1		3	2			
Lasiurus ega	1						
Myotis spp.		3					
Rhogeessa aenea	1	2					

High Quality Low Quality No detection

Figure S1. Captured bat abundance (numbers) each night compared to eDNA detections where high quality detections are highlighted in green and low quality detections in yellow. Detections not made by eDNA are in white. A) depicts data collected from April 24th , 2022 to April 30th , 2022. B) depicts data collected from May 1st, 2022 to May 7th , 2022. Note: eDNA was not collected on April 27th , 2022.

Appendix F: Independent Sample Verification

For independent verification of bat eDNA presence, 5 samples from the experiment were processed independently using different laboratory at Northern Arizona University and alternative markers and bioinformatic approaches. eDNA was extracted from a ~1 cm x 1 cm subsample of filter paper using the DNeasy Blood & Tissue kit. The samples were submerged in kit buffers with previously described modifications to volumes (Clare et al. 2022). Samples were lysed overnight at 56°C. Purification followed the manufacturer's protocol, except that they were eluted to 105 µL. To verify the contents the lab amplified two markers targeting a short section of COI (202 bp insert), using a bat-specific primer set (Walker, Williamson, Sanchez, Sobek, & Chambers, 2016); and a short section of 12S rRNA (~171 bp insert), using a mammal-specific primer set (Ushio et al. 2017). Both primer sets (Table1) were modified with 5' universal tails for the 2-step PCR library preparation method of Coleman et al. (2015). Decontaminated all laboratory surfaces was done with DNA AWAY (Thermo Scientific, Waltham, MA, USA) and then 70% EtOH. For the first PCR step, reagent master-mix was prepared and plated in a DNA-free cleanroom. DNA template from air filters was then added in a UV-treated PCR hood. The samples were amplified with non-template controls (PCR grade water) and a DNA mock community (genomic DNA of nine bat species: *Leptonycteris nivalis, Eptesicus fuscus, Eumops perotis, Lasionycteris noctivagans, Lasiurus cinereus, Myotis occultus, Nyctinomops macrotis, Tadarida brasiliensis, and Euderma maculatum*). COI and 12S markers were amplified separately in 15 µL reaction volumes with 3 µL DNA template, 8.46 µL PCR grade water, 1.5 µL 10X Mg-free PCR buffer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 1.5 mM MgCl2, 0.2 mM each dNTP, 0.2 µM each primer, 0.16 µg/ µL bovine serum albumin (Ambion Ultrapure BSA), and 0.03 U/µL PlatinumTaq DNA polymerase (Invitrogen, Thermo Fisher Scientific). Thermocycling involved an initial denaturation of 94°C for 5 min, followed by 5 cycles of 94°C for 1 min, 45°C for 1.5 min, and 72°C for 1 min. This was followed by 35 cycles of 94°C for 1 min, 60°C for 1.5 min (annealing temperature same for both markers), and 72°C for 1min, concluding with final extension step of 72°C for 10 min. The second PCR step produced indexed, flowcell-ready amplicons from the PCR product of the first step (Colman et al. 2015). Specifically, primers containing 8 bp indices (i.e., tags, barcodes) and Illumina adapters were added to both ends of the amplicon (e.g., dual indexing) by priming and extending from the universal tails. We used a unique index once per air filter sample. This PCR was prepared in 25 µL reaction volumes with purified 2 µL amplicon from the previous PCR step, 12.5 µL 2X Kapa HiFi HotStart ReadyMix (Roche Sequencing, Wilmington, MA, USA), 8.5 µL PCR grade water, and 1 µL each index primer (final concentration: 0.4 µM). Thermocycling included an initial denaturation of 98°C for 2 min, 8 cycles of 98°C for 30 sec, 60°C for 20 sec, and 72°C for 5 min, concluding with a final extension of 72°C for 5 min. Purified libraries were then pooled in equimolar concentrations and sequenced on a MiSeq V2 Micro 300 cycle kit (Illumina, San Diego, CA, USA). This sequencing run contained libraries from other projects that were amplified using the same COI marker. However, air filter samples amplified with 12S were unique to the run.

COI and 12S markers were separated and primers removed from demultiplexed samples using cutadapt 4.0 in paired-end mode (Martin, 2011), discarding untrimmed reads. We

further pre-processed reads using QIIME2 v2022.2 (Bolyen et al., 2018) and custom Tidyverse (Wickham et al., 2019) scripts in R v4.2.1 (R Core Team, 2022). Amplicon sequence variants (ASVs) were generated from the paired-end reads in DADA2 (Callahan et al., 2016). Prior to running DADA2, COI reads were truncated to 125 bases for both R1 and R2 reads. 12S reads were truncated to 125 bases for R1 reads and 105 bases for R2 reads. Additionally, for 12S, poor filtering with default settings of DADA2 led to a subsequent increase in the number of expected errors for both reads to 4.0 (--p-max-ee-f, --p-max-ee-r). For COI, only ASVs with a length of 202 bp were retained. 12S ASVs were not filtered by length due to natural fragment length variation. Integer-minimum thresholds of read abundance (per sample) were empirically determined according to expected features in the mock community (O'Rourke, Bokulich, MacManes, & Foster, 2020). There is no evidence of low abundance errors but as a precautionary measure, ASVs with fewer than 10 reads in a sample were omitted due to signs of tag-jumps after marker separation. Potential tag jumps occurred in up to two reads in seven unrelated libraries of the same run. However, none of these reads ultimately passed the merging stage in DADA2.

Different classification methods were used for COI and 12S ASVs. For COI, a Naïve-Bayes classification was used (i.e., kmer-based classification) against a global reference library of bat species (Walker et al., 2016), which was assembled from the Barcode of Life Database (Ratnasingham & Hebert, 2007). The classifier was trained with a kmer length of 7 (--p-feat-ext-ngram-range = [7,7]) and classified with a confidence threshold of 0.9 in QIIME2 v2022.2. Any COI ASVs not classified to species with the Naïve-Bayes classifier and all 12S ASVs were locally aligned against the National Center for Biotechnology Information's (NCBI) GenBank database (Benson, Karsch-Mizrachi, Lipman, Ostell, & Sayer, 2009), using BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990). Taxonomies were classified from the BLAST results using least common ancestor (LCA) analysis in MEGAN v6 (Huson, Auch, A. F., Qi, J., & Schuster, 2007). The LCA classified with a minimum percent identity of 97%, the top 5% of BLAST results, a minimum support percent of 0.02%, and only accepted hits with a minimum query cover of 90%. All taxonomic assignments were then manually curated to update to current nomenclature.

Supplement References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology*, 8.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2009). GenBank. *Nucleic Acids Research*, *37*(suppl_1), D26–D31. https://doi.org/10.1093/nar/gkn723

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., ... Caporaso, J. G. (2018). *QIIME 2: Reproducible, interactive, scalable, and*

extensible microbiome data science (e27295v2). PeerJ Inc. https://doi.org/10.7287/peerj.preprints.27295v2

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Clare, E. L., Economou, C. K., Bennett, F. J., Dyer, C. E., Adams, K., McRobie, B., Drinkwater, R., Littlefair, J. E. (2022). Measuring biodiversity from DNA in the air. *Current Biology*. https://doi.org/10.1016/j.cub.2021.11.064

Colman, R.E., Schupp, J.M., Hicks, N.D., Smith, D.E., Buchhagen, J.L., Valafar, F., Crudu, V., Romancenco, E., Noroc, E., Jackson, L., Catanzaro, D.G., Rodwell, T.C., Caanzaro, A., Keim, P., Engelthaler, D.M., (2015). Detection of Low-Level Mixed-Population Drug Resistance in Mycobacterium tuberculosis Using High Fidelity Amplicon Sequencing. *PLOS ONE, 10*(5):e0126626. doi:10.1371/journal.pone.0126626.

Huson, D. H., Auch, A. F., Qi, J., Schuster, S. C. (2007). MEGAN analysis of metagenomic data. *Genome Research*, *17*(3), 377–386. https://doi.org/10.1101/gr.5969107

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10–12. https://doi.org/10.14806/ej.17.1.200

O'Rourke, D. R., Bokulich, N. A., MacManes, M. D., Foster, J. T. (2020). A total crapshoot? Evaluating bioinformatic decisions in animal diet metabarcoding analyses. *Ecology and Evolution*, ece3.6594. https://doi.org/10.1002/ece3.6594

R Core Team. (2022). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Ratnasingham, S., Hebert, P. D. N. (2007). bold: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Notes*, *7*(3), 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Ushio, M., Fukuda H., Inoue, T., Makoto, K., Kishida, O., Sato, K., Murata, K., Nikaido, M., Sado, T., Sato, Y., Takeshita, M., Iwasaki, W., Yamanaka, H., Kondoh, M., Miya, M. (2017). Environmental DNA enables detection of terrestrial mammals from forest pond water. *Molecular Ecology Resources*, *17(*6):e63–e75. doi:10.1111/1755-0998.12690.

Walker, F. M., Williamson, C. H. D., Sanchez, D. E., Sobek, C. J., Chambers, C. L. (2016). Species From Feces: Order-Wide Identification of Chiroptera From Guano and Other Non-Invasive Genetic Samples. *PLOS ONE*, *11*(9), e0162342. https://doi.org/10.1371/journal.pone.0162342

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T. L., Miller, E., Bache, S. M., Müller, K., Ooms, J., Robinson, D., Seidel, D. P., Spinu, V., ... Yutani, H. (2019). Welcome to the Tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Appendix G: Figures S2 & S3

Species	Sampler A		Sampler B		Sampler C		Sampler D		Sampler E		Sampler F	
Carollia perspicillata	0	10	0	12	0	0	0	89	0	0	0	0
Glossophaga mutica	25847	79	95389	26797	49	0	6508	75227	8	16713	21574	9989
Natalus mexicanus	0	2	3672	82134	39	11200	17	1177	66429	7241	28605	10556
Saccopteryx bilineata	12	0	0	0	0	38	0	0	0	0	0	0
Sturnira parvidens	0	0	0	2265	29	383	0	6208	0	0	19860	0
Trachops cirrhosus	14	0	1964	0	0	0	14142	0	0	31791	0	0
Alouatta palliata	0	0	0	20390	0	0	0	0	0	0	0	0
Bos taurus	35	51	0	16	144	78	0	2	30865	58	41073	2
Canis spp.	13164	65	3149	699	324	104327	0	3006	15	2395	13	5625
Equus caballus	0	0	0	0	0	0	4100	0	0	0	0	0
Leptodactylus fragilis	0	0	13	0	0	187	0	0	0	5255	0	0
Ototylomys phyllotis	0	0	0	0	0	0	5	0	13282	0	23	0
Ovis aries	36565	127	8254	0	159	0	49444	0	6	0	9	0
Scinax staufferi	0	0	0	0	0	0	0	0	1516	0	0	0
Sus scrofa	55170	0	0	0	14	112	0	0	0	36	0	0
Sylvilagus floridanus	8820	0	0	0	17	0	0	0	0	0	0	0
Trachycephalus typhonius	0	0	3952	0	0	0	0	0	0	0	0	0

Day Night

Figure S2. The total count by sampler (summed across three PCR replicates) for each species detected during (approximately 8:30-15:00 – yellow) and at night (approximately 15:00-8:30 – blue) in the Schoolhouse Cave on April 28th-29th, 2022.

A)

Species	Sampler A						Sampler B						Sampler C					
	R1	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*
Carollia perspicillata	4544	4	0	0	0	0	0	0	0	7	7	5	0	10	0	8193	5	0
Glossophaga mutica	25810	22	15	17	32	0	15082	51501	28806	33	10693	16071	26	53	0	0	0	0
Natalus mexicanus	0	0	0	20	19	0	0	3672	0	24381	39784	17969	0	2	0	705	10495	0
Saccopteryx bilineata	2	0	10	0	0	0	0	0	0	0	0	0	0	0	0	24	0	14
Sturnira parvidens	0	0	0	29	0	0	0	0	0	2265	0	0	0	0	0	0	383	0
Trachops cirrhosus	0	9	5	0	0	0	0	1964	0	0	0	0	0	0	0	0	0	0
Alouatta palliata	0	0	0	0	0	0	0	0	0	20390	0	0	0	0	0	0	0	0
Bos taurus	0	16	19	47	97	0	0	0	0	16	0	0	22	29	0	66	0	12
Canis spp.	0	0	13164	85	239	0	0	0	3149	699	0	0	26	39	0	41744	8233	54350
Equus caballus	0	0	0	0	0	0	0	Text	0	0	0	0	0	0	0	0	0	0
Leptodactylus fragilis	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	0	187	0
Ototylomys phyllotis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ovis aries	21229	6	15330	58	101	0	0	0	8254	0	0	0	55	72	0	0	0	0
Scinax staufferi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sus scrofa	0	55170	0	0	14	0	0	0	0	0	0	0	0	0	0	0	112	0
Sylvilagus floridanus	0	0	8820	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trachycephalus typhonius	0	0	0	0	0	0	0	3952	0	0	0	0	0	0	0	0	0	0

Day Night

B)

Species	Sampler D						Sampler F						Sampler E					
	R1	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*	*R1*	*R2*	*R3*
Carollia perspicillata	0	25	18	7938	17	8	0	0	0	0	0	2462	0	0	0	0	0	0
Glossophaga mutica	0	6	6502	29284	39742	6201	4	4	0	16696	8	9	8	21561	5	9936	31	22
Natalus mexicanus	8	9	0	1171	6	0	45759	20670	0	0	7241	0	47	28553	5	7054	3491	11
Saccopteryx bilineata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sturnira parvidens	0	0	0	6208	0	0	0	0	0	0	0	0	19860	0	0	0	0	0
Trachops cirrhosus	14142	0	0	0	0	0	0	0	0	15446	16462	0	0	0	0	0	0	0
Alouatta palliata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bos taurus	0	0	0	2	0	0	0	30846	19	43	0	15	0	15	41060	0	0	0
Canis spp.	0	0	0	0	0	3006	0	15	0	0	0	2395	0	13	0	0	0	5625
Equus caballus	0	0	4100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leptodactylus fragilis	0	0	0	0	0	0	0	0	0	4181	0	1074	0	0	0	0	0	0
Ototylomys phyllotis	0	0	5	0	0	0	0	4	13278	0	0	0	14	9	0	0	0	0
Ovis aries	27461	21977	6	0	0	0	6	0	0	0	0	0	9	0	0	0	0	0
Scinax staufferi	0	0	0	0	0	0	1516	0	0	0	0	0	0	0	0	0	0	0
Sus scrofa	0	0	0	0	0	0	0	0	0	21	10	5	0	0	0	0	0	0
Sylvilagus floridanus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trachycephalus typhonius	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Day Night

Figure S3. The total read count by sampler (A) samplers A-C, (B) samplers D-E and PCR replicate (R#) for each species detected during the day (approximately 8:30-15:00 – yellow) and at night (approximately 15:00-8:30 – blue) in the Schoolhouse Cave on April 28th-29th.

Appendix H: Published Chapter Collaborative Statements

Student statement on publish chapters and collaborative work

With regards to the date and work included my masters thesis.

The data in both chapters are my own but produced in collaboration with co-authors. In the field of ecology, projects are often collaborative due to their large-scale nature and for safety in the field. All academic professionals as well as local collaborators are included. Both projects described in this thesis were part of annual field trip with a large international research team. As such, other researchers assist with field collections and permits.

For both projects, I was the lead researcher on both published works. I was supervised in project design, collected the data and processed the samples in the lab by my supervisor Dr. Elizabeth Clare. I performed the analysis and bioinformatics on the results and wrote the manuscripts as first author. Below are the contributions from collaborators:

Chapter 2:

Garrett NR, Watkins J, Simmons NB, Fenton MB, Sanchez DE, Froehlich EM, Walker FM, Littlefair JE and Clare EL. 2022. Airborne eDNA documents a diverse and ecologically complex tropical mammal community. Environmental DNA.

JW – provided field assistance and helped collect samples
NBS, MBF – organized permits, coordinated field activities and supervised and approved the analysis
DES, EMF, FMW – independently validated a subset of the data in a secondary lab
JEL – informed and advised the statistical analysis of the data
ELC – supervisor of all work

The final manuscript as edited and approved by all co-authors along with the data.

Citation for the published article:
Garrett, N. R., Watkins, J., Simmons, N. B., Fenton, B., Maeda-Obregon, A., Sanchez, D. E., Froehlich, E. M., Walker, F. M., Littlefair, J. E., & Clare, E. L. (2023). Airborne eDNA documents a diverse and ecologically complex tropical bat and other mammal community. *Environmental DNA*, 5, 350– 362. https://doi.org/10.1002/edn3.385

Chapter 3:

Garrett NR, Watkins J, Francis C, Simmons NB, Ivanova NV, Naaum A, Briscoe A, Drinkwater R, Clare EL. Out of thin air: surveying tropical bat roosts through air sampling of eDNA. PeerJ.

JW – provided field assistance and helped collect samples
CF – took photographs used in figures and supervised or approved the analysis

NBS – organized permits, coordinated field activities and supervised and approved the analysis
NVI, AN, AB – provided access to specialised eDNA facilities and contributed in-kind support including access to commercial clean rooms and sequencing platforms. Supervised or approved the analysis.
RD – informed and advised the statistical analysis of the data
ELC – supervisor of all work.

The final manuscript as edited and approved by all co-authors along with the data.

Citation for the published article:
Garrett NR, Watkins J, Francis CM, Simmons NB, Ivanova N, Naaum A, Briscoe A, Drinkwater R, Clare EL. 2023. Out of thin air: surveying tropical bat roosts through air sampling of eDNA. PeerJ 11:e14772 https://doi.org/10.7717/peerj.14772

Others who played a role outside of academia are listed in the acknowledgements as follows:

Chapter 2:

We wish to thank the staff at Lamanai Field Research Center for all their assistance with sampling logistics and research permits. We also thank colleagues who helped with field work and bat captures during the 2022 field season, and NatureMetrics of London, UK for sequencing data. Particular thanks to Will Clare, Matt Clare, Kaya Courie, Annie Floyd and Owen Floyd who built and tested dozens of prototype filters and Jerry J Grech for the design of our 3D printing.

Chapter 3:

We wish to thank the staff at Lamanai Field Research Center for all their assistance with sampling logistics and research permits. We also thank colleagues who helped with field work and bat captures during the 2022 field season. Thanks to Will Clare, Matt Clare, Kaya Courie, Annie Floyd and Owen Floyd who built and tested the three prototype filters, and Jerry J. Grech for the design of our 3D printing. Thanks to Brock and Sherri Fenton for photographs and field logistics, Helen Haines for access to her attic, and Alejandro Maeda-Obregon for help with the DADA2 pipeline. Helpful comments were provided by two reviewers which greatly enhanced the content of the manuscript.

Nina Garrett MSc Biology Candidate

Supervisor statement on collaborative work.

With regards to the work contained in the thesis of Ms. Nina Garrett.

Both data chapters of this thesis were produced as part of large collaborations. In our field of molecular ecology it is extremely rare for any project or paper to be conducted without collaboration, primarily for safety. As a consequence we always include local and professional collaborators. For the purpose of this thesis, both data chapters use data collected as part of a large international research team which operates annually in the same field site. We rely on assistance and collaboration for permits, field collections and inventories.

I certify that the work in these chapters was produced primarily by Nina Garrett and she is the lead researcher for both projects. She was supervised in project design, collected the data, performed the lab work, analysed the results and wrote both manuscripts as lead author. Other collaborators provided assistance as follows.

Garrett N, Watkins J, Simmons NB, Fenton MB, Sanchez DE, Froehlich EM, Walker FM, Littlefair JE and Clare EL. 2022. Airborne eDNA documents a diverse and ecologically complex tropical mammal community. Environmental DNA.

JW - provided field assistance for the field components of the experiment
NBS, MBF - arranged permits and coordinates field activities and supervised or approved the analysis NMF - coordinates field activities and directs the field team and supervised or approved the analysis
SDE, FEM, FMW - conducted independent validation of a subset of data in a secondary lab location as part of the validation of a new method.
JEL - provided advice on statistical analysis
ELC - supervisor of all work.

All co-authors approved or edited the final manuscript and data for publication.

Citation for the published article:
Garrett, N. R., Watkins, J., Simmons, N. B., Fenton, B., Maeda-Obregon, A., Sanchez, D. E., Froehlich, E. M., Walker, F. M., Littlefair, J. E., & Clare, E. L. (2023). Airborne eDNA documents a diverse and ecologically complex tropical bat and other mammal community. *Environmental DNA*, 5, 350– 362. https://doi.org/10.1002/edn3.385

Garrett NR, Watkins J, Francis C, Simmons NB, Ivanova NV, Naaum A, Briscoe A, Drinkwater R, Clare EL. Out of thin air: surveying tropical bat roosts through air sampling of eDNA. PeerJ.

JW - provided field assistance
CF - took photographs used in figures and and supervised or approved the analysis
NBS - arranged permits and coordinates field activities and supervised or approved the analysis
NVI, AN, AB - provided access to specialised eDNA facilities and contributed in-kind support including access to commercial clean rooms and sequencing platforms. Supervised or approved the analysis.

RD - provided advice on statistical analysis.
ELC - supervisor of all work.

All co-authors approved or edited the final manuscript and data for publication.

Citation for the published article:
Garrett NR, Watkins J, Francis CM, Simmons NB, Ivanova N, Naaum A, Briscoe A, Drinkwater R, Clare EL. 2023. Out of thin air: surveying tropical bat roosts through air sampling of eDNA. PeerJ 11:e14772 https://doi.org/10.7717/peerj.14772

Prof. Elizabeth Clare, Supervisor