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Policy analysis

Molecular genetic analysis of air, water, and soil to detect big brown bats in North America

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ABSTRACT

Cave-hibernating bats are widespread in North America but are facing precipitous population declines due to the impacts of white-nose syndrome (WNS). It is in winter hibernacula that bats are most vulnerable to the fungus that causes WNS, but the locations of over-wintering sites in western North America are largely unknown. This poses a significant challenge for bat monitoring, disease surveillance, and management efforts at the disease front. To advance initiatives to locate bats on the landscape, we developed real-time PCR assays to detect big brown bats (*Eptesicus fuscus*) from environmental DNA samples (eDNA). Three assays were designed, one each for eastern, western, and southern North America, to account for the high intra-specific genetic variability within big brown bats. We demonstrate that these assays can detect bat DNA in environmental samples, including air, water, and soil, and are able to detect target DNA at concentrations as low as 2 copies per reaction. Although the assays are highly sensitive, detections from samples collected in field samples were modest. Our findings suggest that eDNA may provide a much-needed, non-invasive alternative to conventional tools used to detect bats on the landscape but require further research to optimize their field application.

1. Introduction

White-nose syndrome is a devastating, emergent fungal disease of cave-hibernating bats in North America (Blehert et al., 2009; Lorch et al., 2011). First observed in New York in 2006, the fungus has been progressing westward, and is now present in 38 states and 8 Canadian provinces, crossing the Great Plains in recent years (https://www.whitenosesyndrome.org/resources/map, 2020). Early fungus detection and baseline data on bat populations are critical components of disease control and bat population recovery in the face of disease invasion. These efforts are complicated in western North America due to a general lack of information about where bats overwinter (Weller et al., 2018). Furthermore, some effective bat monitoring techniques require contact or close proximity to bats and can be dangerous for personnel and bats, particularly given the emergence of SARS-Cov-2 which has some potential to spread between humans and bats (https://www.fws.gov/southwest/es/Documents/R2ES/AUES_Covid_Bat_Guidance.pdf; accessed 03/04/2021). Thus, natural resource managers in the West have an urgent need for non-invasive tools to locate and monitor bats, and assess their vulnerability to invading Pd.

Existing techniques for bat monitoring, including mist-netting, colony counts, and acoustic monitoring, have established critical baseline and temporal trend data for bat distributions and abundances in North America. Mist netting is inexpensive, transportable and easy to use (Dunn and Ralph, 2004). Colony counts, a method to assess occupancy, abundance, and distribution at summer or winter roosts, provides quick and presumably accurate estimates for species that congregate at known sites. Guano collection at bat roosts can provide additional data on a species' presence/absence, species composition, diet, and pathogen presence (Swift et al., 2018; Walker et al., 2016). Finally, acoustic monitoring uses a combination of frequency, bandwidth, duration, and pulse to determine presence of individual bat species in all seasons and habitats without the need to see or handle bats (Jones and Holderied, 2007).

Despite the clear utility of these methods, each has limitations in application. Mist netting is time consuming, requires specific expertise

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to identify trapped bats, the nets must be monitored frequently, and the mist nets pose risks for *Pd* transmission (MacCarthy et al., 2006). Colony counts require a high level of expertise and can pose occupational hazards for humans. Importantly, while colony counts at hibernacula are highly effective in eastern North America, western bats are rarely found in large, subterranean colonies. For example, 95% of known *Myotis* spp. aggregations in the West contained 10 or fewer individuals (Weller et al., 2018). Thus, the application of this method in the West is limited. Finally, acoustic monitoring of bat calls, while non-invasive, has several weaknesses: calls can be variable within a species based on behavior, sex, age, and body size (Jones and Siemers, 2011), they are not diagnostic for all species, and processing recorded call data requires extensive data management and human verification for species identification (Russo and Voigt, 2016).

Methods that require capture and handling or close proximity to bats are invasive and can compromise bat health and safety. Invasive monitoring techniques pose a risk to both bats and the personnel studying them, especially with respect to the risk of disease transmission (e.g. SARS-Cov-2, rabies, other coronaviruses and lentiviruses, and histoplasmosis). Attention to this risk has been heightened by the emergence of SARS-CoV-2 in humans, which is thought to have originally spilled over from Asian bat species (Andersen et al., 2020; Lau et al., 2020). Given the putative bat origin of SARS-Cov-2, a concern about the susceptibility of North American bats to SARS-Cov-2 in humans has severely hampered bat monitoring efforts (Runge et al., 2020). A recent experiment demonstrated resistance to infection in inoculated big brown bats (*Eptesicus fuscus*) (Hall et al., 2020) and a comparative analysis of the host receptor ACE2 across a broad range of hosts predicted low or very low propensity for binding SARS-Cov-2 in the chiropterans studied (n = 37 species) (Damas et al., 2020). While the risk of reverse spillover to North American bat populations is low, it is not zero and could have profound implications for bats and humans if it were to occur. Thus, there remains an elevated need for additional non-invasive methods for bat monitoring while SARS-CoV-2 continues to spread in human populations.

Environmental DNA (eDNA) sampling is a non-invasive technique that is used to determine species presence within an environment through capturing the DNA that an organism sheds as it passes through mediums including air, water, sediment (Ficetola et al., 2008). To detect eDNA from a single species of interest in environmental samples, highly sensitive real-time PCR (qPCR) assays are often used (Kubista et al., 2006). This approach has been used extensively for aquatic species, including fish (Boothroyd et al., 2016; Carim et al., 2016a, 2019; Mason et al., 2018; Tillotson et al., 2018), amphibians (Evans et al., 2016; Franklin et al., 2018; Goldberg et al., 2018), and mussels (Cho et al., 2016; Dysthe et al., 2018). Recently, scientists and managers have been exploring application of eDNA for monitoring non-aquatic taxa such as rare carnivores (Franklin et al., 2019; Sales et al., 2020a; Sales et al., 2020b) and plants (Johnson et al., 2019). In aquatic environments, eDNA-based monitoring has proved more sensitive (Wilcox et al., 2016) and cost effective (McKelvey et al., 2016) than alternative methods.

While monitoring a volant, terrestrial mammal using eDNA presents unique challenges, we believe the method holds promise for bats because most species aggregate in groups, reuse sites over extended periods, and select enclosed spaces for roosting. These behaviors may concentrate shed DNA in the environment. If applied successfully for bat detection, eDNA methods using qPCR promise unambiguous species identification and are non-invasive. eDNA sample collection requires minimal training, and thus could minimize the time, effort, and skills (e.g. capture and handling, morphological ID) needed to carry out bat monitoring surveys.

Guano sampling is a popular approach to bat monitoring that similarly relies on analyzing DNA from samples found in the environment and is thus non-invasive. It differs from qPCR eDNA tools in that it commonly employs conventional mitochondrial DNA sequencing or metabarcoding (Taberlet et al., 2012) to gather data on individuals and populations (e.g. Walker et al., 2016; Swift et al., 2018). These methods have the advantage that they can detect many species in a single assay (including prey and pathogens, Swift et al., 2018) while qPCR assays detect a single species (but see HTqPCR; Wilcox et al., 2020). However, guano collection requires knowledge of and access to the exact location where the target organism is or was present. A qPCR based eDNA approach offers the potential to survey environmental substrates without reliance on finding guano and precluding the need to enter confined spaces, e.g. air or water exiting a culvert or hibernaculum or surface water at bat foraging or drinking sites. This application is possible using DNA metabarcoding as demonstrated with the 'Species from Feces' approach used to detect bat species in cave sediments in the absence of guano (Walker et al., 2019). However, the qPCR approach is more sensitive when the goal is for targeted detection of specific taxa (Bylemans et al., 2019), which is critical for detection from samples with low target abundance. Furthermore, when pre-existing qPCR assays for a target species already exists, less time and cost are required to perform lab and data analysis than for metabarcoding.

In this study, we designed qPCR assays for the detection of big brown bats (*Eptesicus fuscus*; EPFU) to explore the application of the eDNA approach to bat monitoring. We selected EPFU as our study species because it common, widespread and phylogenetically distant from other North American microbats, facilitating eDNA assay validation. Despite variation in ecological characteristics among bat species, the eDNA technique may be broadly applicable to other chiropterans because most aggregate in groups and use enclosed spaces which may concentrate DNA from urine, guano, and shed hair and skin in the environment (e.g. in soil, air, and water), and many drink and forage over open water depositing DNA through contact or defecation. We designed three assays (to account for regional intra-specific genetic variability) and assessed their ability to detect EPFU DNA in environmental samples collected from a captive EPFU enclosure for proof of principle. In addition, environmental samples were opportunistically collected from field locations and tested to explore potential field applications of the assay. These included water and sediment samples from hibernacula during winter and water samples from potential foraging, drinking, or roosting sites in summer.

2. Material and methods

2.1. Assay development

Three region-specific assays were designed for EPFU to account for the deep genetic divergence between North American EPFU populations observed in previous studies (Neubaum et al., 2007; Turmelle et al., 2011) and in our preliminary analyses of publicly available sequence data. Similar to Turmelle et al. (2011) who based their analyses on the ND2 gene, variation at cytochrome *c* oxidase subunit 1 (COI) was consistent with eastern, western, and southern haplogroups. As such, we constructed assays consistent with this grouping. For in silico validation, COI sequence data from target and non-target species across the EPFU range in North America were collated and aligned in MEGA 7.0.26 (Appendix A). We used DECIPHER (Wright, 2016) to identify COI primer regions and sequences having low variability within EPFU and high variability between EPFU and other North American bat species (see Appendix A for mismatches across species). We then designed a FAM-labeled, minor-groove-binding, non-fluorescent quencher (MBG_NFQ) probe within the selected sequence region by identifying areas that maximize mismatches with non-target species. We used Primer Express 3.0.1 (Life Technologies) to verify that the assays met appropriate annealing conditions (Life Technologies) and used IDT to check for secondary structuring (https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/; see Table 1 for primer and probe sequences).

We tested for both specificity and generality across the range of EPFU in the conterminous United States. We used the NCBI Basic Local Alignment Search Tool (BLAST) to search GenBank (Benson et al., 2005)

Table 1
Environmental DNA assays for detecting big brown bats.

Assay component		Sequence (5′ to 3′)	Tm (°C)	Optimal concentration (nM)	Amplicon length (amplicon position within alignment)
Eastern	Forward primer	ACC TCC CGC TCT ATC TCA ATA TCA	59.1	300	91 bp (420 bp–511 bp)
	Reverse primer	CCA GCA CAG GAA GGG ATA GC	58.4	600	
	Probe	FAM-TCG TCT GAT CTG TTC TAA T-MGBNFQ	68	250	
Southern	Forward primer	G GTA TCT TCA ATC TTA GGA GCC ATT AA	58.3	900	134 bp (366 bp–500 bp)
	Reverse primer	AGGGACAGCAGAAGAAGCACA	58.7	900	
	Probe	FAM-T CTC TCT CAG TAT CAA ACA C-MGBNFQ	70	250	
Western	Forward primer	GGT ATC TTC AAT TCT AGG AGC CAT TAA	58.3	600	134 bp (366 bp–500 bp)
	Reverse primer	AGG GAT AGC AGA AGA AGC ACA GC	60	600	
	Probe	FAM-TCT ATC TCA GTA TCA AAC ACC A-MGBNFQ	69	250	

reference sequences to locate non-target sequence similarity for each sequence component (forward primer, reverse primer, probe; a total of three queries). For the algorithm parameters, all default settings were used except for the maximum target sequences, which was set to 1000 sequences and excluded EPFU. The initial non-target list comprised all the listed species that had a query coverage of greater than 90% (e-value sorted from lowest to highest). We concluded that the assays were likely to be specific because of one or more of the following reasons: (i) no species showed up in two or more query searches, (ii) a close match in one assay component was accompanied in high mismatches in the remaining two, (iii) there was geographic separation between the target species and species that were identified as similar in the BLAST searches.

The in silico searches were followed by in vitro testing. For within-species in vitro testing, 42 putative EPFU tissue samples (wing punches, muscle, extracted DNA) were obtained from various museums, state agencies and academic institutions (Fig. 1, Appendix B). Samples came from 12 U.S. states (AZ, CA, CO, ID, MT, ND, NV, OR, SD, TN, WA, WY) with between 1 and 8 samples per state. All samples used in this study were sequenced to confirm species identification through DNA-based methods. To confirm species identification, we used a forward primer Cox F4 (5′-TCAACCAATCAYAAAGAYATTGGTAC-3′), and a reverse primer Cox R5 (5′-CCAAAGAATCARAAYAAGTGTTGATAT-3′) to generate sequence data from the mitochondrial COI gene (Nadin-Davis et al., 2012) (Eurofins Genomics; Louisville, Kentucky, USA). Poor-quality nucleotide reads were removed from raw sequence files using Sequencher (v5.4.6; Gene Codes Corporation, Ann Arbor, MI). The sequences were queried in GenBank to determine species identity. A number of specimens that were identified in the field as EPFU were identified as a different, non-target species using this method. EPFU are morphologically distinct from other microbats and misidentifications are expected to be rare. Further, these samples amplified with the assays that we designed for EPFU in this study. These results were odd, and seeking to explain them, we noticed that there were 5 mismatches with the forward sequencing primer and EPFU with two of the mismatches within six bases of the 3′ end. We therefore suspected that the COI primers used for species ID amplified EPFU less efficiently than for other bat species whose DNA could have been present due to contamination. To explore this, we re-sequenced the COI region of specimens in this sample set with a different forward primer Cox F6 (5′-TATY-TACTATTTGGYGCTTGAGC-3′), and Cox R5 (as above) (Nadin-Davis

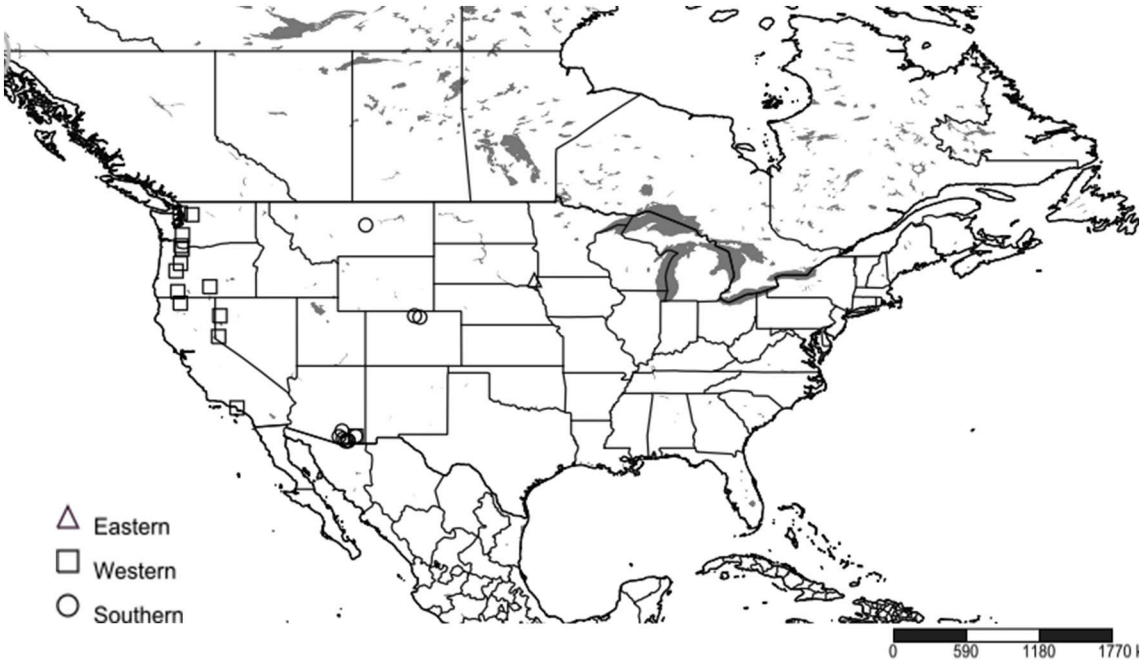

Fig. 1. Map of EPFU samples analyzed using developed eDNA assays. Open squares represent geographic locations that amplified at an earlier cycle threshold using the western assay, open triangles represent locations that amplified earlier using the eastern assay, and open circles represent locations that amplified earlier using the southern assay.

et al., 2012).

To ensure that the assays only detect EPFU and do not detect non-target species, we obtained either extracted DNA or tissues from 28 non-target species selected based on genetic similarity and/or sympatry with EPFU. Tissues were stored in lysis buffer or 95% ethanol and extracted using the DNeasy Blood and Tissue Kit (Qiagen, Inc.) following the manufacturer's protocols. Both target and non-target DNA was analyzed using a QuantStudio 3.0 Real-time PCR Instrument (Life Technologies) in single 15 μL reactions containing 7.5 μL of Environmental Mastermix 2.0 (Life Technologies), 900 nM of the reverse primer, 900 nM of the forward primer, 250 nM of the probe, 2.75 μL of diH_2O, and 4 μL of template DNA. Cycling conditions included an initial hot start of 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. All pipettes, tips, water, etc. were exposed to UV light for 1 h prior to running reactions to minimize contamination. All qPCR reactions were set up under a UV hood.

Primer concentrations were optimized to minimize the number of cycles required for the amplification curve to pass the cycle threshold (C_t) and to produce high relative end-point fluorescence. Forward and reverse primer concentrations were tested (100, 300, 600, and 900 nM, n = 16 combinations), while keeping the probe at 250 nM for all primer combinations following Wilcox et al. (2015). The optimal primer concentrations were as follows: eastern group at 300 nM forward primer and 600 nM reverse primer, western group at 600 nM for both forward and reverse primers, and southern group at 900 nM for both forward and reverse primers. To generate standard curves, DNA extracted from tissue samples were amplified using qPCR (one specimen was selected from each haplogroup). These assays were not multiplexed, and separate standard curves were generated for each region. The qPCR product was purified using GeneJET PCR Purification Kit (Life Technologies) and quantified on a Qubit 2.0 Fluorometer (ThermoFisher Scientific). We tested the sensitivity of the assays by using a standard curve with the following dilution series: 31250, 6250, 1250, 250, 50, 10, and 2 copies per 4 μL of DNA extract, and each reaction was replicated twelve times. The limit of detection (LOD) is defined as "the lowest concentration at which 95% of the positive samples are detected" (Bustin et al., 2009, p. 617). It is used to understand how sensitive the assays are at detecting low concentrations of DNA. A standard curve is used to evaluate the efficiency (if the DNA is doubled with each cycle) of the assays and assess their ability to detect DNA at very low concentrations.

Despite the multiple base-pair mismatches between the non-target and target species, a subset of samples of the non-target species amplified when tested with our EPFU assays (see results below). To examine whether these amplifications were due to true cross-amplification (i.e. genetic similarity of the non-target species to EPFU in the region of the primer and probe set) or due to sample contamination, we Sanger sequenced qPCR product from a subset of these samples. qPCR product from each tissue DNA sample was purified using the GeneJET PCR Purification Kit (Life Technologies), and submitted to Eurofins Genomics (Louisville, Kentucky, USA) for Sanger sequencing using the forward and reverse primers from each respective qPCR assay (Table 1). Raw sequence data were processed in Sequencher (v5.4.6; Gene Codes Corporation, Ann Arbor, MI) to remove poor quality nucleotide reads and only retain reads within each respective target amplicon. Overlapping forward and reverse reads from each respective sample were concatenated when possible and queried using Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the most likely source species. Reads that could not be concatenated were queried individually.

As an additional test, we obtained synthetic DNA sequences covering the entire amplicon locus (gBlocks; Integrated DNA Technologies Inc.) for all non-target species that amplified with our assays (n = 15 species; Appendix C). The gBlocks were reconstituted following the manufacturer's protocol and initially diluted to 0.1 ng/μl (~1.5 billion copies per reaction) before analyzing with each assay following the methods described above for tissue testing. Any gBlock that resulted in amplification was further diluted to approximately 0.0000641 ng/μl (~1 million copies per reaction), and 0.000002 ng/μl (~31,250 copies per reaction). We selected these concentrations because they are higher than we would expect in environmental samples and provide a conservative test of the specificity of the assay.

Finally, we analyzed a complete *M. lucifugus* whole genome sequence to confirm that there were no other areas of the complete genome that might cross amplify. Using the nucleotide blast search option of "aligning two or more sequences" on GenBank, we compared each assay component (see Table 1 for assay sequences) individually against the *M. lucifugus* (accession number: NW_005871090.1) to see if there was any amplification across the entire genome. For the algorithm parameters, all default settings were used except for the maximum target sequences, which was set to 1000 sequences.

2.2. Environmental sampling

Environmental samples were collected from an enclosure housing a big brown bat colony for proof of principle, and from the natural habitat of big brown bats to explore potential applications of eDNA tools for bat monitoring.

2.2.1. Captive animals

Air and water samples were taken in a captive enclosure housing approximately 30 EPFU at the Northeast Ohio Medical University (NEOMED). Air was filtered using a QuickTake 30 vacuum air pump (SKC, PA) with Via-Cell cartridge for sampling bioaerosols ≥1.56 μm, placed 1 m off the ground in the center of the enclosure (facility volume ~54,000 L; 12 × 16 ft × 10 ft high). The lighting within the enclosure simulated 12 h of daylight and 12 h of darkness. Captive bats roosted under blankets on the wall when they were not active. To assess whether bats shed detectable levels of DNA in the air in real-time, samples were collected during both dark and light periods of the day as a proxy for activity level, assuming bats were less active in the light. This testing was non-invasive and did not require additional IACUC considerations. Four samples were collected during the dark period (one sample over 1 h; 900 L of air, and the remaining samples over 20-minute time intervals; 300 L of air). Three air samples were collected during the light period (over 20-minute time intervals; 300 L of air). Via-Cell cartridges were refrigerated until extraction and the water filter was stored dry on desiccant and frozen at −20 °C until extraction. One 740 mL water sample was collected from drinking water provided for the bats and filtered with a glass fiber filter (1.5 μm pore size; Whatman) using a peristaltic pump (Geotech).

2.2.2. Active season sampling

Sampling occurred opportunistically during the "active season" (summer) at waterbodies associated with bat activity, e.g. drinking and foraging, or roosting in Montana (n = 3 sites; Appendix D). For active season sampling, water was filtered directly from two mountain streams and a large river according to previously established aquatic eDNA sampling protocols (Carim et al., 2016c). Briefly, water was filtered onsite through 1.5 μm glass fiber filters (see Appendix D for filtered volume quantities) using a peristaltic pump, and samples were stored in a plastic bag with silica desiccant until genetic analysis. The Hellgate Gulch site (site = 5, Appendix D) was chosen to coincide with a mist-netting effort set to capture bats during evening emergence over a potential drinking area. Four temporally spaced water samples were filtered approximately 30 m upstream of the mist nets. The site was chosen based on proximity to the mist-nets and did not have characteristics conducive to bat foraging or drinking activity. Specifically, the water surface was riffled, and branches hung over the creek which could deter a bat approach. Despite this, we deemed it possible that bat DNA could flow from upstream reaches visited by bats given the overall amount of bat activity observed in the drainage. The Davis Creek site (MPG site; Appendix D) was located at a very small, slow-moving seep

on a road at which reproductive female EPFU had been captured consistently during the reproductive season (between April and July in Montana). This stream was sampled in September and EPFU were not documented using the site the morning of sampling. Three samples were filtered on site after dawn. A site on the Missouri River (BS1; flowrate of approx. 5480 cfs) was sampled in the afternoon downstream of a bridge which held a large, little brown bat (*M. lucifugus*) maternity colony (n > 600). The presence of big brown bats within this colony was not suspected. All active season water filters were dried on desiccant and stored at room temperature or frozen at −20 °C. EPFU presence in the vicinity at the time of sampling was documented for one of eight active season samples (collected at the Hellgate Gulch site).

2.2.3. Inactive season sampling

Inactive season sampling was conducted opportunistically coinciding with winter hibernacula colony counts at two tunnels and a mine in Wisconsin (three sites and 15 samples in total).

One site (site 3, Appendix D) was visited twice, once in November and later in March of the 2018–2019 hibernation season. Multiple EPFU aggregations were observed near the entrance of this tunnel (totalling n = 51 and n = 215 EPFU in November and March, respectively) roosting on either side of the tunnel wall, at a height of ~1.75 m. Water was scooped directly under and/or within 5 m downstream of the bats from running water that ran along both sides of the tunnel and out onto the landscape. On the second visit, samples were collected downstream and outside of the hibernaculum to assess whether EPFU could be detected without entering the site. Finally, to assess soil as an environmental medium for bat detection, paired sediment samples were collected from inside the hibernaculum during this visit. Sediment (~5.0 g) was scraped off the surface of the ground with a sterile tongue depressor, placed into a plastic bag, and transported on ice to the National Wildlife Health Center (United States Geological Survey; USGS) for *Pd* detection. DNA extracts were later obtained from USGS for this study. No EPFU were observed at sites 1 or 2 in the year of sampling. EPFU historically occupied site 1, however following the invasion of WNS this site was mostly depopulated of all bat species. Only 2 individual bats (PESU) were observed at sampling. At site 2, bats historically roosted over water at this site though EPFU had not been observed previously. Four total bats were observed at ~5 m away from sampling (*Myotis lucifugus*, n = 2; *Perimyotis subflavus*, n = 1; *Myotis* species, n = 1).

Samples were collected as above with the following modifications. To minimize disturbance to the bats, water was scooped from water sources inside the hibernacula (pools or stream) and transported outside of the hibernacula to be filtered. To minimize the risk of transporting viable *Pd* spores across state lines, water filters were either stored in ATL lysis buffer (QIAGEN, Inc.) or on desiccant and heat-inactivated by incubation at 55 °C for at least 20 min.

2.3. DNA extraction

DNA was extracted from all samples using half the filters with the DNeasy Blood and Tissue Kit (Qiagen, Inc.), as described in Carim et al. (2016c). The final elution had a volume of 100 μL. Extractions were carried out in an area designated solely for this purpose, and the samples were paired with a negative extraction control to test for contamination; the highest number of samples extracted within a batch was 12. All qPCR reactions had a minimum of one qPCR negative control per 95 wells. The DNA extraction area was strictly controlled; technicians were not allowed to handle PCR products before entering, and lab space was cleaned with bleach solution regularly. To extract DNA from Via-Cell cartridges, cartridge media was dissolved in ~5.0 mL of distilled water and filtered through a glass filter and processed as above.

2.4. Testing eDNA samples

Each eDNA sample was run in triplicate with the following reaction volumes: 7.5 μL of 2× Environmental Mastermix 2.0 (Life Technologies), optimized primer concentrations (Table 1), 250 nM probe, 0.75 μL of 20× EPFU assay (see Table 1 for concentrations), 1.5 μL of 10× internal positive control (IPC) assay (Life Technologies), 0.30 μL of 50× IPC DNA (Life Technologies), and the remainder with deionized water. To each reaction, 4 μL of DNA template was added. Samples were determined to be "inhibited" if there was at least a one C_t delay relative to the no-template control. A no-template control (distilled water), as well as a positive control (big-brown bat DNA) was included in each qPCR run. All reported DNA copy numbers were rounded up to the nearest integer. Copy numbers were averaged among technical replicates of each sample and subsequently rounded up to the nearest integer. To confirm that amplifications were due to the detection of EPFU DNA, qPCR replicates (only wells that were positive) of each individual sample were pooled and verified via sanger sequencing.

3. Results

Species identification using mitochondrial DNA sequencing confirmed that 38 of the 42 tissues that were identified in the field as EPFU based on morphology were EPFU. Four individuals were identified as EPFU in the field but were different bat species based on mitochondrial DNA from DNA barcoding. These were *M. lucifugus*, *L. noctivagans*, *M. yumanensis*, and *P. subflavus*. Most of these misidentified species were collected and submitted for white nose syndrome or rabies diagnostics and came from multiple sources. Collectively, the eastern, western, and southern assays detected the target COI DNA locus (herein referred to as DNA) from the 38 tissue samples genetically confirmed as EPFU, while failing to detect DNA from negative controls (Appendix A).

The EPFU assays also detected the target sequence in 15 of the 28 non-target species in at least one of the samples assayed (Fig. 1, Table 2). Across all non-target species that amplified, the minimum number of mismatches in the forward primer, probe, and reverse primer were 2, 3, and 2, respectively, while the largest number of mismatches were 6, 7, and 8, respectively (Appendix A). Quantitative PCR assays do not typically amplify given mismatches at these frequencies (Gand et al., 2020), and therefore we suspected that the non-target samples were contaminated with trace amounts of EPFU DNA, which could have originated during sample collection, storage, subsampling of tissue, DNA extraction, or qPCR set-up. Sanger sequence data from the qPCR product of non-target species produced reads that were consistent with EPFU, while sequence data of the larger amplicon (as performed for 'species identification using mitochondrial DNA') produced reads that were consistent with the non-target species.

Our eastern and southern assays failed to detect DNA in the 15 non-target species gBlock samples at the highest concentration tested (0.1 ng/μl), however, amplification curves were present in nine of the 15 species gBlocks tested with the western assay. These curves were shallow and late compared to the positive control, and the curve shapes were distinct from curves resulting from known target samples. This assay did not detect DNA in the gBlocks samples when further diluted to concentrations still above what we would expect in an environmental sample.

Finally, when we compared the nucleotide differences between the *M. lucifugus* whole genome and the assay components, we found 10, 13, and 14 mismatches in the eastern, southern, and western forward primers, respectively, 4, 7, 8 mismatches in the eastern, southern, and western probe region, and 7, 8, and 9 mismatches in the western, eastern, and southern reverse primers, respectively (see Table 3). There was an average of six matches that occurred at the 5′ end of the assay component (minimum = 0, maximum = 13), and an average of 3 mismatches that occurred in the 3′ end of the assay component (minimum = 0, maximum = 11) (Table 3).

The standard curve analysis had an efficiency of 98.39% for the eastern haplogroup ($R^2 = 0.993$, y-intercept = 37.935, slope = −3.361), an efficiency of 98.01% for the western haplogroup ($R^2 = 0.995$, y-

Table 2
Non-target species tested, number of individuals tested within the species, and number of assays (eastern, western, southern) of false positives across all individuals, species assigned based on mitochondrial DNA identifications. Each non-target sample was only run one time.

Species name based on mitochondrial DNA	Number of individuals tested	Eastern amplifications	Western amplifications	Southern amplifications
Antrozous pallidus	4	0	1	0
Corynorhinus rafinesquii	3	0	0	0
Corynorhinus townsendii	8	2	1	0
Euderma maculatum	1	0	0	0
Lasiurus blossevilli	1	0	0	1
Lasiurus cinereus	11	7	4	2
Lasiurus intermedius	2	0	0	0
Lasionycteris noctivagans	10	3	3	0
Lasiurus seminolus	3	0	0	0
Myotis auriculus	3	0	0	0
Myotis californicus	5	0	1	0
Myotis ciliolabrum	2	0	0	0
Myotis evotis[a]	7	3	1	0
Myotis grisescens	3	1	0	0
Myotis leibii	1	0	0	0
Myotis lucifugus	9	3	5	2
Myotis septentrionalis	8	2	2	0
Myotis sodalis	7	2	4	1
Myotis sp.	1	1	1	1
Myotis thysanodes[a]	2	0	0	0
Myotis velifer	7	0	1	1
Myotis volans	7	1	2	0
Myotis yumanensis	6	0	1	0
Nycticeius humeralis	9	0	2	0
Parastrellus hesperus	1	0	0	0
Perimyotis subflavus	8	3	4	1
Pipistrellus hesperus	2	0	0	0
Tadarida brasiliensis	3	0	0	0

[a] These specimens were field IDd as MYEV or MYTH though our genetic ID methods can only confirm that they are either MYEV OR MYTH due to their genetic similarity. The names within the table are consistent with field identification so that separation can still be maintained.

Table 3
Blast search of the *Myotis lucifugus* whole genome (accession number: NW_005871090.1) against the each of assay components. The nucleotide sequences highlighted in blue indicate regions within EPFU assays that are an exact match to *M. lucifugus*.

Component	Sequence Assay (5′ to 3′)	Match
Eastern_F-primer	ACCTCCCGC TCTATCTCAATATCA	14/24
Eastern_R-primer	CCAGCACAGGAAGGGATAGC	13/20
Eastern_probe	TCGTCTGATCTGTTCTAAT	15/19
Southern_F-primer	GGTATCTTCAATCTTAGGAGCCATTAA	14/27
Southern_R-primer	AGGGACAGCAGAAGAAGCACA	13/21
Southern_probe	TCTCTCTCAGTATCAAACAC	13/20
Western_F-primer	GGT ATC TTC AAT TCT AGG AGC CAT TAA	13/27
Western_R-primer	AGGGATAGCAGAAGAAGCACAGC	14/23
Western_probe	TCT ATC TCA GTA TCA AAC ACC A	14/22

intercept = 37.994, slope = −3.371), and an efficiency of 98.82% for the southern haplogroup (R^2 = 0.992, y-intercept = 38.728, slope = −3.351). The limit of detection, defined here as the lowest concentration with >95% amplification success (following Bustin et al., 2009 and Klymus et al., 2019) for the southern and western assays were at 10 copies per reaction, and the limit of detection for the eastern assay were 2 copies per reaction. At two copies per reaction, DNA was detected in twelve of twelve replicates for the eastern assay, ten of twelve replicates for the western assay, and eight of twelve replicates for the southern assay.

Using our assays, EPFU DNA was detected in ex-situ as well as in-situ environmental samples (Table 4, Appendix B). All EPFU-positive eDNA detections were successfully verified via sanger sequencing. In the captive bat enclosure, the 740 mL drinking water sample had a mean value of 11,080 copies per reaction (all triplicates amplified). Six of seven air samples from the enclosure amplified in at least one replicate. The small quantities of target DNA detected from the air samples precluded a comparison between active and inactive periods.

Of eight water samples collected in-situ during the active season, a single sample amplified with our western assay. The single positive detection came from Hellgate Gulch, the only active season site at which EPFU presence was documented in the vicinity during sampling. At this site four water samples were collected upstream of a mist-netting effort, temporally spaced over 1.5 h. One sample amplified (in one of three

Table 4
Number of positive amplifications with the EPFU qPCR assay at each site sampled, for each environmental sample type (denominator = number of site replicates). N/a represents unsampled sites for that environmental matrix. Shaded rows indicate sites at which EPFU presence was verified at time of sampling.

Site name	Site number	EPFU occupied	Number of positive amplifications for water	Number of positive amplifications for sediment	Number of positive amplifications for air
Wis-1	1	Yes (n = 215)	0/5	1/5	n/a
Wis-2	2	Not detected	0/1	n/a	n/a
Wis-3	3	No detected	0/2	n/a	n/a
MPG ranch	4	Unknown	0/3	n/a	n/a
Missouri river	5	Unknown/not suspected	0/1	n/a	n/a
Hellgate Gulch	6	Yes	1/4	n/a	n/a
NEOMED (captive)	7	Yes (n ~ 30)	1/1	n/a	6/7

assay replicates). This result is consistent with the mist-netting data, which detected two total EPFU over the survey night, one within 12 min of the time at which the EPFU-positive water was sampled and the other before water sampling began. Abundance, levels of activity, and recency of activity of EPFU were unknown and EPFU presence was not suspected at the other two active season sampling sites, Davis Creek and the Missouri River. No detections were made at these sites.

Three sites were surveyed during the inactive period, though EPFU were only observed at one of these. Despite that approximately 215 EPFU were observed roosting near or downstream at a distance from sampling, EPFU DNA was not detected in seven water samples taken at the known occupied site. Our assay did detect EPFU DNA in one of five soil eDNA samples taken at this site. The positive soil sample was the farthest from the entrance (~22 m) of the five soil samples collected, and within 3 m of the farthest observed EPFU roost. No EPFU detections were made at the two remaining hibernacula sampled, consistent with visual count data. Lastly, none of the negative template controls amplified.

4. Discussion

This is the first study to use bats as a focal species for eDNA detection with qPCR. We designed three EPFU assays to target the major haplogroups within eastern, western, and southern North America. The big brown bat assays developed here successfully detected DNA from EPFU tissues with high sensitivity within haplogroups and high specificity. While DNA was detected in a number of non-target species samples, amplicon sequencing and subsequent testing on synthetic DNA sequences suggested that this cross-amplification was due to contamination. We demonstrated that eDNA can be applied for detecting bats using air, soil, and water samples, albeit with limited success in field samples used in this study.

While there is substantial precedent in plant and microbial systems (Hirst, 1952; West and Kimber, 2015; Yoo et al., 2017; Johnson et al., 2019), to our knowledge, there is only one published study reporting detection of airborne mammalian DNA (Clare et al., 2021). In our study, six of the seven filtered air samples successfully detected bat DNA in the air. However, eDNA concentrations were low despite the 30 bats being housed in the vivarium. One limitation to our approach may be that the sampling protocol and equipment we used were optimized for fungal spore collection, not the capture of sloughed DNA from macrobiota. The use of equipment designed to capture small molecules and/or sample over extended periods (e.g., over hours or days) might improve DNA capture by increasing the volume of air sampled or increasing the probability of the target passing in the vicinity of the sampler. An optimized air sampling method may provide a better approach for sampling bats than collecting water from lotic systems for eDNA, particularly when bats are not active, as it is allows for greater volumes of the sampling matrix to be concentrated. Additionally, it does not require a water source, making it more useful across a diversity of habitats. This approach holds promise for monitoring bats and *Pd* without the need to enter hibernacula or other enclosed roosting sites. However, because air has barely been explored as an eDNA source, effective field methods will require further development.

Species-specific qPCR assays for EPFU and other bat species have uses beyond occupancy monitoring. qPCR assays can be used for species identity of degraded carcasses or guano at roost locations when targeting species of concern (Korstian et al., 2015). Species-specific assays can also be used to determine species identity of bushmeat trade in locations where there are hunting pressures for bat species, such as the flying fox in Indonesia (Sheherazade and Tsang, 2015). Despite the benefits to using species-specific qPCR assays for detecting targets at low densities, several limitations do exist. For species that exhibit high intraspecific variation, haplogroups can go unaccounted for when building an assay, potentially leading to false negative results. High amounts of intraspecific genetic diversity are documented among more heavily studied bat species such as little brown bats (*M. lucifugus*; Vonhof et al., 2015), and long-legged myotis (*M. volans*; Vonhof et al., 2015). For EPFU, another distinct EPFU haplogroup has been documented in Southern Mexico, Venezuela, Puerto Rico, Dominican Republic (Turmelle et al., 2011). Few genetic sequences exist in the public domain to assess whether our assays would be expected to amplify EPFU DNA from these regions. Before our assays can be reliably applied to these regions, local EPFU specimens would need to be collected and validated. Lastly, secondary contact between EPFU haplogroups exists in some areas, such as Colorado (Neubaum et al., 2007); a multiplex with the eastern, western, and southern assays may be appropriate in areas such areas to ensure efficient EPFU detection.

Here and in other studies we have found that obtaining uncontaminated tissues to validate assays from laboratories and museums that keep tissues from both the target and related species is often difficult (Rodgers, 2017). The extreme sensitivity of qPCR means that even single copies of target species DNA could lead to a false positive amplification if present in a non-target specimen. Typical collection, storage, and laboratory protocols are generally not designed with this level of sensitivity in mind and repositories generally do not have protocols in place to ensure high levels of separation between samples and/or DNA from different species. In addition, low level contamination originally confounded results of mitochondrial sequencing of the COI barcoding gene region of target specimens in this study. Specimens identified in the field as EPFU were identified as *Myotis* sp. via sanger sequencing of COI using the Cox F4 and Cox R5 primer combination. However, on in silico inspection we found numerous base-pair mismatches in the forward primer sequence when aligned with EPFU sequences, and fewer mismatches to *Myotis* sp. As a result, the primers had a higher affinity for *Myotis* sp. than EPFU and preferentially amplified contaminating DNA.

To navigate this highly contaminated environment for qPCR assay development, we used synthetic sequences to test whether cross-amplification occurred due to true presence of the target sequence in the homologous region of the non-target genomes or if the signal was a false detection associated with contamination (Carim et al., 2016b). We believe that use of synthetic sequences to check for cross amplification when clean tissues are unavailable is a valid approach to support specificity under these circumstances. The lack of cross-amplification with the synthetic sequences coupled with the high number of mismatches associated with those non-target species that cross-amplified, indicated that cross amplification was due to contamination of target tissue rather than a lack of assay specificity. This conclusion was supported by amplicon sequence data from qPCR products generated by cross-amplification in non-target samples with the EPFU assays, all of which were EPFU. Further, when using synthetic sequences to test assay specificity there is a small chance of missing a matching sequence that occurs elsewhere in the genome of the non-target species. To explore this possibility, we analyzed a complete *M. lucifugus* whole genome sequence and confirmed that it would be unlikely for areas of the complete genome to cross amplify because of the overall high mismatches in either the 5′ and the 3′ ends of the assay components.

The assays detected eDNA of big brown bat with limited success during both the active and the inactive season, even when bats were present. Existing approaches to assess bat occupancy and abundance require an understanding of when and where bats concentrate in their environment, e.g., by placing acoustic monitoring equipment at water sources during their peak hours of activity in the summer. We found that when we applied the assays with this same strategy during active season water sampling, we were able to detect EPFU in a stream (sample HGCT4) during nightly emergence. Paired mist netting indicated that EPFU were present around the time of sampling, as determined by a physical capture downstream within 12 min of the collection of the positive water sample. While this data point is promising, the strength of the detection was weak and occurred in only one of three replicate assays. Improved placement of water sampling sites, e.g., at or just downstream from the pools where bats are observed skimming the

water, is likely to improve the success of this application.

The failure to detect EPFU in stream samples collected from under and downstream from torpid EPFU aggregations roosting above the water during winter sampling was troubling. Given the large concentration of individuals and the presumed duration of their occupancy, we anticipated that their DNA would be deposited in the environment. Indeed it was, and EPFU DNA was detected in a sediment sample at this site, consistent with another study which previously documented bat DNA in sediment (Walker et al., 2019). The failure to detect EPFU in water samples at this site may be due to the short residence time of water in lotic systems. Given a point source of DNA and, the brief time period captured by our scooping sampling method (probably less than 30 s), this is likely insufficient for DNA deposition when bats are dormant.

A framework already exists to monitor bat presence and abundance over time and in response to acute stressors like WNS using colony counts, acoustic protocols, capture and other approaches (NABat; North American Bat Monitoring Program; Loeb et al., 2015). While these approaches are effective, they have limitations including some risk to the safety of personnel and the bats under study. The suspension of activities because of the perceived threat of bidirectional transmission of SARS-CoV-2 between humans and North American bats has elevated the need for non-invasive methods to monitor bat populations. eDNA sampling is simple, does not require advanced technical skills for identification, and decreases risk to bats and humans while providing high levels of specificity for species identification.

Results from our study indicate that detecting bats from air and water is feasible. We envision that with future research and development focused on field application it could be used to determine occupancy and or species composition in confined spaces (e.g. caves, attics, snags), at bridges, culverts, and talus, and at any water body accessed by bats for food or drinking. Future efforts should focus on developing robust field protocols for reliable detection of target bat species on the landscape, improving safety for both bats and humans.

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CRediT authorship contribution statement

Natasha R. Serrao contributed to the methodology, project administration, investigation, validation, and writing. Julie B. Weckworth contributed to the methodology, project administration, investigation, validation, and writing. Kevin S. McKelvey contributed to the methodology, validation, and writing. Joseph C. Dysthe contributed to the methodology, investigation, validation. Michael K. Schwartz contributed to the investigation, methodology, project administration, writing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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