Development of a Bat Guano and Acoustic Sampling Testing Protocol to Identify Species Occupying VDOT Bridges

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Over half of Virginia's extant bat species, including six imperiled bat species, have been documented as using bridges as day or night roosts. To prevent or minimize harm to these species, the Virginia Department of Transportation (VDOT) performs surveys to detect bat use in transportation structures when essential infrastructure maintenance must occur. The current indicators used by VDOT to inspect bridges for bat use include staining, guano piles, and the presence of live bats. Notwithstanding, without trained personnel, it can be difficult to positively identify species use. Without the ability to confidently identify roosting species, regulatory agencies cannot discount transportation structure use by a sensitive species, such as the federally endangered gray bat (*Myotis grisescens*).

Most acoustic detection survey methods allow for the identification of bat species active in an area but cannot confirm that a species is using a transportation structure as a day or night roost with certainty. Because acoustic detection methods are relatively inexpensive and not labor intensive, they can be used to monitor areas for an extended period to determine the likelihood of bat presence and provide region-wide predictive modeling to assess potential risk. However, acoustic monitoring alone cannot confirm roost use, so a subsequent, genetics-based approach could confirm which species have roosted in a structure.

In this project, we tested a combined approach using intensive acoustics in conjunction with DNA barcoding of guano found at the sampled bridges to detect the species present in the areas around 40 bridges in southwestern Virginia's Bristol District. From March to November 2019, we observed bat activity with acoustic sampling throughout the Clinch, Powell, Holston, Big Sandy, and New River watersheds. Gray bat activity at the bridges was correlated with proximity to the known summer maternity roost in the Bristol area and mean cave density in the surrounding landscape. Combined with pilot acoustic data from 2018 and a partial continuation in 2020, we observed high year-to-year variations in gray bat activity. We found that a long acoustic sampling duration is necessary to discern the monthly presence and relative abundance patterns of imperiled bat species (focusing on the six imperiled species documented as using bridges) across the year from emergence to the initiation of hibernation. The spatiotemporal patterns that we observed can help VDOT assess the risk to gray bats and other bat species from transportation structure management activities.

In total, 283 guano samples were collected from 29 bridges for subsequent DNA analysis. Although 245 of the samples were amplified, only 77 (27% of the collected samples) were of sufficient quality to find a species match. Nine bridges had guano with DNA that matched big brown bats (*Eptesicus fuscus*), 12 bridges had guano matching gray bats, and three bridges had guano matching the federally threatened northern long-eared bat (*Myotis septentrionalis*). The bat species at all the sites with guano-derived DNA were also recorded acoustically. For guano DNA analysis, additional work-refining techniques will be needed; however, as proof of concept, the combined approach to bat sampling that we developed may aid VDOT managers in assessing bat use of bridges, which is particularly valuable in areas such as the New River drainage into which the gray bat population is newly expanding.

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ABSTRACT

Over half of Virginia's extant bat species, including six imperiled bat species, have been documented as using bridges as day or night roosts. To prevent or minimize harm to these species, the Virginia Department of Transportation (VDOT) performs surveys to detect bat use in transportation structures when essential infrastructure maintenance must occur. The current indicators used by VDOT to inspect bridges for bat use include staining, guano piles, and the presence of live bats. Notwithstanding those practices, it can be difficult to positively identify species use without trained personnel,. Without the ability to confidently identify roosting species, regulatory agencies cannot discount transportation structure use by a sensitive species, such as the federally endangered gray bat (*Myotis grisescens*).

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In this project, we tested a combined approach using intensive acoustics in conjunction with DNA barcoding of guano found at the sampled bridges to detect the species present in the areas around 40 bridges in southwestern Virginia's Bristol District. From March to November 2019, we observed bat activity with acoustic sampling throughout the Clinch, Powell, Holston, Big Sandy, and New River watersheds. Gray bat activity at the bridges was correlated with proximity to the known summer maternity roost in the Bristol area and mean cave density in the surrounding landscape. Combined with pilot acoustic data from 2018 and a partial continuation of this data collection in 2020, we observed high year-to-year variations in gray bat activity. We found that a long acoustic sampling duration is necessary to discern the monthly presence and relative abundance patterns of imperiled bat species (focusing on the six imperiled species documented as using bridges) over the year from emergence to the initiation of hibernation. The spatiotemporal patterns that we observed with acoustics can help VDOT assess the risk to gray bats and other bat species from transportation structure management activities.

In total, 283 guano samples were collected from 29 bridges for subsequent DNA analysis. Although 245 of the samples were amplified, only 77 (27% of the collected samples) were of sufficient quality to find a species match. Nine bridges had guano with DNA that matched big brown bats (*Eptesicus fuscus*), 12 bridges had guano matching gray bats, and three bridges had guano matching the federally threatened northern long-eared bat (*Myotis septentrionalis*). The bat species at all the sites with guano-derived DNA were also recorded acoustically. For guano DNA analysis, additional work refining techniques will be needed. As proof of concept, however, the combined approach to bat sampling that we developed may aid VDOT managers in assessing bat use of bridges, which is particularly valuable in areas into which the gray bat population is newly expanding, such as the New River drainage.

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INTRODUCTION

Bats are a diverse group of mammals and an integral component of Virginia's biodiversity, providing important ecosystem services such as agricultural and forest insect pest control and terrestrial and aquatic nutrient cycling (Kunz et al., 2011). In temperate zones, most bat species hibernate during winter or undertake long seasonal migrations to warmer regions and have low fecundity (e.g., one to two offspring per annual reproductive cycle) and low annual recruitment, which results in high sensitivity to summer day roosting and foraging habitat loss or disturbance (Silvis et al., 2016). At present, several of Virginia's cave-hibernating bats are being threatened with widespread extirpation from the impacts of a fungal disease that causes white-nose syndrome (WNS), whereas migratory bats are vulnerable to mortality associated with wind energy development (Powers et al., 2015; True et al., 2021). Four bat species in Virginia have a listed status under the Endangered Species Act: the endangered Indiana bat (*Myotis sodalis*), the endangered gray bat (*Myotis grisescens*), the endangered Virginia big-eared bat (*Corynorhinus townsendii virginianus*), and the threatened northern long-eared bat (*Myotis septentrionalis;* Powers et al., 2015; Reynolds et al., 2016). Moreover, the formerly abundant little brown bat (*Myotis lucifugus*) and tricolored bat (*Perimyotis subflavus*) have been petitioned for possible listing and are currently under status review by the U.S. Fish and Wildlife Service (USFWS, 2019, 2020). For listed species that roost during the summer maternity season in forests, such as the Indiana bat and northern long-eared bat, management actions that result in tree/snag removal, such as for forestry, surface mining, and highway construction, may necessitate minimization and mitigation efforts to prevent or reduce take levels (i.e., seasonal clearing restrictions; Ford et al., 2021; Silvis et al., 2016). These species, along with the year-round cave-obligate gray bat, have adapted by using anthropogenic structures such as bridges that mimic suitable tree or cave day roosts (Johnson et al., 2002). Because of this, the Virginia Department of Transportation

(VDOT) regularly inspects transportation structures but particularly before any planned structure maintenance begins to avoid take of protected bats.

Survey measures to help minimize the impact on bats are particularly critical in the Tennessee River Valley drainage of southwestern Virginia, where federally endangered gray bats regularly day and night roost in bridges (C. Kelly, North Carolina Wildlife Resources Commission, personal communication; Johnson et al., 2002; Powers et al., 2016). Other Virginia bats with protected status or of high conservation concern that have been documented as using bridges are the Indiana bat, the northern-long eared bat, the little brown bat, and the tricolored bat. More common species such as Rafinesque's big-eared bat (*Corynorhinus rafinesquii*), the big brown bat (*Eptesicus fuscus*), the southeastern myotis (*Myotis austroriparius*), the eastern small-footed bat (*Myotis leibii*), and the evening bat (*Nycticeius humeralis*) also regularly use transportation structures as roosts in Virginia and elsewhere (Cervone et al., 2016; Geluso et al., 2018; Keeley & Tuttle, 1999). Inspecting a bridge prior to management activities for evidence of bat use (i.e., the presence of guano deposits or structure staining) does not provide the species-specific confirmation necessary to fully facilitate planning to minimize potential impacts. As such, regulatory agencies such as the USFWS and Virginia Department of Wildlife Resources (VDWR) cannot discount bridge use by a listed species if the transportation structure occurs within a species' known distribution.

Prior to the advent of WNS, the presence of listed species was generally determined through mist-net captures at or adjacent to project areas. However, with the severe population decline of many species and subsequent low detection probability when using live-capture techniques (Deeley et al., 2021a; Nocera et al., 2019a), the USFWS has developed acoustic monitoring protocols for the Indiana bat and northern long-eared bat to guide regulatory assessment (Niver et al., 2014). Acoustic monitoring protocols provide ways to identify and document the foraging activities of bat species and offer cost-effective methods to determine local bat species occupancy (Ford et al., 2005, 2016). With long-term passive acoustic sampling in multiple locations, habitat use and seasonal presence patterns are often apparent, showing how activity varies in space and time (Gorman et al., in review; Nocera et al., 2020).

Preliminary work in the Clinch–Powell system in southwestern Virginia from 2018 suggested that most gray bat activity, and presumably actual abundance, is concentrated within the Clinch and Powell watersheds near the Tennessee state line in proximity to a known hibernaculum and maternity roost in the city of Bristol on the Virginia–Tennessee line. However, by midsummer, gray bat presence becomes widely dispersed within the drainage area before declining through the fall as bats begin moving southwards toward their putative winter hibernaculum in Hawkins County, Tennessee. Acoustic monitoring and subsequent live captures have shown that during mid and late summer, there is significant presence of gray bats in the adjacent New River drainage area to the northeast of the Clinch–Powell system. Acoustic monitoring obviously provides evidence of local presence, but it can also assist with broader determinations of landscape-level distribution and abundance, allowing managers to assess risk both spatially and temporally (Barr et al., 2021). Johnson et al. (2010a, b) demonstrated the value of acoustic monitoring for determining gray bat activity patterns relative to streams and the characteristics of the surrounding landscape within the species' historical distribution as well as within the "new" expanded range in northwest Georgia. Unfortunately, because software identifying bat species from acoustic monitoring is based on search-phase (foraging) echolocation pulses as the diagnostic character, this method cannot be used to determine

conclusively whether either day or night roosting by any particular species occurred at a transportation structure (Britzke et al., 2002).

Advances in species identification through DNA-based analysis of deposited guano can definitively document species-specific use of transportation structures, such as day or night roosts, with a high rate of correct classification (>90%; Walker et al., 2016). The application of DNA barcoding utilizes a taxonomic database of species-specific DNA sequences (Hebert et al., 2003; Kress & Erickson, 2012) as a reference for the identification and analysis of individuals of unknown taxonomic affinity (Ivanova et al., 2007). The genetic identification of vertebrates is best accomplished through the amplification and sequencing of the cytochrome oxidase I – subunit 3 (*COI-3*) gene of mitochondrial DNA (Ivanova et al., 2007; Weigt et al., 2012). Walker et al. (2016) developed an order-wide DNA mini-barcode assay targeting fecal samples based on *COI* variations, which proved highly discriminatory within the bat order Chiroptera (approximately 92% species-level identification of barcoded species). Using *COI* sequences mined from GenBank and new sequences from collected samples, Korstian et al. (2016) tested the use of the *COI* locus for the DNA barcoding of many bat species found in the United States and found that 80% of the species examined had distinct barcodes, including those that are of high conservation concern in Virginia (i.e., the little brown bat, northern long-eared bat, and tricolored bat). Brown et al. (2017) screened sequences from the mitochondrial *16S* ribosomal subunit to create a DNA sequence database for the 16 bat species known to occur in neighboring Tennessee and demonstrated the usefulness of this barcoding gene to identify bats such as the little brown bat and northern long-eared bat from their guano.

Similar to acoustics however, species determination from DNA analysis has limitations. Whereas guano testing can confirm the use of a transportation structure, in most instances it cannot determine when a bat used the site as a roost. Accordingly, the development of a monitoring approach that combines acoustic monitoring to determine temporal as well as spatial landscape-level presence with DNA assays of guano to determine roost use could provide transportation managers with a complementary, two-phase assessment technique to gauge risk. Operationally, the deployment of acoustics combined with guano collection may reduce the need for exhaustive transportation structure examinations and may provide sufficient information to inform regulatory agencies, especially where the combined techniques show that the presence of bats is unlikely.

PURPOSE AND SCOPE

The aim for our project was to test a protocol for detecting bat species roosting in bridges using a combination of long-term acoustic monitoring and DNA barcoding of guano found at bridge sites in southwestern Virginia where the presence of gray bats was highly likely. Specifically, we sought: (1) to assess the accuracy of a DNA-based identification protocol from guano using ongoing watershed-level bat acoustic surveys or mist-netting as a baseline comparison; (2) to correlate bat species occupancy (with an emphasis on gray bats) as derived from guano assessments with relative activity observed using acoustic monitoring at each site; and (3) to develop models of multi-bat species presence/predicted probability of occurrence relative to day of year, bridge type, riparian characteristics, and surrounding landscape metrics singularly with DNA results and acoustic results and in combination with both techniques within and adjacent to the distribution of the gray bat. VDOT will use these findings and, in

coordination with the USFWS, refine these protocols as a means of verifying the presence/absence of protected bat species at VDOT structures.

METHODS

Bridge Selection

For acoustic and guano sampling, we selected bridges within the Tennessee River Valley watersheds (Clinch, Powell, and Holston watersheds) along with portions of the adjacent New River and Big Sandy watersheds in Virginia where gray bat as well as other imperiled bat species occurrences are known or suspected on the basis of VDWR, USFWS, VDOT, and Virginia Tech records. We included a variety of bridges representing common structure types and materials as well as locations that encompassed the length and width of the sampled watersheds across a range of stream-order and landscape configurations (i.e., forested, mixed, agricultural, and developed) that have been shown to influence bat presence and activity (Johnson et al., 2008). Information about each potential bridge was supplied by VDOT, and additional supplementary information for selected bridges was acquired from VDOT (VDOT, 2019).

To maximize opportunities for retrieving bat guano, we prioritized the selection of bridges where signs of bat use had been found during past inspections, whereas bridges with no previous reports of bat use were chosen to represent areas and other variables important to our acoustic monitoring, such as bridge type and landscape configuration. We identified a total of 41 bridges within VDOT's Bristol District in southwestern Virginia in 2019 (*Figure 1*). We chose to repeat the pilot work from 2018 acoustic sampling at 12 bridges in the Clinch and Powell watersheds and added one bridge in the upper Clinch River watershed, six bridges in the Powell River watershed, eight bridges in the Holston River watershed, five in the Big Sandy watershed, and nine bridges in the New River watershed.
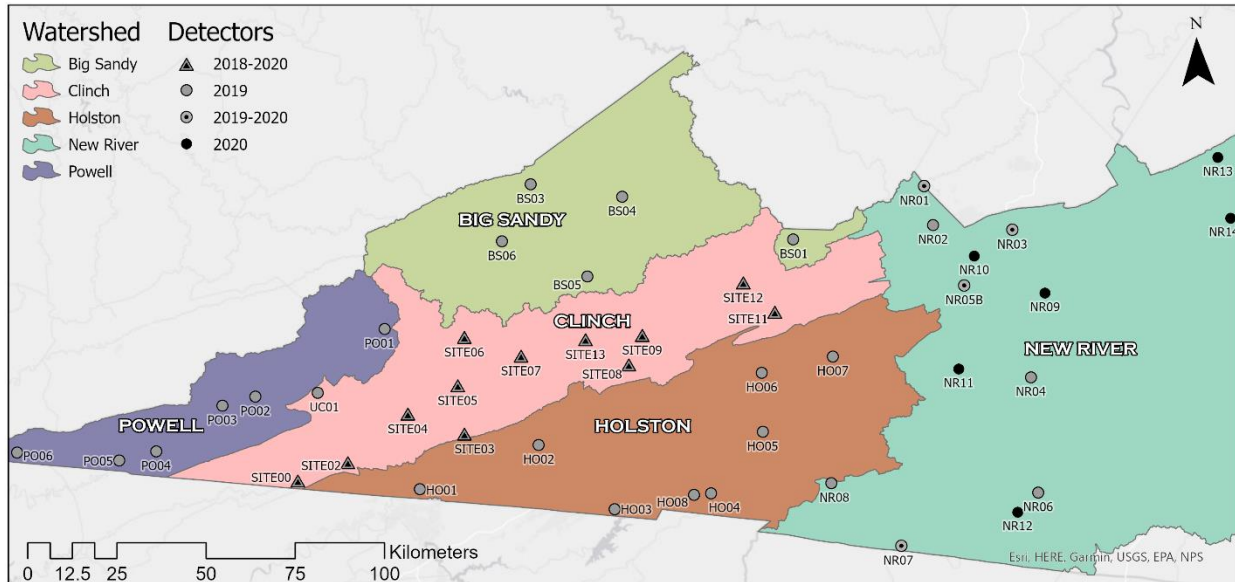
Figure 1. Locations of the selected bridge sites for the 2018–2020 acoustic detector deployments over five watersheds in VDOT's Bristol District, Virginia

Acoustic Sampling

At each selected bridge, we placed one Wildlife Acoustics SM-4 zero-crossing/frequency division detector with an SMM-U1 omni-directional microphone (Wildlife Acoustics, Maynard, MA) pointed along the stream, in line with the methods described by Austin et al. (2018) and Coleman et al. (2014), to continuously record bat activity between March and November 2019. Because bridge use can occur throughout the March–November period when bats are present on the landscape, we exceeded the normal time frame (eight to nine nights) currently required for an acoustic level of effort with a 95% probability of detection of threatened and endangered bats in eastern North America (Niver et al., 2014). The only exceptions were five sites in 2020 where we deployed Wildlife Acoustics SM-4 full-spectrum version detectors provided by the Tennessee Valley Authority for use in the region (SITE00, SITE02, SITE03, SITE04, and SITE05; *Figure 1*). Microphones were mounted external to the detector and elevated above the vegetation clutter on 3-m telescoping poles. The call files were then processed through the USFWS and U.S. Geological Survey-approved Kaleidoscope 5.1 (Wildlife Acoustics, Maynard, MA) for nightly species-specific identification, which was set to match the species likely to be found in the area: the Virginia big-eared bat, the big brown bat, the eastern red bat (*Lasiurus borealis*), the hoary bat (*Lasiurus cinereus*), the silver-haired bat (*Lasionycteris noctivagans*), the gray bat, the eastern small-footed bat, the little brown bat, the northern long-eared bat, the Indiana bat, and the tricolored bat. All the call files identified to the species, irrespective of the nightly maximum likelihood estimator scores, were retained for the watershed-wide monthly activity level modeling (Nocera et al., 2020).

Bridge Inspections for Bat Use

Each bridge that we sampled was inspected as completely as safety would allow. Due to examinations being performed mostly without the aid of specialized equipment, portions of the larger bridges, such as the central I-beams and the underside of the expansion joints, were not

accessible for thorough inspection. During inspection, we followed the bridge and culvert bat-use survey methods provided by the Georgia Department of Transportation and Georgia's Department of Natural Resources (2018). At the bridges, we searched for visible staining and the presence of guano. We also inspected crevices with lights and borescope cameras for extant bats and documented audible bat vocalizations.

Guano Collection

When guano was observed and accessible during our inspections, we collected guano pellets into 1.5-mL collection tubes with nitrile-gloved hands. To avoid cross-contamination, we changed gloves between sample collections if contact was made with a pellet. If isolated pellets were observed, we put them into individual collection tubes; when sampling guano pellets from larger piles, we placed multiple pellets in the same tube, prioritizing pellets from the top of the pile to collect the most recently deposited feces. We then added 95% ethanol to each collection tube and subsequently froze the samples prior to DNA extraction. Although we prioritized fresh guano that did not appear desiccated, we had no way of estimating guano freshness/age with high certainty.

Genetic Processing

We performed DNA extraction from the collected guano using the QIAmp DNA Stool Mini Kit (Qiagen, Valencia, CA) in line with the manufacturer's protocols. We used polymerase chain reaction (PCR) methods to amplify the extracted DNA using the *Mysp* 1 and *Mysp* 2 primers from the *16S* ribosomal subunit (Brown et al., 2017). We used a 22-µL reaction consisting of 5× PCR Platinum^{TM} buffer, 25-mM $MgCl_2$, 2.5-mM deoxynucleotide triphosphate blend, 2 U Platinum *Taq* polymerase^{TM} (Thermo Fisher Scientific, Waltham, MA), 1-µg bovine serum albumin, 5-µM *Mysp* 1 and 5-µM *Mysp* 2 (Integrated DNA Technologies, Coralville, IA; Brown et al. 2017), 13.3-µL deionized H_2O, and 2.2-µL extracted guano DNA. The reaction mixture was then placed in a T-100 thermocycler (BioRad, Hercules, CA) to follow the procedure described by Brown et al. (2017), except we ran all the samples with an annealing temperature of 48°C as suggested for samples that do not amplify at higher temperatures. We confirmed DNA amplification by subjecting the PCR products to electrophoresis in a 2% agarose Tris/Borate/Ethylenediaminetetraacetic acid gel. For each amplified DNA sample, we prepared two separate 13-µL reactions for sequencing by combining 4-µL of the amplified DNA and 3-µL of either the forward or reverse primer. The reactions were then sent to Virginia Tech's Fralin Genomics Sequencing Center for bidirectional Sanger sequencing. We then aligned the forward and reverse sequences with one another and assembled the sequences via the De Novo method using Geneious Prime 2021.1 software (Biomatters, Inc., San Diego, CA). To match the assembled samples to the corresponding species, we compared the nucleotide sequences to the records found in the GenBank database using the Basic Local Alignment Search Tool (Altschul et al., 1990; Kress & Erickson, 2012).

Mist-Netting

To confirm the presence of gray bats in the New River watershed and collect fresh guano samples from a known species to use as control for DNA barcoding, we mist-netted in the Clinch River watershed for one night in July 2019 and in the New River watershed for one night in July 2019 and four nights in August 2019. We set double- and triple-high mist nets (Avinet, Inc., Dryden, NY) across streams and trails in locations near our acoustic sites in the New River, which had had the most gray bat activity a month prior to netting, as well as at two sites not directly associated with an acoustic detector but within the watershed (*Figure 2*). The mist nets remained open for three to five hours after sunset. For each captured bat, we recorded the species, life stage (adult or juvenile by degree of epiphyseal fusion), sex, weight, right forearm length, reproductive condition, and evidence of WNS wing damage (Brunet-Rossinni & Wilkinson, 2009; Haarsma, 2008; Reichard & Kunz, 2009). We attached aluminum alloy bands with uniquely serialized identification numbers to the forearms of all the captured big brown bats, tricolored bats, and bats in the genus *Myotis* (right forearms of males and left forearms of females; Porzana, Ltd., Icklesham, UK). We affixed a 0.27-g high-frequency radio transmitter in the 150.000–151.999 MHz range (Holohil Inc., Carp, Canada) between the scapulae of the captured gray bats (up to two bats per site) using Perma-Type© Surgical Cement (Perma-Type Co., Inc., Plainville, CT). We used TRX-2000 radio telemetry receivers and three-element Yagi antennas (Wildlife Materials Inc., Murphysboro, IL) to track the bats to their day roosts. The tracking was conducted after sunrise and continued for up to five days or until the roost was found. Once a roost was located, we documented the roost type and recorded the GPS coordinates.
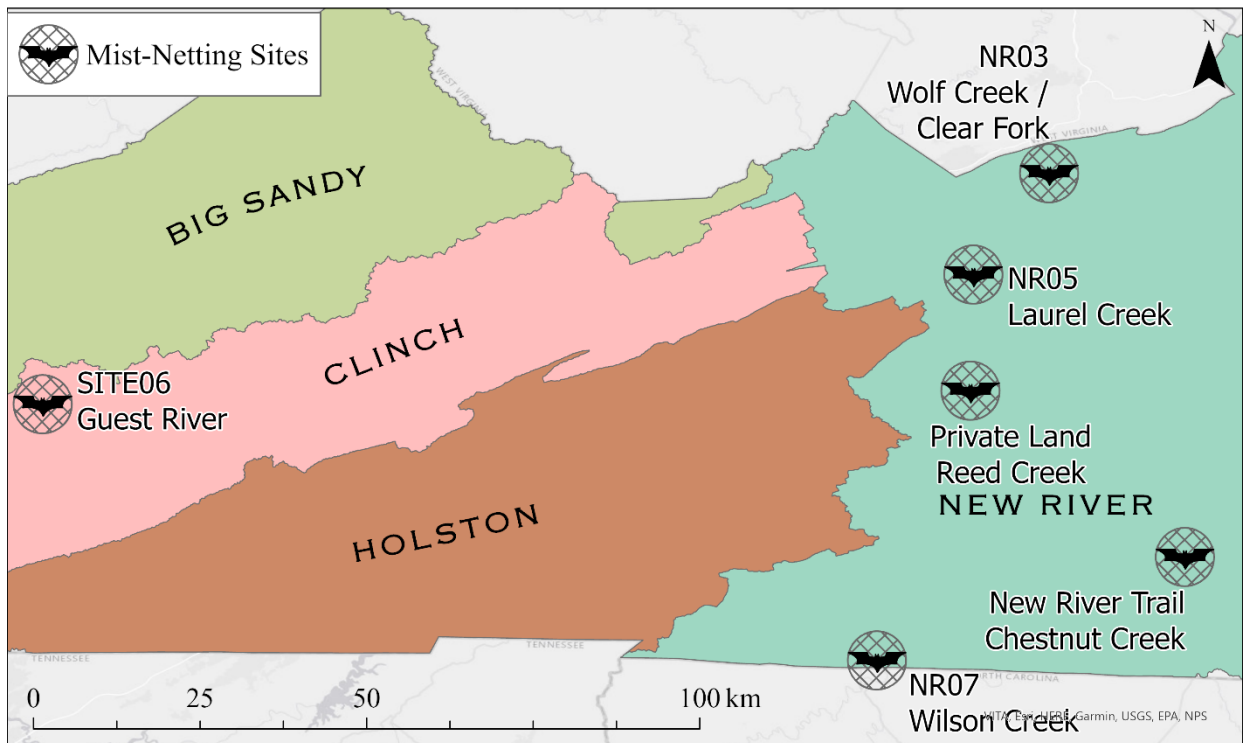
Figure 2. Bat mist-netting sites in VDOT's Bristol District, Virginia, in 2019

Statistical Analyses

Generalized Linear Mixed Model: 2019 Gray Bat Activity

We used nightly summary outputs of the call files classified as gray bats by the program Kaleidoscope 5.1 (Wildlife Acoustics, Inc., Maynard, MA) from our call dataset of nine months in 2019 at 40 bridges (without filtering the call files by maximum likelihood estimator). We used the sum of the nightly gray bat call files as our response variable and 18 candidate predictor variables, which were derived from either the landscape or time of year (*Table A1*). We checked for correlations between the candidate predictor variables using the corrplot package in program R and determined any variable correlations $<|0.6|$ as acceptable (Wei & Simko, 2021). Variable pairs that exceeded the limit bounds were not used together in any of the candidate models. To help the models run smoothly, certain variables (variables representing distances and mean cave density) were centered and scaled using the scale function in base R, which subtracts the mean of the values from each value and divides the result by the standard deviation of the values. For all the candidate models created, we used the detector "site" as a random variable. We created a total of 30 generalized linear mixed models (*Table A2*) comprising single variable models, several a priori variable combination models, and a null model using the package lme4 in program R (Bates et al., 2015). We used the package AICcmodavg in R to rank the candidate models with Akaike information criterion (AIC), and we considered models within 2 ΔAICc units (i.e., closely ranked models) as competing models (Jorge et al., 2021; Mazerolle, 2020), which therefore accounted for response variable performance that had potential biological merit (Burnham & Anderson, 2002, 2004).

Occupancy Analysis Using Guano Sample Identification

For each bat species that we identified from guano, we used the presence (1) or absence (0) of the species at a bridge as determined by the DNA barcoding of the guano to assess the occupancy probability using the same landscape metrics previously mentioned but with the addition of bridge-specific metrics, including bridge width and length (m), average daily traffic, bridge type (parallel box beam, steel I-beam, pre-stressed girder, flat slab/box, cast-in-place, or trapezoidal box), and bridge underdeck material (concrete or corrugated steel). As a way of testing for potential competitive exclusion, we also added the presence of a heterospecific roosting species (i.e., gray bats for big brown bat occupancy models and vice versa) as a variable; their presence was confirmed either with DNA barcoding or visual observation during inspections. Because the time of presence cannot be accurately inferred from guano, we could not use any observation-level variables related to the date or weather. Instead, we used the percentage of high-quality bases in each DNA sequence to predict detection probability. Each set of candidate occupancy models was created with the occu function in R package unmarked and included single-variable models, several a priori variable combination models, and a null model (Fiske & Chandler, 2011). We used the package AICcmodavg in R to rank candidate models with AIC and considered models within two ΔAICc units as competing.

RESULTS AND DISCUSSION

Acoustics

In a previous study, we recorded bat activity between April 3 and November 3, 2018, at 12 sites in the Clinch River watershed within VDOT's Bristol District for a total of 2,483 nights of sampling effort (K. Powers, Radford University, unpublished data). Between March 10 and November 8, 2019, we recorded bat activity at 41 bridges in four watersheds within VDOT's Bristol District for a total of 8,619 nights of sampling effort. The Holston, Powell, and Big Sandy watersheds were sampled only in 2019. In 2020, we recorded bat activity between March 6 and November 19 at 12 sites in the Clinch River watershed and nine sites in the New River watershed (four repeated from 2019 and six additional sites) for a total of 5,017 nights of sampling effort. The call files from these 16,119 nights of sampling were classified by Kaleidoscope 5.1 software as "Noise," "No ID," or as one of the 12 bat species expected to occur in southwestern Virginia.

Of the species of high conservation concern, the relative activity of the Virginia big-eared bat (average number of calls recorded per night; *Figure 3*) was very low throughout all the watersheds in all three years. In 2018, activity in the Clinch River watershed peaked in May, whereas in 2019 and 2020, it peaked in July. In the New River watershed, the sites that were monitored only in 2019 had very low average activity that peaked in May. In the 2019 sites that we repeated in 2020, activity peaked in July 2019 and April 2020 and was consistently higher each month in 2019 compared to 2020, except for October 2020. The New River sites that were monitored only in 2020 had similar levels of activity, which peaked in July and August. In 2019, the highest mean activity of Virginia big-eared bats occurred in June in the Powell River watershed and remained at a similar level in July but dropped precipitously in August. Similar activity levels were detected in the Big Sandy watershed in 2019, with activity peaks in June. Activity declined thereafter. However, we note that due to the species' very low echolocation call amplitude, the detection probability for Virginia big-eared bats using acoustic methodologies is extremely poor.

As expected for the heavily WNS-impacted *Myotis* species, mean nightly activity was very low. Little brown bat activity (*Figure 4*) in the Clinch River watershed was relatively low across all three years of sampling but peaked in May in 2018 and 2020 and in April in 2019. In 2020, little brown bat activity in the Clinch River watershed was higher than in previous years, and the species remained more active through October than in previous years. In 2019, little brown bats were most active in the Holston River watershed in June, and in the Big Sandy watershed, activity both started and peaked in April. Little brown bats arrived in the Powell River watershed in April in 2019 (rather than March as in the nearby watersheds), and activity peaked in June. The mean nightly activity in the New River watershed was overall higher in 2019 than in 2020 and peaked in March in 2019 compared to the activity peak seen in August in 2020. Activity in the New River sites that were monitored only during 2019 peaked in August, and at those monitored only in 2020, activity peaked in September. Similarly, the mean nightly northern long-eared bat activity (*Figure 5*) was relatively low through all the sampled watersheds and remained near the same levels for all three years of sampling. In the Clinch River watershed, activity peaked in April 2018, July 2019, and October 2020. The mean activity peaked in May 2019 in the Holston River watershed and in July 2019 in the Powell River watershed. Northern long-eared bat activity in the New River watershed sites, where monitoring was repeated in 2019 and 2020, was higher in 2019 and peaked in July 2019. The New River sites that were monitored

only in 2019 had very low levels of activity that peaked in August, whereas the sites added in 2020 had higher levels than most watersheds and an activity peak in June. The Big Sandy watershed in 2019 had the highest mean nightly northern long-eared bat activity, which peaked in June. The mean nightly Indiana bat activity (*Figure 6*) was also relatively low in all the sampled watersheds during all three years of sampling. In the Clinch River watershed, Indiana bat activity peaked in April in 2018 and 2019 and in March in 2020. The mean nightly activity in the Clinch River watershed was highest in 2019, and for all three years, activity dropped significantly by July and August and tapered off further through October. Activity was also slightly higher in 2019 compared to 2020 in all months, except for May in the New River watershed, where it peaked in March in 2019 and May in 2020. The New River sites where monitoring was discontinued after 2019 had low activity levels that peaked in September. The sites added in 2020 had similar levels and peaked in May. In the Big Sandy watershed in 2019, Indiana bats were mostly active in April and had very low activity levels for the remainder of the sampled months.
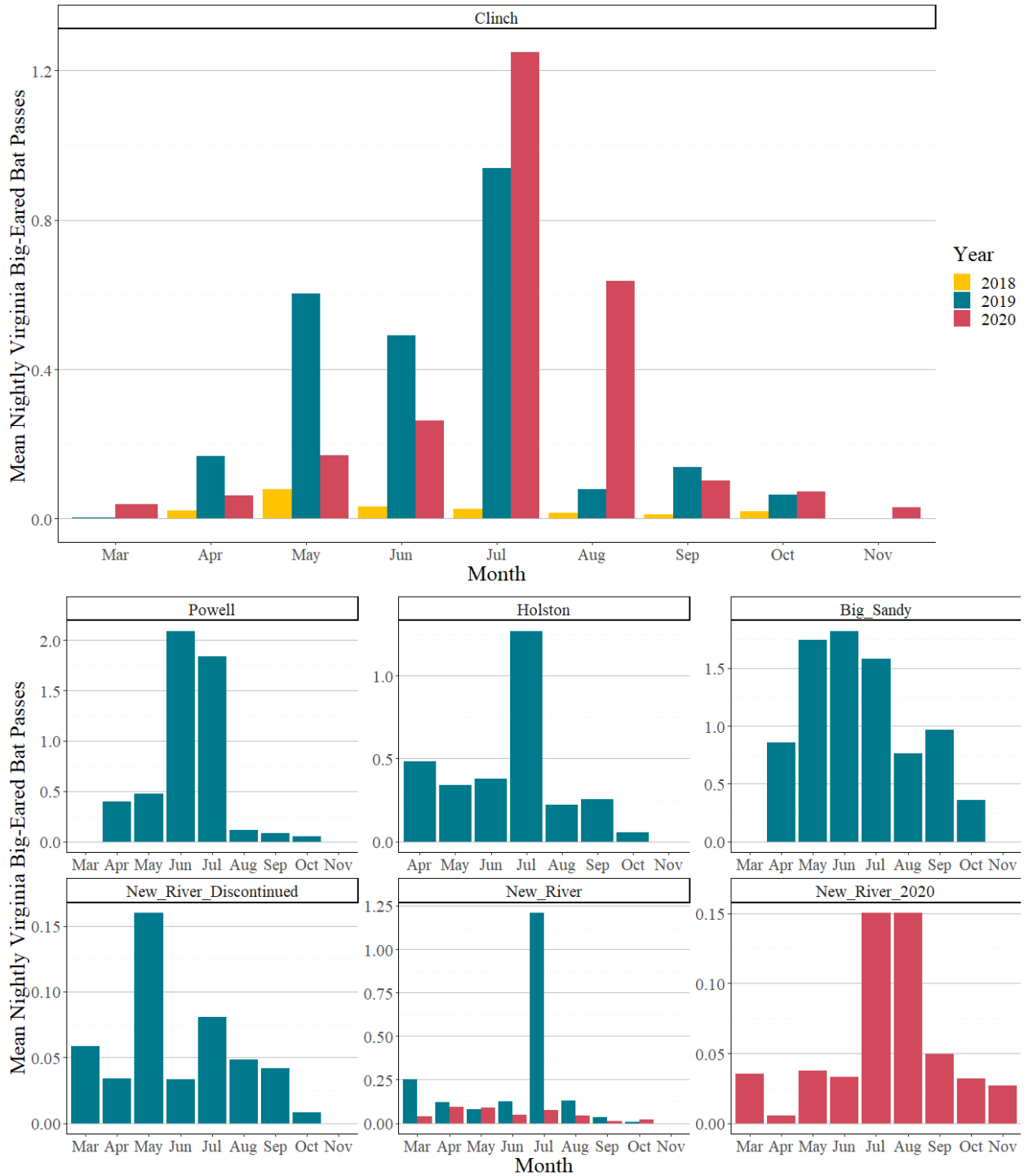
Figure 3. Mean nightly acoustic activity of Virginia big-eared bats (*Corynorhinus townsendii virginianus*) per month and watershed in VDOT's Bristol District, Virginia, in 2018–2020. The sites in the New River Watershed were divided into sites that were monitored only in 2019 ("New_River_Discontinued"), sites that were monitored in 2019 and 2020 ("New River"), and sites that were monitored only in 2020 ("New_River_2020"). Note that the *y*-axis scale is unique for each graph.
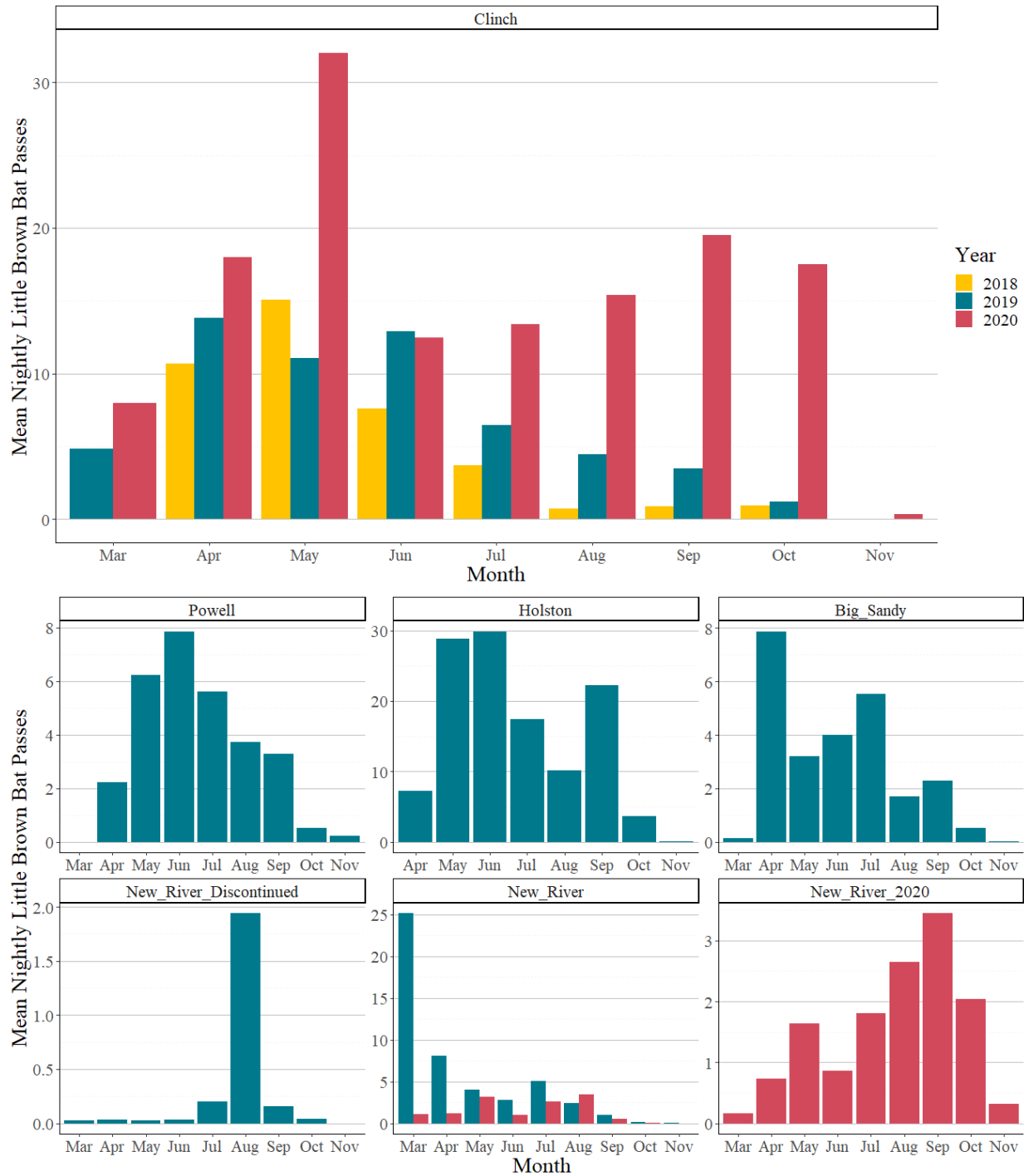
Figure 4. Mean nightly acoustic activity of little brown bats (*Myotis lucifugus*) per month and watershed in VDOT's Bristol District, Virginia, in 2018–2020. The sites in the New River Watershed were divided into sites that were monitored only in 2019 ("New_River_Discontinued"), sites that were monitored in 2019 and 2020 ("New_River"), and sites that were monitored only in 2020 ("New_River_2020"). Note that the *y*-axis scale is unique for each graph.
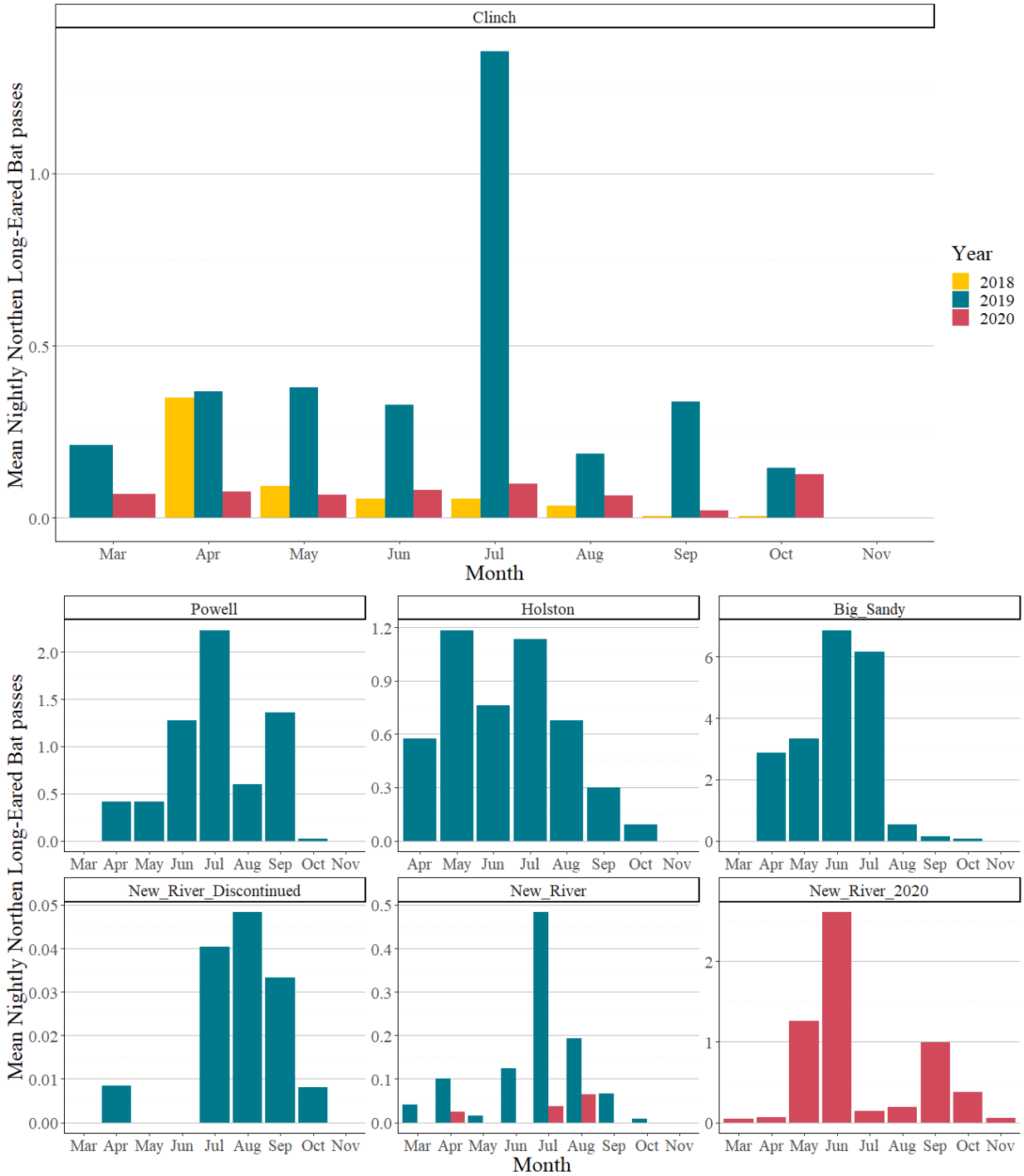
Figure 5. Mean nightly acoustic activity of northern long-eared bats (*Myotis septentrionalis*) per month and watershed in VDOT's Bristol District, Virginia, in 2018–2020. The sites in the New River Watershed were divided into sites that were monitored only in 2019 ("New_River_Discontinued"), sites that were monitored in 2019 and 2020 ("New_River"), and sites that were monitored only in 2020 ("New_River_2020"). Note that the *y*-axis scale is unique for each graph.

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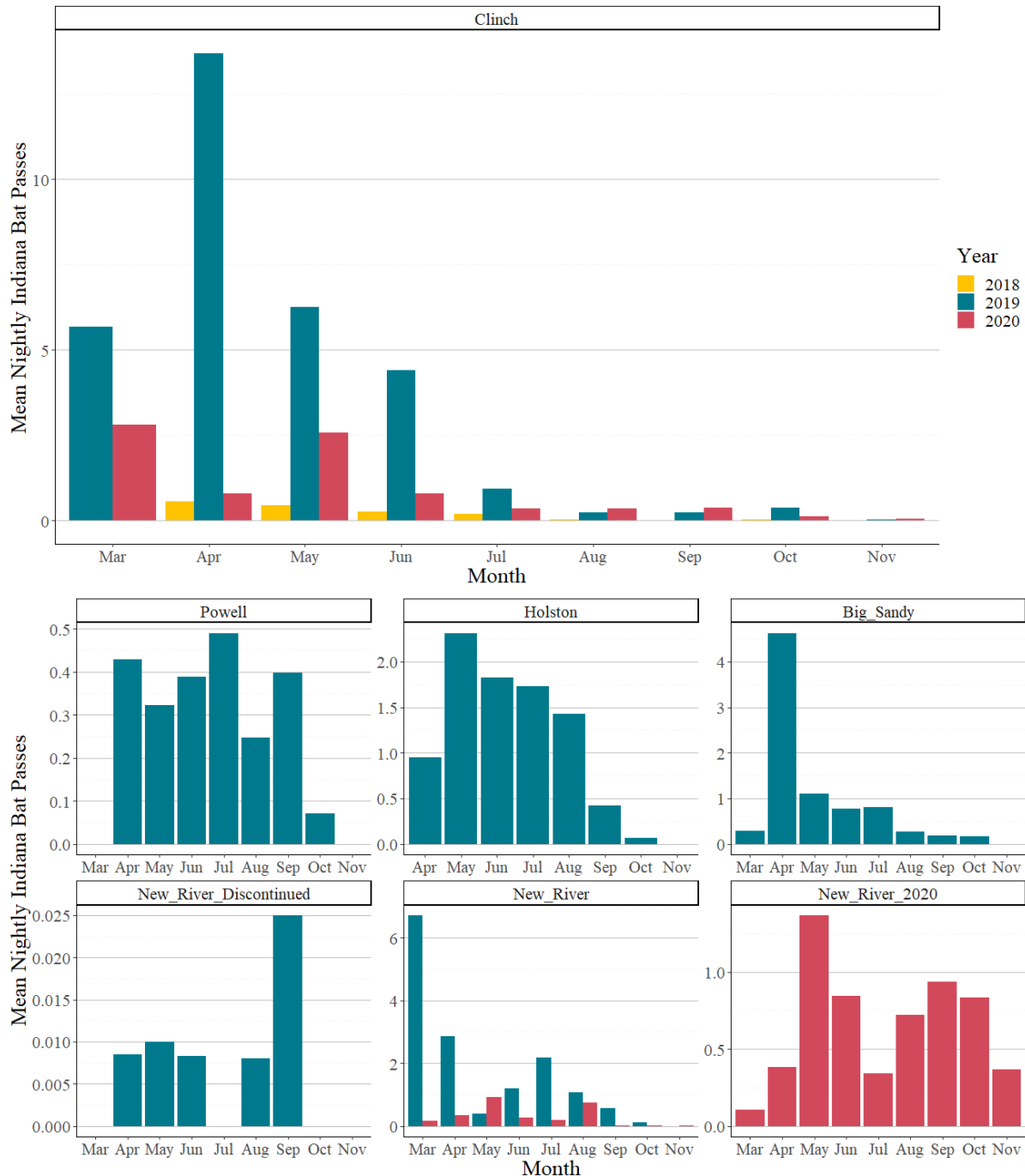
Figure 6. Mean nightly acoustic activity of Indiana bats (*Myotis sodalis*) per month and watershed in VDOT's Bristol District, Virginia, in 2018–2020. The sites in the New River Watershed were divided into sites that were monitored only in 2019 ("New_River_Discontinued"), sites that were monitored in 2019 and 2020 ("New_River"), and sites that were monitored only in 2020 ("New_River_2020"). Note that the *y*-axis scale is unique for each graph.

Despite also being a heavily WNS-impacted species, the mean nightly activity of the tricolored bats (*Figure 7*) in the Clinch River watershed was relatively high in all three years and increased by approximately 41% from 2018 to 2019 and by approximately 71% between 2019 and 2020. Over the whole survey period, tricolored bats had the highest overall mean activity among the imperiled species, particularly in the Clinch River watershed. In the Big Sandy

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watershed in 2019, activity remained somewhat constant after peaking in April and again in August before declining in October. Activity in the Holston River watershed in 2019 was highly ephemeral, with a small peak in April and a large peak in September, perhaps indicating the beginning of fall swarming and migration. In the Powell River watershed, tricolored bat activity peaked in May and remained relatively high in June, before declining until another peak was observed in September.

Figure 7. Mean nightly acoustic activity of tricolored bats (*Perimyotis subflavus*) per month and watershed in VDOT's Bristol District, Virginia, in 2018–2020. The sites in the New River Watershed were divided into sites that were monitored only in 2019 ("New_River_Discontinued"), sites that were monitored in 2019 and 2020 ("New_River"), and sites that were monitored only in 2020 ("New_River_2020"). Note that the *y*-axis scale is unique for each graph.
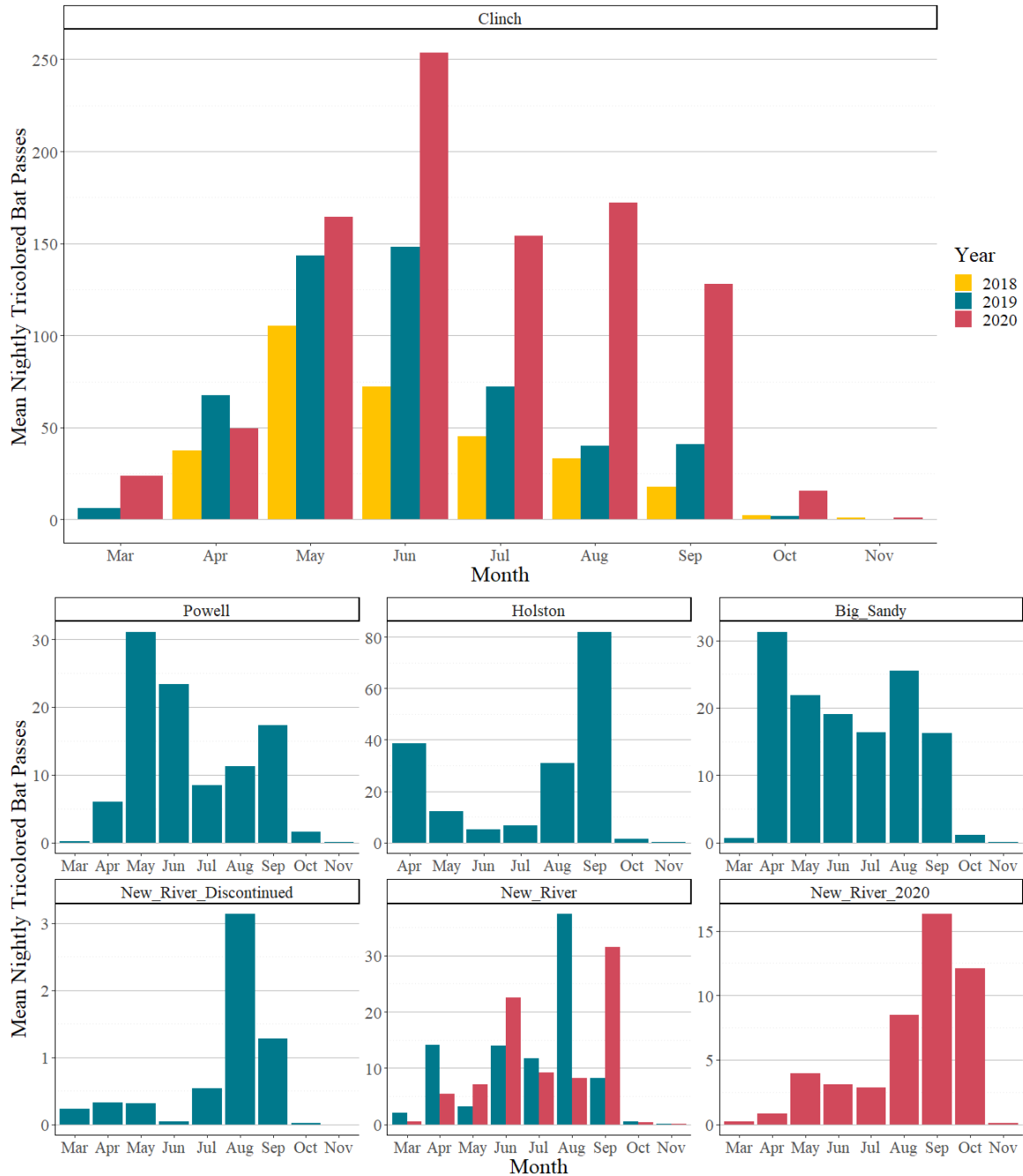
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The gray bat activity levels in the Clinch River watershed had similar temporal patterns to those of the other species but showed an increasing trend over the three survey years (*Figure 8*). The mean nightly gray bat activity was highest in the Clinch River watershed, as expected due to the proximity to the maternity colony in Bristol. It peaked during May in all three years of sampling (*Figure 9*), increasing by approximately 79% between May 2018 and May 2019 and by approximately 76% between May 2019 and May 2020. Activity in the Clinch River watershed peaked again during September in all three years. The peak was most pronounced in 2020 when the mean nightly activity was similar to that in May and June and, unlike in previous years, remained relatively high during October (*Figure 9*). The Powell River watershed had the next-highest gray bat mean nightly activity (*Figure 9*), which also peaked in May, followed by the activity in the Holston River watershed, which peaked in September (both watersheds were sampled only in 2019). Both the New River and the Big Sandy watersheds are on the northern periphery and outside of the gray bat's current distribution, respectively (*Figure 10*; USFWS, 2020). Accordingly, the mean nightly activity within these watersheds was lower than that in the other watersheds sampled. The mean nightly gray bat activity in the Big Sandy watershed (also sampled only in 2019) peaked in June and again in August, whereas in the New River watershed, activity peaked in August in both 2019 and 2020. In the New River sites that we monitored in both years, activity was higher in 2019 compared to 2020, except for September and November 2020. Activity at the 2019-exclusive New River sites peaked in August at very low levels, whereas at our added 2020 sites, gray bat activity peaked in September and had slightly higher levels than the group of New River sites that were monitored in both years.
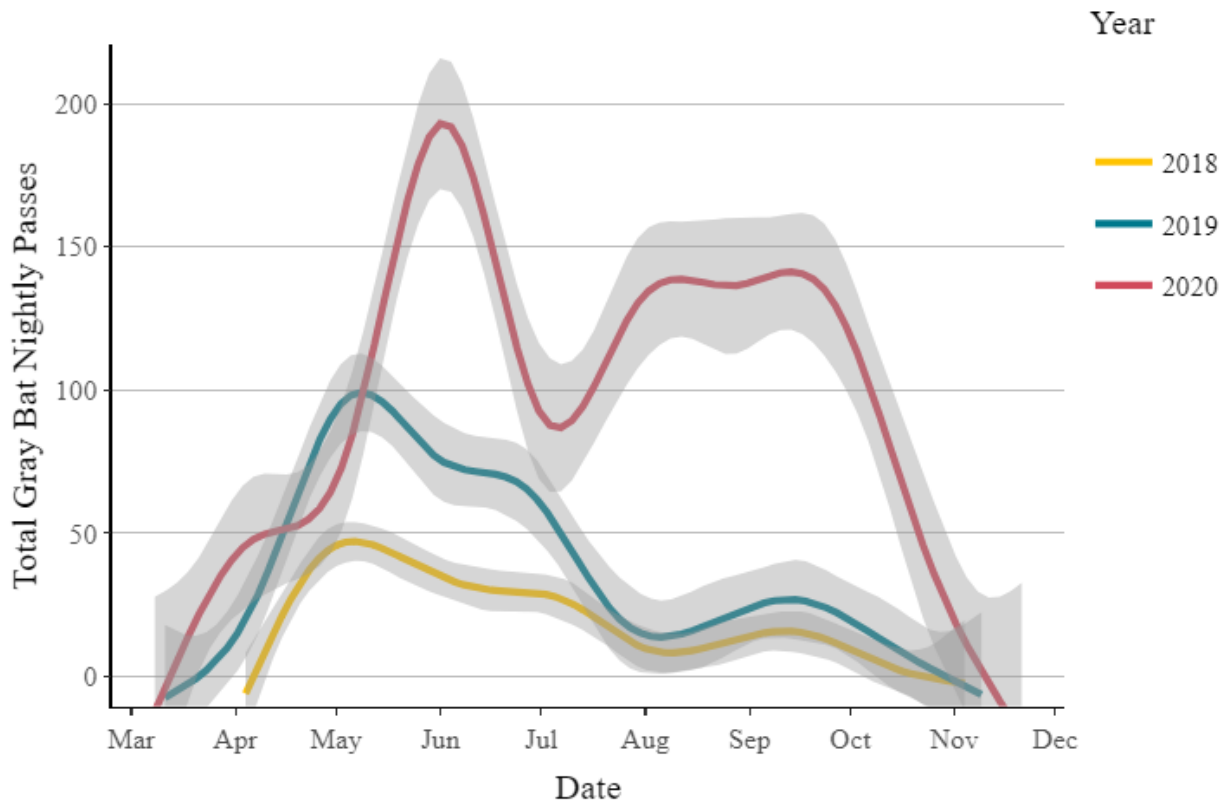
Figure 8. Trend lines showing total nightly gray bat (*Myotis grisescens*) call files and 95% credible intervals from the 12 sites in the Clinch River Watershed in the Bristol District, Virginia, in 2018–2020. Predictive trend lines were generated using a general additive model smoothing method using the package ggplot2 in program R.
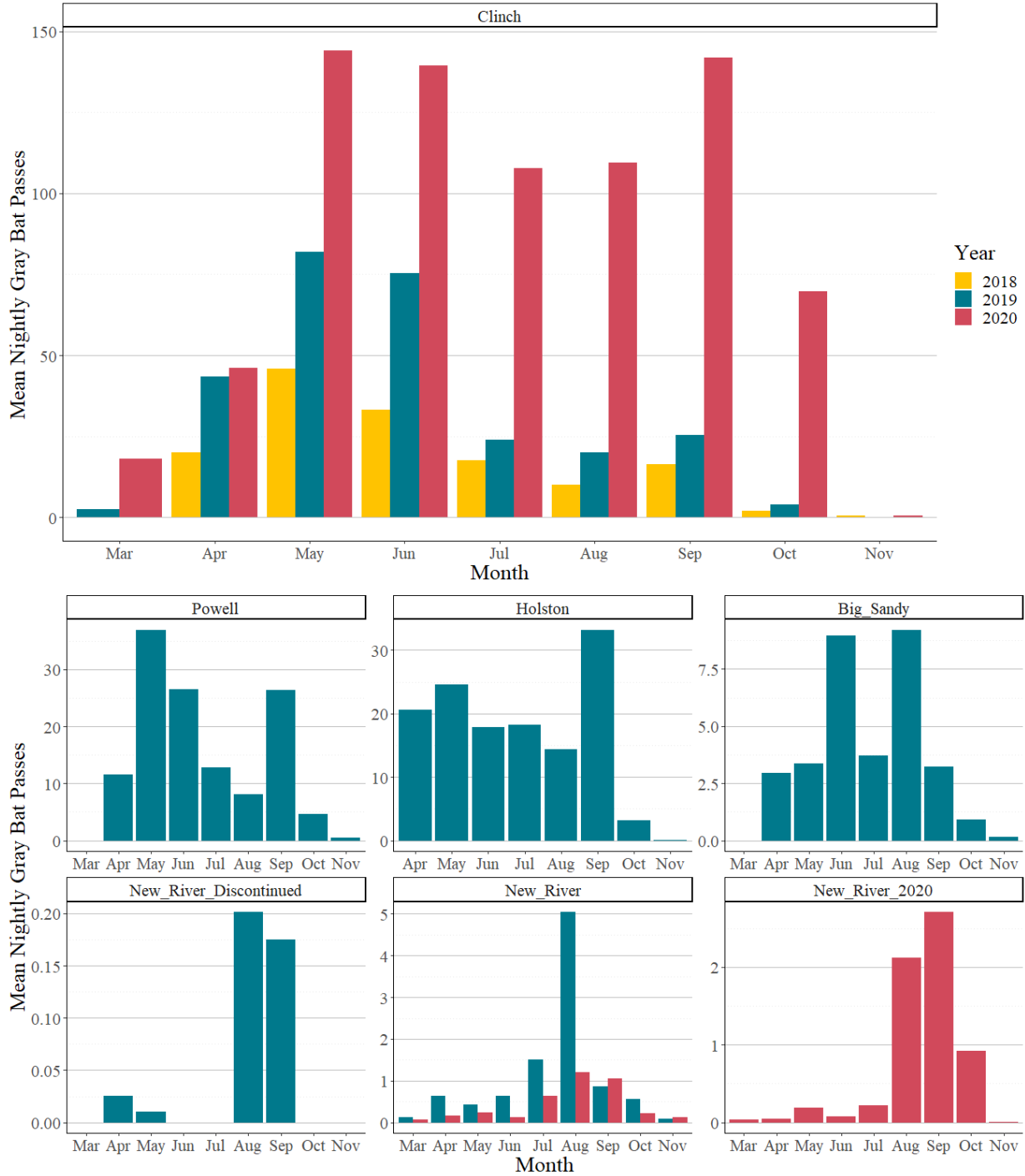
16

Figure 9. Mean nightly acoustic activity of gray bats (*Myotis grisescens*) per month and watershed in VDOT's Bristol District, Virginia, in 2018–2020. The sites in the New River Watershed were divided into sites that were monitored only in 2019 ("New_River_Discontinued"), sites that were monitored in 2019 and 2020 ("New_River"), and that were sites monitored only in 2020 ("New_River_2020"). Note that the *y*-axis scale is unique for each graph.
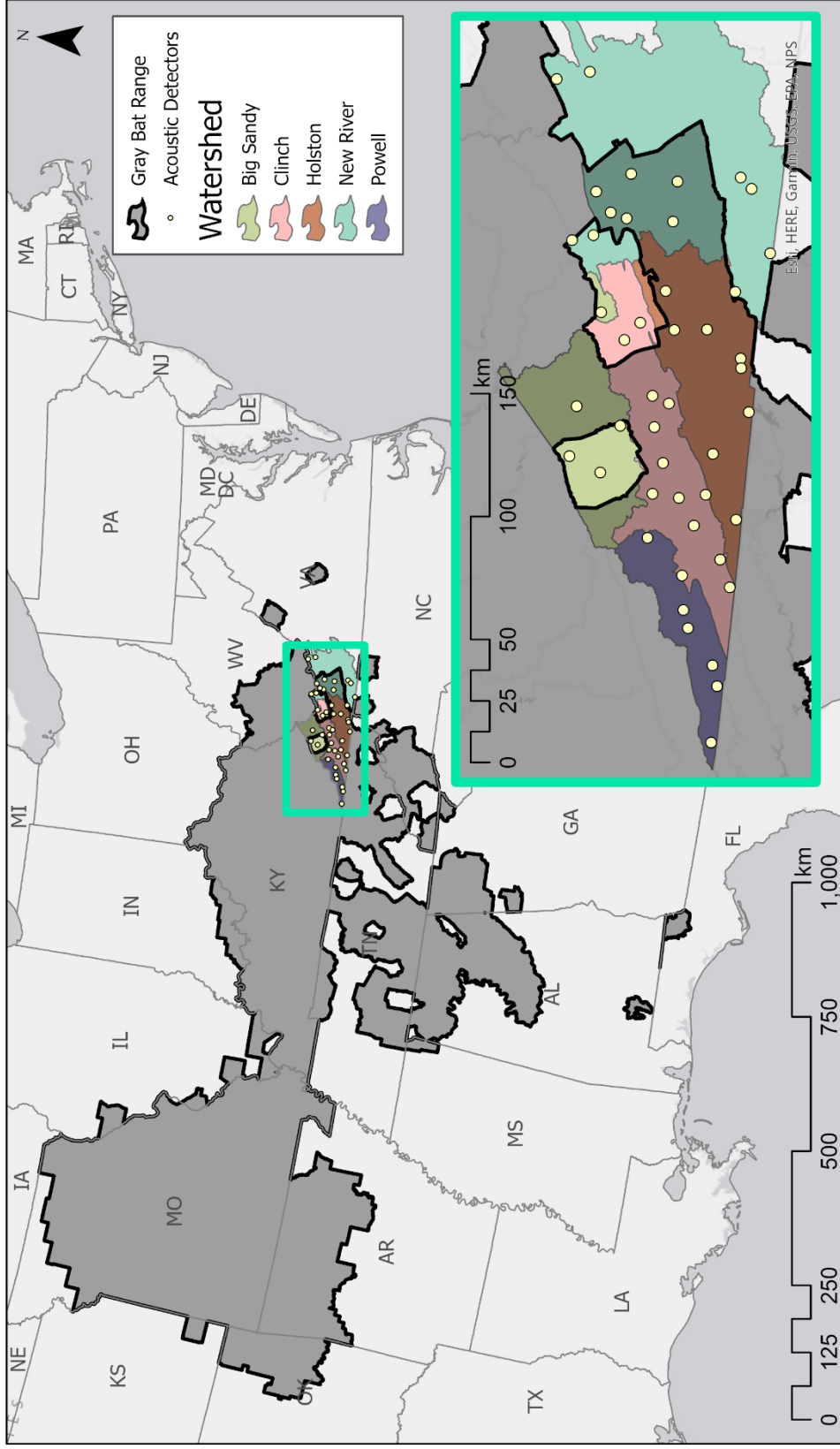
Figure 10. Gray bat (*Myotis grisescens*) range distribution (U.S. Fish and Wildlife Service, 2020) in relation to southwestern Virginia's watersheds. The study area is highlighted in the inset rectangle.

Discussion of Acoustic Results

The low Virginia big-eared bat activity levels across all the sampling periods in southwestern Virginia were expected. Acoustic monitoring is not an ideal method for this species; in addition to their overall rarity in the region, they are considered "whispering bats" due to their tendency to echolocate at very low amplitudes (Loeb et al., 2011). The higher mean activity in the Clinch River watershed in 2020 than in previous years could be due in part to the deployment of five full-spectrum acoustic detectors in the watershed in 2020, whereas zero-crossing detectors were used in previous years (SITE00, SITE02, SITE03, SITE04, and SITE05; *Figure 1*). Full-spectrum acoustics are more sensitive to low-amplitude bats in the genus *Corynorhinus* (Comer et al., 2014); however, these detectors require more frequent battery replacement and expansive digital data storage and are thus unsuited for our long duration work.

As expected, in all three years of recording, the overall activity was very low for little brown bats, northern long-eared bats, and Indiana bats, which is consistent with their decline due to WNS in Virginia, the surrounding states, and elsewhere (Austin et al., 2018, 2019; Deeley et al., 2021a; Frick et al., 2010; Nocera et al., 2019b; Powers et al., 2015). Although individuals from these species are still present on the landscape, acoustic monitoring alone cannot determine their population sizes, whether they are forming maternity colonies, or if they are reproductively successful (Deeley et al., 2021a). There is at least one known recurring little brown bat maternity colony in the New River watershed (R. Reynolds, VDWR, personal communication), to which the peak in little brown bat activity in the New River watershed in May (when the bats were pregnant) may be attributable. However, members of the colony likely remained close to the roost from parturition to the beginning of pup volancy and were therefore not within range of our detectors (Henry et al., 2002). Research conducted at Fort Drum, New York (Ford et al., 2011; Nocera et al., 2019b) showed that the little brown bat, Indiana bat, northern long-eared bat, and tricolored bat activity levels post-WNS were generally similar to those we observed in southwestern Virginia. However, the activity levels of these species in New York were higher in late summer, whereas in our study area, these species had higher overall activity levels in earlier months, perhaps indicating that these species are not widely successful in reproduction in southwestern Virginia.

In southwestern Virginia, Indiana bat activity generally peaked in spring, and northern long-eared bat activity generally peaked in summer. In contrast, recent acoustic monitoring in coastal Virginia (De La Cruz et al., 2020) found that Indiana bat activity peaked in late summer and fall, and northern long-eared bat activity peaked in spring in coastal Virginia as they migrated from wintering in eastern North Carolina to spend summer around the District of Colombia metro area. However, similar to southwestern Virginia, the coastal study also observed relatively low activity from WNS-impacted Indiana bats and northern long-eared bats (De La Cruz et al., 2020; Frick et al., 2010; Powers et al., 2015). As observed in other species, summer peaks in northern long-eared bat activity could indicate maternity activity and therefore successful reproduction (Deeley et al., 2021b), whereas Indiana bat spring activity peaks may indicate the area is near hibernacula or between hibernacula and potential maternity sites.

The temporal patterns that we documented of all acoustic bat activity, and *Myotis* spp. in particular, in southwestern Virginia were similar to those documented in previous years in the central Appalachians by Muthersbaugh et al. (2019a, b), with variable activity starting as early as mid-March and lasting until mid-November. Although we did not use temperature as a variable in our models, it could be a factor in how late into autumn bat activity continues. Warmer

autumn months can allow bats to forage longer and build up a larger energy storage, which in turn can increase the probability of survival during hibernation (Frick et al., 2012). We do note that total nightly *Myotis* spp. call files in March and November 2020 were higher than in the corresponding months in 2019 (by 6,152 in March and by 139 in November), as were the average monthly temperatures for those months in 2020 in southwestern Virginia (~4°C in both months; National Oceanic and Atmospheric Administration, 2021). As the climate changes and temperatures in autumn months rise, bat activity patterns may change, and surveying outside the expected time frame for bats should be considered (Odom & Ford, 2020).

By October of each year tricolored bats had mostly gone into hibernation or migrated out of the Clinch River watershed; a similar pattern was seen in the New River watershed in 2019. However, in 2020, tricolored bats were still active in October, mostly attributable to observations at an added site (NR11, *Figure 1*), which may suggest that they were overwintering in a local hibernaculum. In addition to traditional hibernacula such as caves, tricolored bats have often been found overwintering in culverts, perhaps a strategy that allows portions of their population to avoid exposure to WNS. Furthermore, because some tricolored bats in the New River watershed were active in late fall, this species could potentially be occupying transportation structures, particularly culverts, throughout the winter months (Bernard et al., 2019; Leivers et al., 2019; Lutsch, 2019; Meierhofer et al., 2019).

Gray bat spring migration from hibernacula occurs from March to May and fall migration between late August and mid-November (Sasse, 2019), which was reflected in our activity maps (*Figure 11*). Northward dispersal in July and August is likely due to dispersal by both juveniles and adults from the known maternity colony site in Bristol, and the increase in activity concentration in September may be due to fall swarming in the area or near staging caves in the region prior to returning to the presumed hibernacula in Tennessee. Consistent activity in the Big Sandy watershed may indicate a smaller colony in the area or individuals from a Kentucky or possibly West Virginia-centered overwintering population. It may be that gray bats have historically been present in the New River and Big Sandy watersheds but were never detected, particularly before the widespread use of acoustic sampling, or that the arrival of the species may be a recent development due to competitive release following the decline of the once-abundant little brown bat (Powers et al., 2016). Competitive release and niche/habitat use change have been observed in other WNS-affected bat communities in the United States formerly dominated by little brown bats (Jachowski et al., 2014). It is also possible that the three gray bats that we captured in the New River drainage in August were early migrants. These transitional times are when gray bats have been shown to be more generalist in their roost selection, roosting in concrete barriers and even trees (Samoray et al., 2020; Sasse, 2019). If the gray bat population size increases and sympatric species affected by WNS continue to decline or change in distributional patterns in Virginia, it is plausible that gray bats may continue to show expansion into watersheds beyond their historic distribution. This is particularly true for the New River Valley because of the large number of potentially suitable summer roost and winter hibernacula caves in the New River Valley (Culver et al., 1999; *Figure 10*). Our study offers a systematic, region-wide acoustic survey providing a regional view of the movement of gray bats across the landscape over time and estimates activity within and beyond the upper Tennessee River watershed.
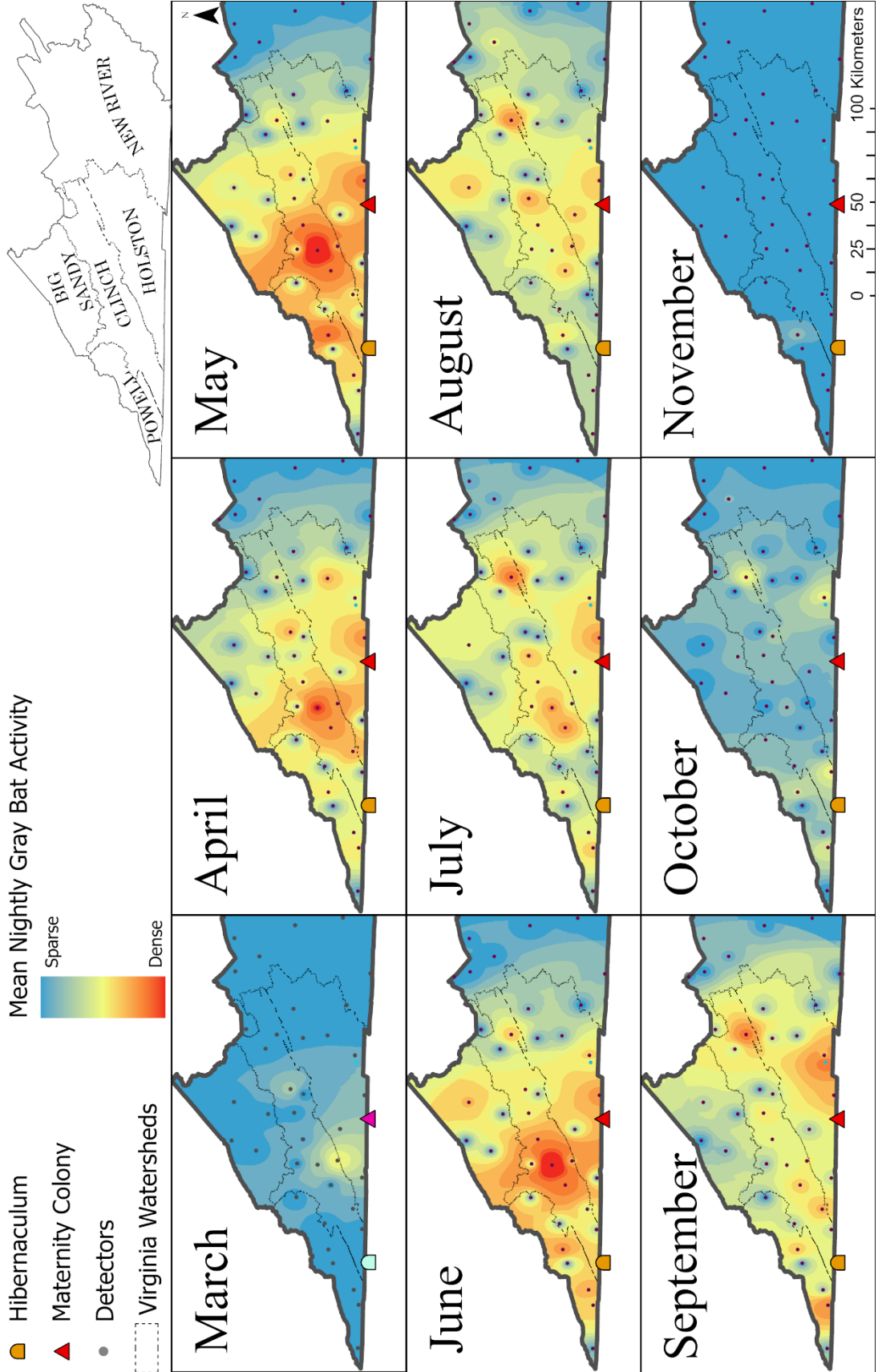
Figure 11. Heatmaps showing mean nightly gray bat (*Myotis grisescens*) activity in VDOT's Bristol District in 2019 using mean nightly call files per month processed with the inverse distance weighted interpolation tool

Monitoring the regional distribution and potential expansion of the gray bat would provide additional useful information as gray bat is a listed species that frequents transportation structures. Additional acoustic and netting efforts earlier in summer in the New River watershed could help clarify whether this area is being used by this species throughout summer and would provide insights into the potential for permanently established populations.

Bridge Inspections for Bat Use and Guano Collection

Thirty-one of the 39 bridges that we inspected had signs of bat use. We directly observed bats at 13 of the bridges we inspected; 11 of these bridges had roosting big brown bats, two bridges had small-footed bats, one bridge had a tricolored bat, and one bridge had a gray bat (*Table A3*). We were able collect guano from 24 detector sites and obtained a mean of 6.58 (± 1.06) samples per site. Because some acoustic detectors were in areas with two to four bridges, we inspected multiple bridges in some sites and collected guano from a total of 29 bridges. Most of the guano collected was from under bridge ends. Areas commonly bearing guano piles, such as pier caps, were inaccessible without specialized equipment and were therefore not sampled. We also acquired fresh guano from a known gray bat bachelor cave in Lee County through the Virginia Department of Conservation and Recreation, and we obtained several fresh gray bat guano samples from mist-netting live captures for use as controls for the validation of the resulting species identification.

Genetic Processing

We successfully extracted DNA from a total of 293 composite guano samples: 283 from guano samples collected under bridges, six from guano acquired from live captures of gray bats by mist-netting in the region, and four from guano collected at a gray bat summer roost cave in Lee County by the Virginia Department of Conservation and Recreation. Of these, 240 samples amplified; however, only 70 samples were of sufficiently high quality and length in base pairs to assemble into one contiguous sequence after DNA sequencing. We identified three bat species from the guano at 15 collection sites: gray bats, big brown bats, and northern long-eared bats (*Figure 12, Table A4*). All the species identified at their respective sites were also acoustically recorded at those sites. The samples that never amplified via PCR may have been too degraded or may possibly have originated from an unrelated species with a similar guano pellet appearance and size, such as a rodent. Several of the samples that amplified but did not assemble had the same species identification for both the forward and reverse sequences ($n = 13$), and some assembled sequences were not matched with any species in GenBank ($n = 11$). However, five were matched with the same species for both the reverse and forward sequences of those samples (in all five samples, the species was northern long-eared bat). Despite some assembled sample sequences having no match, it is likely that the species assignment was correct in instances where both the forward and reverse sequences composing the assembled sequence matched the same species. We were able to obtain a species match for an average of 27% of the samples per site. This success rate could likely be improved upon with adjustments to the PCR reaction or thermocycling parameters, by adding a step using a PCR purification kit to improve subsequent DNA sequence quality, or by adopting less stringent criteria for determining a species match.
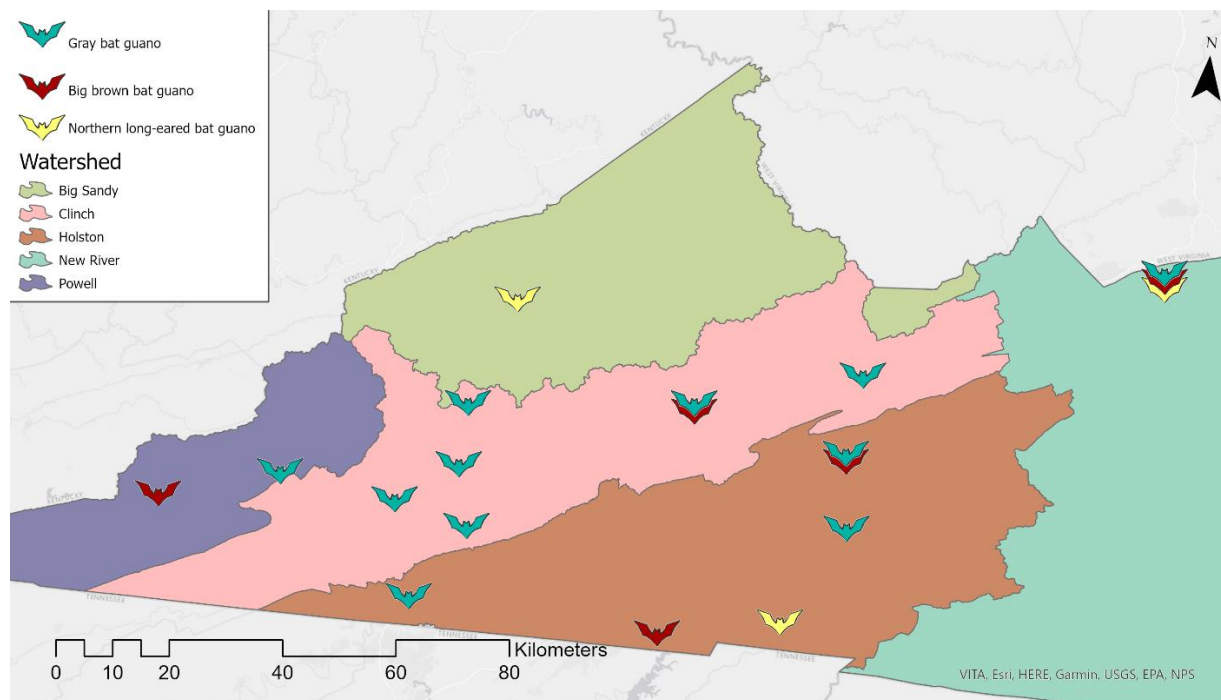
Figure 12. Map of VDOT's Bristol District bridge locations with confirmed roosting species identified using DNA barcoding with DNA extracted from guano collected at bridge sites in 2019 (information on the Federal ID of these bridges in *Appendix*, *Table A*). The identified species included gray bats (*Myotis grisescens*), northern long-eared bats (*Myotis septentrionali*s), and big brown bats (*Eptesicus fuscus*).

Discussion of DNA Barcoding Results

In the case of several of our fresh guano samples with an incorrect species identification, the "universal" primer more closely matched the target annealing sequences for the species incorrectly identified. Because we knew the correct species for the fresh guano, we discovered that a lower annealing temperature should have been used to allow more permissive annealing. The correct species identification followed thereafter, and we applied that annealing temperature going forward. During the initial tests of different PCR procedures, we included a small set of samples multiple times through variations of PCR protocols, and on one occasion obtained different species identifications from the same sample processed in different ways. The samples that we collected under bridges were of unknown age and may have unavoidably been exposed to DNA from multiple species, bat or otherwise, which is a consideration for future sampling. By using a different sequencing method, such as next-generation sequencing in combination with multifaceted DNA metabarcoding, it may be possible to find multiple species from individual or pooled samples, whereas with the Sanger sequencing we applied, we were likely to identify only the most common DNA within each sample (Swift et al., 2018; Walker et al., 2016, 2019). More complex metabarcoding methods could also reveal more information about the diet, parasites, and the sex of roosting bats (Guan et al., 2020). Although Sanger sequencing does not give the most complete results, its straightforward application and lower cost make it an attractive option for future VDOT guano sampling. A way to mitigate this inconsistency can be to collect and process more samples when using the Sanger approach to overcome the procedure's inherent low likelihood for species identification due to unavoidable issues with the quality of the starting material. Additionally, it may be worthwhile to test whether amplifying samples under multiple protocols may result in a substantial number of samples returning a different species match. For

example, when collecting samples to be processed with Sanger sequencing, it may be more appropriate to collect more single-pellet samples and to try to sample as many different bridge areas as possible as different species may have different preferences for roosting locations on the same structure.

Although obtaining a species identification from guano offers good confirmation of species using transportation structures, it is important to note that it cannot be determined whether the species is absent if that species has not been identified via DNA barcoding. Nonetheless, with expanded spatial sampling, DNA barcoding could be a useful tool at least in part for confirming species' use of transportation structures. Such an approach could provide additional clarity as to what structures or discrete areas are preferred by different bat species, particularly when matched with either acoustic occupancy or relative activity data from the same location. When collecting guano, fresher samples from the tops of piles should be prioritized; however, we observed that guano of unknown age can still provide results. Nevertheless, a fresh sample is more likely to provide a higher quality sequence and a good DNA template with a complete *COI* sequence to amplify. Although research has shown that, except in cases where it is covered in mold, older bat guano can be viable for DNA extraction (Walker et al., 2019), work with most mammalian taxa suggests otherwise (Wultsch et al., 2015).

Mist-Netting

During mist-netting in the Clinch River watershed (SITE06; *Figure 2*) on July 25, 2019, we captured one female and three male adult gray bats, a female juvenile little brown bat, and a female adult tricolored bat. None of the bats caught that night were reproductively active (i.e., there was no evidence of post-lactation for the females or descended testes for the males). We radio-tracked the female gray bat and one of the male gray bats. We failed to detect either gray bat at their respective day roosts before sunset. Subsequent tracking after sunset allowed us to detect the male while it was actively foraging along the Guest River near the bridge where it was captured (SITE06; July 26 starting at 21:20). The female gray bat was never located.

During mist-netting in the New River watershed on August 6, 2019 (Reed Creek*; Figure 2*), we captured a male adult big brown bat, one male adult and four female juvenile eastern red bats, and a post-lactating female adult gray bat, which we tracked to a small wooden bridge on private property over Reed Creek, approximately 1.6 km from the capture site. The female was roosting in a horizontal, partially open metal pipe under the long edge of the bridge (*Figure 13*).

Figure 13. Post-lactating female gray bat (*Myotis grisescens*) day roost in a pipe under a bridge above Reed Creek in the New River Watershed on August 7, 2019. On the left is a perpendicular view showing the radio-tagged bat's (A) antenna and (B) banded forearm; on the right is a photo taken parallel to the bridge showing the bridge and pipe structure. The white arrow points to the roost location within the pipe.

On August 12, 2019, we mist-netted at the Wolf Creek and Clear Fork confluence near site NR03 (*Figure 2*) and captured a male juvenile big brown bat, two male adult eastern red bats, and a male adult gray bat. All the captured bats were non-reproductive. We were able to track the male gray bat to a day roost in an abandoned brick building approximately 120 m from the capture site (*Figure 14*). The rest of our 2019 mist-netting efforts only produced big brown or eastern red bat captures (data available on request).

Figure 14. Roost location of a non-reproductive adult male gray bat (*Myotis grisescens*) on August 13, 2019, Rocky Gap, Bland County, Virginia, near Wolf Creek in the New River Watershed

Discussion of Mist-Netting Results

Our mist-netting efforts in the New River watershed in 2019 confirmed acoustic findings of gray bat presence that year and allowed us to collect fresh guano from captured gray bats, which was used as control samples for DNA barcoding. Our netting efforts were constrained to late summer, after juvenile volancy; however, activity increased further into fall, and some activity was recorded in early spring (*Figure 11*). More netting efforts at different times of the active season (between early March and early November) could reveal more demographic information on the bats using the area in different seasons and indicate whether they are successfully reproducing (i.e., presence of pregnant or lactating females beyond the known maternity colony site in Bristol). Such a result would provide stronger evidence of potential range expansion. Gray bat activity changes spatially depending on the time of year, and the roost types that bats choose (i.e., caves, transportation structures, buildings, and even trees) change seasonally based on their reproductive and migratory status (Samoray et al., 2020; Sasse, 2019). Mist-netting also allows the opportunity to tag and track bats to day roosts and may provide better insights into their transportation structure use. However, tracking gray bats can be particularly challenging as they have large, long, and linear foraging ranges (up to 70 km; LaVal et al., 1977), as opposed to other *Myotis* species that occur in Virginia (Menzel et al., 2005; Silvis et al., 2016), and can potentially day roost long distances from where they were captured, thus beyond the transmitter reception range for current tracking technology. Second, because gray bats are largely obligate cave dwellers, if they are roosting in a cave or cave-like structure, radio signals from transmitters are typically blocked even in close proximity to the observer (Amelon et al., 2009).

Statistical Analyses

Acoustic Data: Generalized Linear Mixed Model: 2019 Gray Bat Activity

Our top AICc-ranked (AICc weight of 0.325; *Table 1)* generalized linear mixed model (GLMM) for gray bat activity included acoustic (bridge) site as a random variable, distance to the Bristol area maternity colony, day of year, and an interaction of mean cave density within 2 km^2 with volancy period (pre- or post- June 15, the expected date when juveniles become volant; Orndorff et al., 2019). Our top model suggested that gray bat activity in the region is likely to be higher prior to juvenile volancy in midsummer, in areas of higher surrounding cave density, and closer to the Bristol maternity colony site (*Table 1, Figures 15-16)*. Only four other models were ranked within two AICc units of the top model, and they were all the same as the top model with the exception of an additional variable for each (distance to hibernaculum, minimum landform index, % developed area cover within 2 km^2, and % low vegetation cover within 2 km^2; AICc weights of 0.2361, 0.1423, 0.1386, and 0.1216, respectively); thus we considered them less parsimonious and less informative (*Table A2*).

Discussion of Generalized Linear Mixed Model Results

Our top GLMM (*Table 1*) predicted that gray bat activity would be highly concentrated and more local prior to pup volancy, as often observed with other species (Deeley et al., 2021a; Nocera et al., 2019b), especially closer to the maternity colony and in areas with higher cave density (*Figure 15-16*). Once pups became volant, activity became dispersed over a larger area in southwestern Virginia. However, our GLMM predicted that, all other factors being equal, areas with higher cave density would still have higher concentrations of gray bat activity (*Figure 16*), which is expected as areas with more caves can be used by more bachelor colonies or smaller maternity colonies. Therefore, post-volancy areas with an abundance of roosting choices could be more likely to contain individual bats that are both foraging and exploring potential roosts rather than merely exhibiting migratory behavior.

Table 1. Summary of the Best Competing Generalized Linear Mixed Models as Ranked with AICc Predicting Gray Bat (*Myotis grisescens*) Activity Using Acoustic Data Recorded in 2019 within VDOT's Bristol District, Virginia

Variable	Parameter Estimate	Standard Error	*z*-value	*P*-value
Intercept	−0.11	0.29	−0.37	0.714
Volancy (PRE)	0.02	0.05	0.29	0.770
Mean Cave Density	0.96	0.24	3.95	<0.01
Distance to Maternity Colony	−2.12	0.44	−4.85	<0.01
Day of the Year	−0.96	0.07	−14.34	<0.01
Volancy (PRE) * Mean Cave Density	0.2	0.05	4.16	<0.01
Pseudo-R² (Fixed Effects)	0.53			
Pseudo-R² (Total)	0.95			
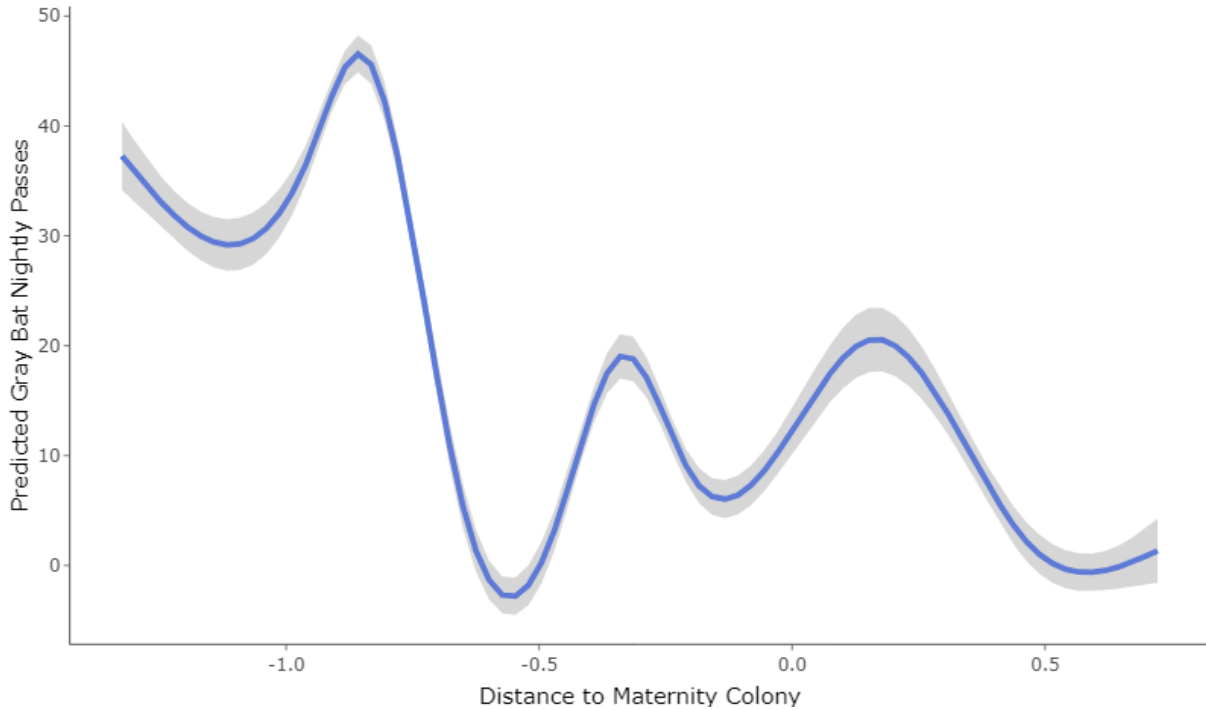
Figure 15. Gray bat (*Myotis grisescens*) predicted acoustic activity lines with 95% credible intervals by distance to the maternity colony. Predicted with our highest AICc-ranked generalized linear model built using 2019 acoustic data from VDOT's Bristol District, Virginia. The range of distance values on the *x*-axis has been centered and scaled using the scale function in program R.
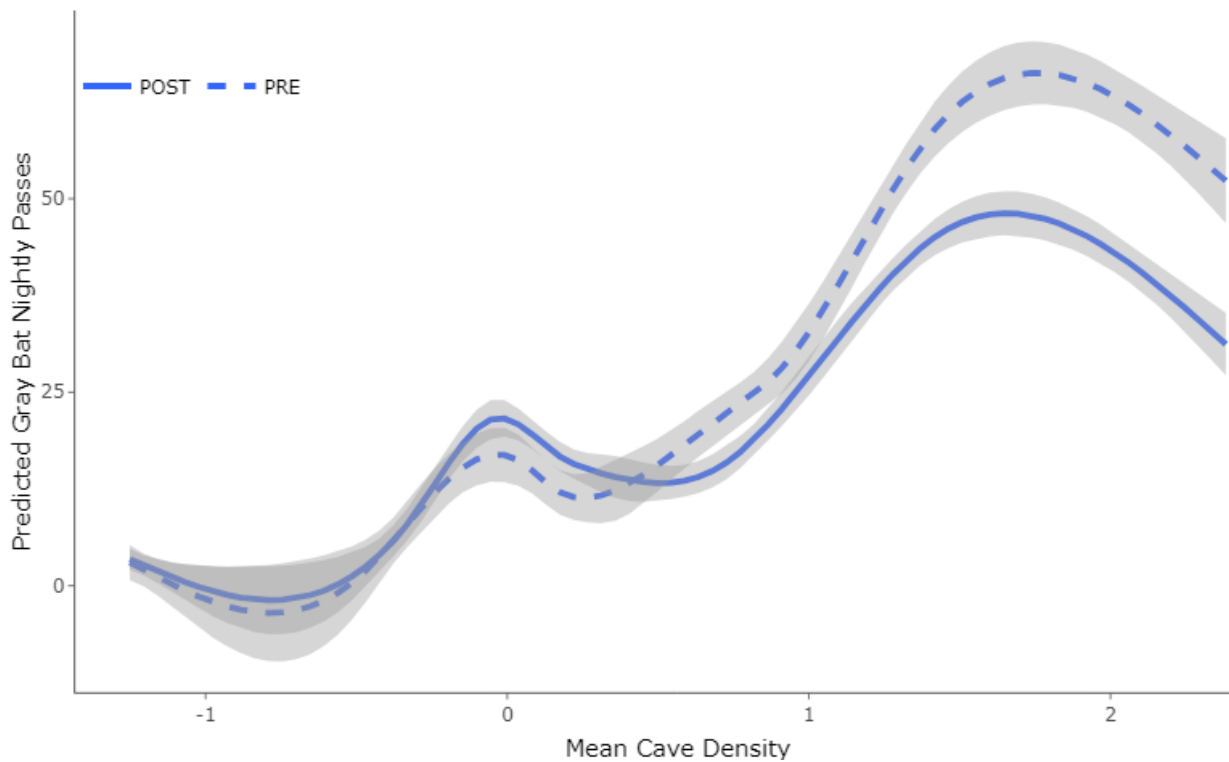
Figure 16. Gray bat (*Myotis grisescens*) predicted acoustic activity lines with 95% credible intervals before (PRE) and after juvenile volancy (POST) by mean cave density. Predicted with our highest AICc-ranked generalized linear mixed model built using 2019 acoustic data from VDOT's Bristol District, Virginia. The range of distance values on the *x*-axis has been centered and scaled using the scale function in program R.

Bridge Occupancy Analysis From Guano Sample DNA Barcoding Species Identification

We found positive occupancy data for three bat species identified using DNA barcoding with DNA extracted from guano samples; however, because of the limited number of observations, we modeled the occupancy of only big brown and gray bats. Positive big brown bat occupancy was observed in nine of 29 bridges sampled from a total of 25 of 240 guano samples (10.4%). Positive gray bat occupancy was found in 12 of 29 bridges sampled from a total of 32 of 240 guano samples (13.3%). Lastly, positive northern long-eared bat occurrence was found in four of 29 bridges sampled from a total of six of 240 guano samples (2.5%), which we determined was insufficient for occupancy analysis.

The best-ranked model for big brown bat occupancy contained the percentage of high-quality bases per sequence (% HQ) to predict detection probability and percentage of karst (% karst) within a 2-km radius to predict occupancy. Percent high-quality sequence had a direct relationship with detection probability for big brown bats using DNA from guano (*Table 2; Figure 17*). The percentage of karst within a 2-km radius had an inverse relationship with big brown bat occupancy (*Table 2; Figure 17*), and though the *P*-value was >0.05, adding % karst to the model made it outcompete the model that consisted of % HQ alone, suggesting that it contributed to the model fit. Among the variable combinations that we compared, no other models fell within 2 ΔAICc units of the top model for big brown bat occupancy (*Table A5*).

Table 2. Model Summary of the Top Big Brown Bat (*Eptesicus fuscus*) Occupancy Model Based on Occupancy Observed Using DNA From Guano Collected in VDOT's Bristol District in 2019

Variable	Parameter Estimate	Standard Error	*z*-value	*P*-value
Occupancy				
(Intercept)	27.9	21.7	1.29	0.198
% Karst	−29.5	22.4	−1.32	0.187
Detection				
(Intercept)	−4.38	0.622	−7.05	<0.01
% High-Quality Sequence	6.78	1.027	6.60	<0.01
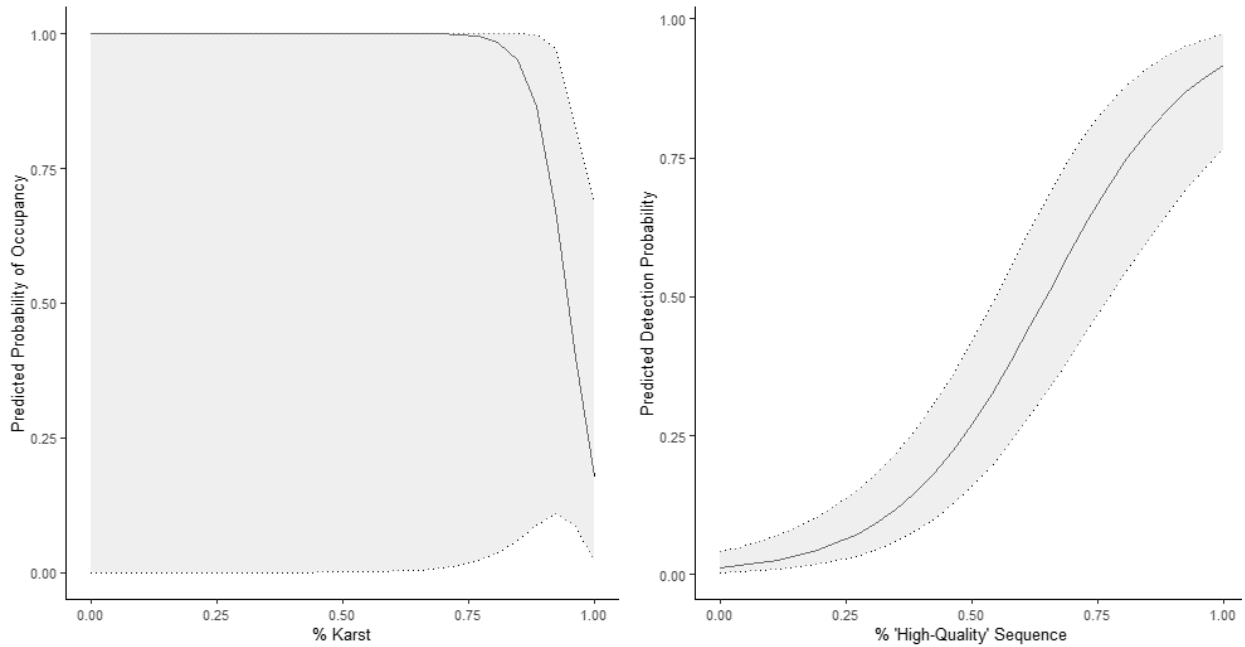
Figure 17. Predicted probability of big brown bat (*Eptesicus fuscus*) occupancy by percentage of karst within a 2-km radius (left panel) and predicted detection probability of big brown bats from guano samples collected in VDOT's Bristol District, Virginia, in 2019 by the proportion of high-quality DNA sequence (right panel) according to the top AICc-ranked occupancy model. The 95% confidence intervals are shown in gray.

All competing models for gray bat occupancy included % HQ as a detection probability predictor, and in all models, the relationship was positively correlated (*Table 3; Table A6; Figures 18-20*). The best-ranked model for gray bat occupancy also included % karst within a 2-km radius, which was directly correlated with gray bat occupancy probability (*Table 3; Figure 18*). There were two competing models: the second-best contained bridge width (*Table 3; Figure 19*), and the third-best contained mean cave density within a 2-km radius (*Table 3; Figure 20*).

Table 3. Model Summaries of the Three Competing Gray Bat (*Myotis grisescens*) Occupancy Models Based on Occupancy Derived From DNA From Guano Collected in VDOT's Bristol District, Virginia, in 2019

Variable	Parameter Estimate	Standard Error	*z*-value	*P*-value
Best-Ranked Gray Bat Occupancy Model				
Occupancy				
(Intercept)	−6.23	4.50	−1.39	0.166
% Karst	8.79	5.51	1.59	0.111
Detection				
(Intercept)	−2.62	0.44	−5.95	<0.01
% High-Quality Sequence	3.65	0.75	4.88	<0.01
Second-Best Gray Bat Occupancy Model				
Occupancy				
(Intercept)	237.33	651.000	0.36	0.715
Bridge Width (m)	−5.46	15.000	−0.36	0.716
Detection				
(Intercept)	−3.01	0.345	−8.70	<0.01
% High-Quality Sequence	4.08	0.664	6.14	<0.01

(Table 3 Cont.)				
Variable	**Parameter Estimate**	**Standard Error**	***z*-value**	***P*-value**
Third-Best Gray Bat Occupancy Model				
Occupancy				
(Intercept)	−3.20	3.090	−1.04	0.300
Mean Cave Density	26.0	21.800	1.19	0.233
Detection				
(Intercept)	−2.77	0.445	−6.23	<0.01
% High-Quality Sequence	3.82	0.741	5.16	<0.01
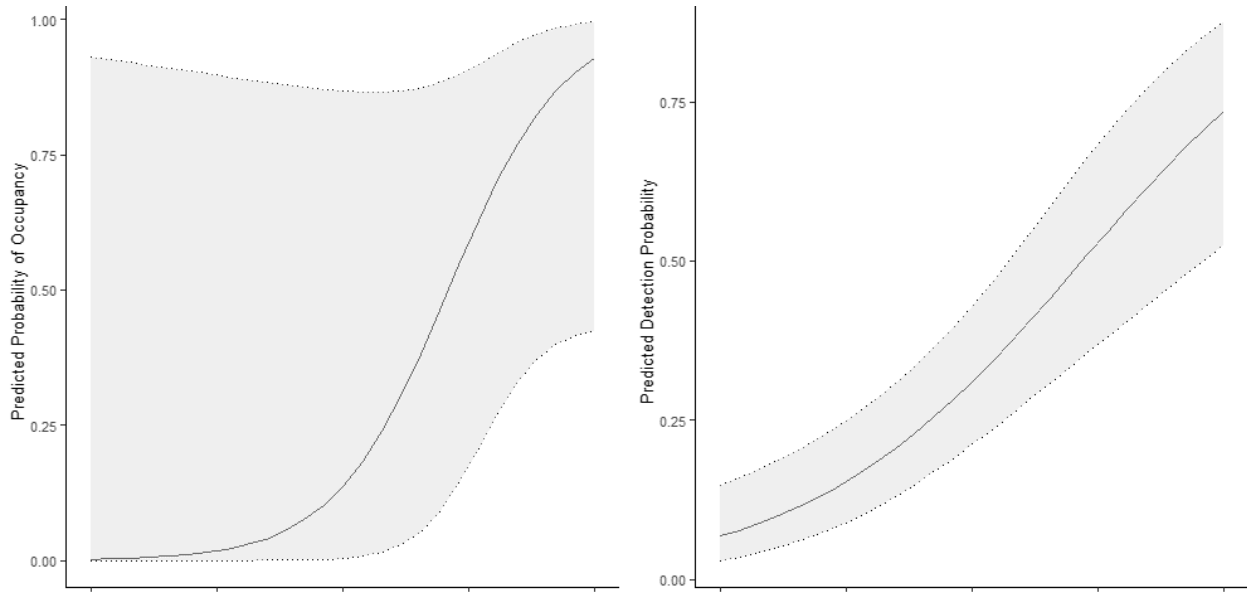
Figure 18. Predicted probability of gray bat (*Myotis grisescens*) occupancy by the percentage of karst within a 2-km radius (left panel) and predicted detection probability of gray bats from guano samples collected in VDOT's Bristol District, Virginia, in 2019 by proportion of high-quality DNA sequence (right panel) using the top AICc-ranked occupancy model. The 95% confidence intervals are shown in gray.
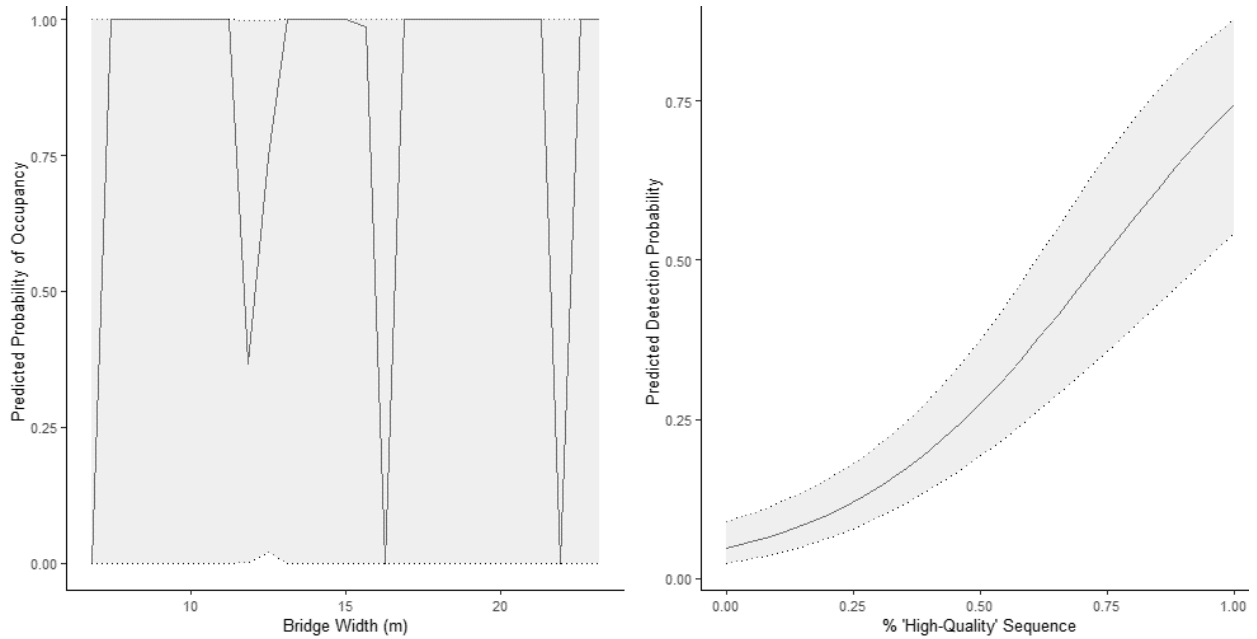
Figure 19. Predicted probability of gray bat (*Myotis grisescens*) occupancy by bridge width (left panel) and predicted detection probability of gray bats from guano samples collected in VDOT's Bristol District, Virginia, in 2019 by proportion of high-quality DNA sequence (right panel) according to the second-best AICc-ranked occupancy model. The 95% confidence intervals are shown in gray.
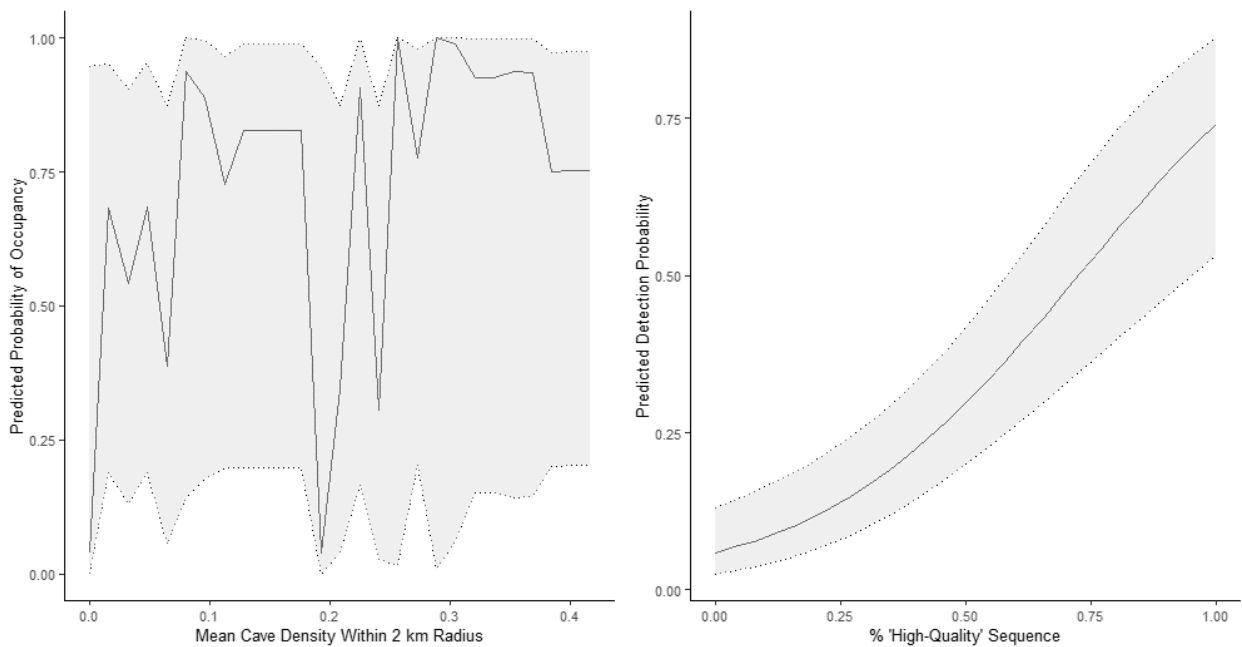
Figure 20. Predicted probability of gray bat (*Myotis grisescens*) occupancy according to mean cave density within a 2-km radius (left panel) and predicted detection probability of gray bats from guano samples collected in VDOT's Bristol District, Virginia, in 2019 by proportion of high-quality DNA sequence (right panel) according to the third-best AICc-ranked occupancy model. The 95% confidence intervals are shown in gray.

Discussion of Occupancy Analysis From Guano Results

The positive relationship to % karst within a 2-km radius was expected for a cave-obligate bat such as the gray bat, and even though the effect size was small to moderate, this trend was biologically consistent for the species (*Table 3; Figure 18*). With more occupancy data from bridges and caves, along with acoustic data to gauge timing, it may be possible to determine whether gray bats in areas with less karst or caves may be more likely to use alternatives to caves, such as transportation structures, especially after juveniles become volant and during seasonal migration. Conversely, the inverse relationship between big brown bat occupancy probability and karst was not as biologically clear although this species is considered a habitat generalist and uses a variety of roost types in summer that would not necessarily be related to karst presence (Agosta, 2002). The *P*-value for the karst variable in the big brown bat occupancy model was slightly higher than that in the gray bat model and had a wider confidence interval. Nonetheless, the addition of the karst variable improved the model fit enough to outrank the % HQ despite the added model complexity (*Table 3; Figure 18*).

The only bridge-specific variable that was retained in our top supported models was bridge width as a predictor of gray bat occupancy probability; however, the relationship was not conclusive. Despite this model being ranked second, this individual parameter had an estimate with a large standard error whereby the bounds on the estimate crossed zero (*Table 3*). Examining the prediction plot (*Figure 19*) the relationship appears equivocal, and the confidence interval was maximized. Nonetheless, these same bridge-specific parameters may still prove to be of some significance for occupancy with additional bridge features (i.e., bridge height above water) or accounting for other potential variable interactions.

The third best-ranked gray bat occupancy model included mean cave density within a 2-km radius, which, similar to % karst, was also biologically plausible for this cave-obligate species. The variable mean cave density was also included in the top gray bat GLMM model using relative activity from acoustic data. However, in the occupancy model, the relationship was weaker and the confidence interval wider than in the GLMM relative activity analysis (*Tables 1 and 3; Figures 16 and 20*). This result could be a function of the occupancy model being based on less data, or perhaps mean cave density has a stronger effect on the relative activity of gray bats than the likelihood of using bridges as roosts. Nocera et al. (2018) found similar incongruencies of meaningful covariates between occupancy and relative activity models for little brown bats, whereby occupancy was not strongly correlated to the same covariates that influenced relative activity; this actually proved to be more useful for understanding where greater concentrations of little brown bats occurred on the landscape.

All the competing models showed that, when using guano, the % HQ DNA sequence influenced the detectability of both big brown bats and gray bats. Unsurprisingly, the detection probability for both gray bats and big brown bats improved with higher quality sequences. Unfortunately, our exploratory occupancy models were built on small sample sizes because many of our sequences were not of sufficient quality to yield reliable species identification. Invariably, these models could be improved with occupancy data from additional high-quality sequenced samples, which could possibly be attained from fresher guano samples or from previously extracted samples with additional adjustments to the PCR protocol.

CONCLUSIONS

- *Acoustic monitoring at a bridge site from spring to fall in combination with multiple guano samples over that period from that site can provide an assessment of bat use with relatively high confidence, which can inform VDOT structure maintenance decisions in the following year.* In areas such as the New River drainage, where gray bat presence is expanding or the occurrence of other listed or sensitive species are routinely observed, this combined non-invasive approach of using acoustics and DNA barcoding may help VDOT better assess where potential risks to bats may occur.

- *With limited structure occupancy data from guano, the only bridge-specific parameter that was shown to influence occupancy was bridge width; however, the effect was rather weak and would benefit from more occupancy data.*

- *Even if the DNA barcoding from guano samples in this project did not identify a species of concern, the presence of species cannot be wholly discounted based on the lack of guano confirmation alone.* However, the absence of species identification from guano samples combined with negative data returned by acoustic monitoring would provide strong weight of evidence of the low likelihood of transportation structure use by a bat species (e.g., gray bats). Collecting more samples from as many areas on a bridge as possible would allow for a higher confidence level although costs may be a factor to consider.

Acoustics

- *The relative activity of various sensitive species revealed through long-term monitoring emphasizes the need to consider the transitory spring and fall seasons as well as the maternity seasons of bats generally, and gray bats specifically, relative to the use of transportation structures.*

- *Tricolored bat activity detected in late fall in this project may indicate their continued presence into hibernation in the area; hence, the use of culverts and other structures in the winter months is a possibility that should be considered.*

- *Gray bats have been documented outside the limits of their previously presumed range in Virginia in the Big Sandy and New River watersheds, especially before maternity season and after pups are volant (mid-July), which provides additional evidence that regional range expansion is occurring.*

DNA Barcoding

- *DNA barcoding can provide information on bat species roosting at a structure and may be most useful in areas where sensitive bat species ranges are expanding.*

- *Challenges to DNA amplification and sequence quality occurred in this project (e.g., a species match was found for 27% of the collected samples). More frequent field collections, the development and use of species-specific PCR primers, and adjustments of PCR amplification protocols to achieve a higher success rate are required before this method can become operationally mature. Once the protocols have been set, it will be possible to process guano relatively quickly.*

RECOMMENDATIONS

1. *In the next update of VDOT Environmental Division's "Preliminary Bat Inventory Guidelines for Bridges and Buildings" by VDOT's Biological Resources Program manager, the guidance should reflect the findings in this study regarding time of year restrictions.* Specifically, for structures that show signs of bat use, the window in which bats are considered to be on the landscape and potentially occupying structures beyond the maternity season should be extended into mid-November. Maintenance of structures should be avoided to the extent feasible between mid-March through mid-November.

2. *VDOT's Biological Resources Program Manager should coordinate with the Central Office Maintenance Division staff to update the Maintenance Division's Best Practices Manual regarding structure inspections.* Specifically, culverts with structure numbers should be inspected for bat presence by bridge maintenance staff or consultants prior to maintenance in all seasons of the year.

3. *The Virginia Transportation Research Council should convey the findings in this report to USFWS and VDWR for their consideration in future updates to bat survey protocol followed by DOTs.* Specifically, the following findings from this study should be considered in bat survey protocol updates to improve the accuracy and efficiency of surveys:

   - *In areas proximate to the range of listed bat species that have been observed using bridges, acoustic detectors should be placed near bridges for one full year or March through mid-November at a minimum to determine if and when these species may be present.* If year-round monitoring is not feasible, efforts in early spring, early and mid-summer, and perhaps early fall within an area would be contributory.

   - *If bat guano analysis is added to the approved methods for bat surveys, guano sampling and DNA barcoding should be limited to sites that have acoustic activity.* Findings of low acoustic activity and no guano suggests that it can be reasonably assumed there is no bat use of a structure and a lessened need for site monitoring. Findings of high acoustic activity and some guano presence may indicate sites where winter work or employment of deterrents is warranted.

   - *If bat guano analysis is added to the approved methods for bat surveys, DOTs can take advantage of full bridge inspections by collecting fresh guano using tarps or other collectors prior to maintenance activities to collect samples whenever possible, including hard-to-reach areas requiring specialized equipment.* This will provide a higher likelihood of yielding better quality DNA templates for species identification. However, it can be difficult to predict where bats will roost for all possible locations, especially in cases where bats are intermittently using structures during migration.

IMPLEMENTATION AND BENEFITS

Implementation

With regard to the time of year guidance in Recommendation 1, VDOT's Biological Resources Program Manager will add these best practices to *VDOT's Preliminary Bat Inventory Guidelines for Bridges and Buildings* during its next update (by the spring of 2023).

With regard to the culvert inspection guidance in Recommendation 2, VDOT's Biological Resources Program Manager will coordinate with the Central Office Maintenance Division staff to add this best practice to the Maintenance Division's Best Practices Manual. This addition will be initiated by January 2022. This change will also be reflected in the updated version of *VDOT's Preliminary Bat Inventory Guidelines for Bridges and Buildings*.

With regard to the bat survey protocol in Recommendation 3, survey protocols are determined by the USFWS and are included in the *USFWS Rangewide Indiana Bat Survey Guidelines* (USFWS, 2019). If the survey protocol is modified to reflect findings from this research and/or other studies, VDOT's Biological Resources Program Manager will document these changes in a subsequent update of *VDOT's Preliminary Bat Inventory Guidelines for Bridges and Buildings* and/or other appropriate District Environmental guidance documents.

Benefits

Implementing Recommendation 1 will help better inform VDOT's best practices on the timing of structure maintenance activities so VDOT can continue to comply with state and federal requirements to avoid take of protected bat species.

Implementing Recommendation 2 will ensure that large culverts (in addition to bridges) occupied by bats are surveyed prior to maintenance activities, thereby facilitating VDOT's continued compliance with state and federal requirements to avoid take of protected bat species.

Implementing Recommendation 3 will ensure that regulatory staff are aware of the findings and recommendations from this study and will consider using them to inform decisions regarding updates to their bat survey protocol. VDOT Environmental staff will continue following any bat monitoring and survey guidelines issued by USFWS. This will increase the accuracy of bat species' identification and support VDOT's continued compliance with state and federal requirements to avoid take of protected bat species.

ACKNOWLEDGMENTS

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APPENDIX: SUPPLEMENTARY TABLES

Table A1. Parameters Used for Analysis

Parameter	Scale	Description	ArcGIS Pro Layer	ArcGIS Pro Tool(s)
Latitude	decimal degrees	Latitude of each site's location	-	-
Volancy	PRE/POST	Volancy considered before or after June 15	-	-
Distance to Hibernaculum	meters[a]	Nearest known hibernaculum in Tennessee	-	-
Distance to Maternity Colony	meters[a]	Known maternity colony in Bristol, Virginia	-	-
Day of Year	transformed Julian day	Transformed Julian day (cosine of degrees derived from Julian day [(Julian day*360)/365] using the function cos_d in R package aspace)	-	-
Stream Order	Strahler number	Stream order of the nearest stream to each detector as an approximation of stream width	USGS National Hydrography Dataset Version 2	"Near"
% Forest	%	Percentage of forest cover within 2-km^2 buffer around detector. Deciduous, evergreen, and mixed forest types were combined.	Nature Conservancy Terrestrial Habitat Map for the Northeast U.S. and Atlantic Canada	"Tabulate Area"
% Low Veg	%	Percentage of low vegetation cover within 2-km^2 buffer around detector. Shrub/scrub, herbaceous, hay/pasture, and cultivated crops were combined to make low vegetation.	Nature Conservancy Terrestrial Habitat Map for the Northeast U.S. and Atlantic Canada	"Tabulate Area"
% Developed	%	Percentage of developed cover within 2 km^2 buffer around detector. Combined low, medium, and high intensity development.	Nature Conservancy Terrestrial Habitat Map for the Northeast U.S. and Atlantic Canada	"Tabulate Area"
% Karst	%	Percentage of karst within 2-km^2 buffer around detector. Karst was a combination of evaporites, gypsum, evaporite basins, sandstone karst, and carbonates.	USGS Karst in the United States: A Digital Map Compilation and Database map	"Tabulate Area"
MCD	numeric[a]	Average number of caves within 2-km^2 buffer around detector. Number of known caves was available for cells on a 20-km^2 grid from the Appalachian Landscape Conservation Cooperative. Caves were assumed to fall only within karst areas, and the karst layer was divided by and merged with the same 20-km^2 grid. Mean cave density of each karst portion of a grid cell was calculated by dividing the cave number values inside the karst portions by the area of karst. All the mean cave density values that fell	Appalachian Landscape Conservation Cooperative USGS Karst in the United States: A Digital Map Compilation and Database Map	-

Parameter	Scale	Description	ArcGIS Pro Layer	ArcGIS Pro Tool(s)
		within a 2-km^2 buffer around each detector were then averaged.		
Min Elevation	meters	Minimum elevation value within 2-km^2 buffer around detector	USGS 1/3 Arc Second 3DEP tiles	"Zonal Statistics"
Max Elevation	meters	Maximum elevation value within 2-km^2 buffer around detector	USGS 1/3 Arc Second 3DEP tiles	"Zonal Statistics
Mean Elevation	meters	Mean of elevation values within 2-km^2 buffer around detector	USGS 1/3 Arc Second 3DEP tiles	"Zonal Statistics"
LFI: the difference between the elevation at a pixel and the mean elevation of pixels within a 1-km^2 buffer			USGS 1/3 Arc Second 3DEP tiles	"Focal statistics" & "Raster calculator"
Min LFI	meters	Minimum LFI value within 2-km^2 buffer around detector	LFI layer created from USGS 1/3 Arc Second 3DEP	"Zonal Statistics"
Max LFI	meters	Maximum LFI value within 2-km^2 buffer around detector	LFI layer created from USGS 1/3 Arc second 3DEP	"Zonal Statistics"
Mean LFI	meters	Mean of LFI values within 2-km^2 buffer around detector	LFI layer created from USGS 1/3 Arc Second 3DEP	"Zonal Statistics"
Effort[a]	numeric	Number of amplified guano samples collected at a site	-	-
Bridge Type[a]	-	Flat slab/box, parallel box beam, cast in place, steel I-beam, pre-stressed girder, or wood	-	-
Under-Deck Material[a]	-	Concrete, corrugated steel, or wood	-	-
Bridge Length[a]	meters	Length of bridge as available from the VDOT website	-	-
Bridge Width[a]	meters	Width of bridge as available from the VDOT website	-	-
Average Daily Traffic[a]	numeric	Average daily traffic at each bridge as available from the VDOT website	-	-
Year Built[a]	year	Year each bridge was built as available from the VDOT website	-	-
Big Brown Bat Occupancy[a]	binary (0,1)	Whether big brown bat guano was identified at a site (for occupancy models of gray bats)	-	-
Gray Bat Occupancy[a]	binary (0,1)	Whether gray bat guano was identified at a site (for occupancy models of big brown bats)	-	-
% HQ[a]	%	Percentage of sample sequence that is high quality (automatically generated in Geneious Prime software)	-	-

[a] Parameters used only in the occupancy analysis from the guano species identification.
Note. 3 DEP, 3D Elevation Program; HQ, high quality; MCD, mean cave density; LFI, landform index; USGS, U.S. Geological Survey

Table A2. AICc Table of Candidate Generalized Linear Mixed Models Predicting Gray Bat Acoustic Activity Using Acoustic Data Collected From VDOT's Bristol District, Virginia, in 2019

Model	K	AICc	Δ AICc	AICc Wt
Volancy * MCD + Day of Year + Distance to Maternity Colony	8	41351	0	0.325
Volancy * MCD + Day of Year + Distance to Maternity Colony + Distance to Hibernaculum	9	41352	0.6391	0.2361
Volancy * MCD + Day of Year + Distance to Maternity Colony + Minimum LFI	9	41353	1.651	0.1423
Volancy * MCD + Day of Year + Distance to Maternity Colony + % Developed	9	41353	1.705	0.1386
Volancy * MCD + Day of Year + Distance to Maternity Colony + % Low Vegetation	9	41353	1.967	0.1216
Volancy * MCD + Day of Year + Stream Order + Distance to Hibernaculum + Distance to Maternity Colony + % Developed + Minimum LFI + % Forest	13	41358	6.386	0.01334
Volancy * MCD + Day of Year + Stream Order + Distance to Hibernaculum + Distance to Maternity Colony + % Developed + Mon LFI + % Karst	13	41358	6.677	0.01153
Volancy * MCD + Day of Year + Stream Order + Distance to Hibernaculum + Distance to Maternity Colony + % Developed + Minimum LFI + % Low Vegetation	13	41358	6.852	0.01057
MCD + Day of Year + Distance to Maternity Colony	6	41366	14.44	0.000238
MCD + Day of Year + Distance to Maternity Colony + Distance to Hibernaculum	7	41366	15.09	0.000172
MCD + Day of Year + Distance to Maternity Colony + Volancy	7	41367	15.34	0.000152
MCD + Day of Year + Distance to Maternity Colony + Minimum LFI	7	41367	16.06	0.000106
MCD + Day of Year + Distance to Maternity Colony + % Developed	7	41367	16.12	0.000103
MCD + Day of Year + Distance to Maternity Colony + % Low Vegetation	7	41368	16.41	8.87e-05
Volancy + MCD + Day of Year + Stream Order + Distance to Hibernaculum + Distance to Maternity Colony + % Developed + Minimum LFI + % Forest	12	41373	21.78	6.07e-06
Volancy + MCD + Day of Year + Stream Order + Distance to Hibernaculum + Distance to Maternity Colony + % Developed + Minimum LFI + % Karst	12	41373	21.91	5.68e-06
Volancy + MCD + Day of Year + Stream Order + Distance to Hibernaculum + Distance to Maternity Colony + % Developed + Minimum LFI + % Low Vegetation	12	41373	22.22	4.87e-06
Volancy * MCD	6	41567	215.7	4.79e-48
Distance to Maternity Colony	4	41577	226	2.69e-50
Distance to Hibernaculum	4	41580	229.1	5.72e-51
MCD	4	41581	229.6	4.47e-51
% Karst	4	41586	235.3	2.65e-52
Latitude	4	41588	236.8	1.23e-52
% Low Vegetation	4	41591	239.4	3.4e-53
Null Model	3	41592	240.8	1.69e-53
% Forest	4	41592	240.8	1.65e-53
Volancy	4	41592	241.2	1.4e-53
% Developed	4	41593	241.3	1.31e-53
Minimum LFI	4	41593	242.2	8.29e-54
Stream Order	4	41593	242.2	8.11e-54
Maximum Elevation	4	41594	242.6	6.96e-54

Note. MCD, mean cave density within a 2-km radius. LFI, landform index

Table A3. Information About the Sampled Bridges

SITE ID	Bridge FedID	Bridge Type	Underdeck Material	Inspection Date	Bat Use	Bird Use	Inspection Comments	Bat SPP Seen Roosting	# S	# PCR	# Guano ID EPFU	# Guano ID MYGR	# Guano ID MYSE
BS01	18563	flat slab/box	concrete	8/15/2019	NO	YES			0	0	0	0	0
BS03	5840	N/A	N/A	N/A	N/A	N/A			0	0	0	0	0
BS04	4003	parallel box beam	concrete	11/9/2019	YES	NO	10+ EPFU in cracks on road surface and guardrails	EPFU	0	0	0	0	0
BS05	5830	cast in place	concrete	8/16/2019	YES	YES	5 live EPFU roosting on surface SE area under deck	EPFU	7	0	0	0	0
BS06	5826	steel I-beam	corrugated steel	11/9/2019	YES	NO	4/30/19: MYLE in guardrail, 3+ EPFU 5/30/2019: MYLE in guardrail 7/7/19: 6 MYLE & 2 EPFU in guardrail crevices 11/9/19: EPFU in guardrail at NW expansion joint	EPFU, MYLE	19	11	0	0	2
HO01	16880	steel I-beam	concrete	7/29/2019	YES	YES	6–8 EPFU in guardrail (north side of bridge)	EPFU	12	12	0	7	0
HO02	19045	steel I-beam	concrete	7/19/2019	N/A	N/A	N/A		0	0	0	0	0
HO03	19088	steel I-beam	concrete	7/19/2019	YES	YES			9	9	1	0	0
HO04	18980	steel I-beam	concrete	7/30/2019	YES	NO			17	16	0	0	6
HO05	17426	steel I-beam	concrete	7/30/2019	YES	N/A	Scattered guano on I-beams, no large piles		5	5	0	1	0
HO06	17601	cast in place	concrete	7/31/2019	YES	YES	Swallow nests under expansion joints (north) 7/8/2019: MYGR juvenile female roosting in guard rail crevice & 3+ EPFU in guardrail (SE of bridge)	MYGR	14	13	2	6	0

(Table A3 Cont.)

SITE ID	Bridge FedID	Bridge Type	Underdeck Material	Inspection Date	Bat Use	Bird Use	Inspection Comments	Bat SPP Seen Roosting	# S	# PCR	# Guano ID EPFU	# Guano ID MYGR	# Guano ID MYSE
HO07	17389	cast in place	concrete	7/31/2019	YES	YES	7/8/2019: EPFU flew from bridge at dusk (visual + Echometer ID)	EPFU	15	15	0	0	7
HO08	18870	steel I-beam	corrugated steel & concrete	7/30/2019	YES	YES	Many EPFU roosting under deck above bike trail CMI netting: MYGR captured nearby	EPFU	17	12	0	0	0
NR01[a]	18493	parallel box beam	concrete	8/15/2019	NO	YES			0	0	0	0	0
NR02	18526	pre-stressed girder	concrete	11/6/2019	YES	YES			0	0	0	0	0
NR03[a]	2992	steel I-beam	concrete	8/14/2019	YES	NO	Guano on NW pier cap		5	5	1	0	1
NR03[a]	3022	steel I-beam	concrete	8/14/2019	YES	N/A	Guano on NE pier cap		13	13	9	3	0
NR03[a]	3023	steel I-beam	concrete	8/14/2019	YES	NO	Guano on SW pier cap 7/23/2019: MYLE under deck on open surface (SE side)	MYLE	11	11	5	3	1
NR03[a]	2993	steel I-beam	concrete	8/14/2019	YES	NO			11	11	3	3	0
NR04	19709	steel I-beam	corrugated steel	7/23/2019	YES	YES			0	0	0	0	0
NR05	19596	N/A	N/A	N/A	N/A	N/A			0	0	0	0	0
NR05B[a]	3095	cast in place	concrete	7/31/2019	NO	NO			0	0	0	0	0
NR06	8777	cast in place	concrete	11/10/2019	NO	YES	4 bird nests, unknown spp		0	0	0	0	0
NR07[a]	8775	pre-stressed girder	corrugated steel	7/24/2019	YES	YES	Swallow nests under deck, north side		4	4	1	0	0
NR08	8915	steel I-beam	wood	11/10/2019	NO	NO			0	0	0	0	0

(Table A3 Cont.)

SITE ID	Bridge FedID	Bridge Type	Underdeck Material	Inspection Date	Bat Use	Bird Use	Inspection Comments	Bat SPP Seen Roosting	# S	# PCR	# Guano ID EPFU	# Guano ID MYGR	# Guano ID MYSE
PO01	19255	steel I-beam	both corrugated steel & concrete	11/8/2019	YES	NO			2	1	0	0	0
PO02	26612	steel I-beam	corrugated steel	11/8/2019	YES	YES	Large guano piles under expansion joints 3 EPFU under SE of deck	EPFU	7	7	0	0	0
PO03	10709	steel I-beam	corrugated steel	11/8/2019	YES	YES	Guano pile on pier cap & under expansion joint (over railroad)		3	3	1	0	1
PO04	10975	steel I-beam	concrete	11/7/2019	YES	YES			0	0	0	0	0
PO05	10893	steel I-beam	corrugated steel	11/7/2019	YES	YES			1	1	0	0	0
PO06	24147	steel I-beam	concrete	11/8/2019	NO	NO			0	0	0	0	0
SITE00[b]	16709	wood	wood	N/A	N/A	N/A			0	0	0	0	0
SITE02[b]	16712	steel I-beam	corrugated steel	steel I-beam	NO	NO			0	0	0	0	0
SITE03[b]	16604	steel I-beam	corrugated steel	7/8/2019	YES	NO			13	12	0	2	0
SITE04[b]	16612	steel I-beam	concrete	7/29/2019	YES	YES	Dead PESU on south end wall surface (under bridge deck)	PESU	8	8	0	1	2
SITE05[b]	16590	steel I-beam	concrete	7/19/2019	YES	YES	8/23/2019: EPFU in guardrail crevice	EPFU	4	4	0	3	0
SITE06[b]	19328	cast in place	concrete	11/8/2019	YES	NO			3	3	0	0	0
SITE07[b]	25321	steel I-beam	corrugated steel	11/9/2019	YES	YES			17	17	0	0	0
SITE07[b]	16323	steel I-beam	concrete	11/9/2019	NO	YES			7	7	0	0	0
SITE08[b]	16305	steel I-beam	concrete	N/A	N/A	N/A			0	0	0	0	0

(Table A3 Cont.)

SITE ID	Bridge FedID	Bridge Type	Underdeck Material	Inspection Date	Bat Use	Bird Use	Inspection Comments	Bat SPP Seen Roosting	# S	# PCR	# Guano ID EPFU	# Guano ID MYGR	# Guano ID MYSE
SITE09[b]	25687	steel I-beam	corrugated steel	11/10/2019	YES	NO	3 EPFU in nearby culvert		9	9	2	1	0
SITE11[b]	18486	cast in place	concrete	11/6/2019	YES	YES	Guano under SW expansion joint 6/2/2019: EPFU in guardrails	EPFU	4	4	0	1	0
SITE12[b]	18519	steel I-beam	concrete	6/11/2019	YES	YES	Guano pile under old nest		9	9	0	0	0
SITE13[b]	16516	N/A	N/A	N/A	N/A	N/A			0	0	0	0	0
UC01	10696	steel I-beam	concrete	11/8/2019	YES	YES			6	6	0	0	0
UC01	10697	steel I-beam	concrete	11/8/2019	YES	YES	Guano piles under eastern expansion joint 7/7/2019: bat squeaks/chatter detected		31	17	0	1	0

[a] Sampled in 2019 and 2020
[b] Sampled in 2018, 2019, and 2020

Note. SITE ID refers to the detector site ID, which in some cases was near more than one bridge. Bridge FID is the federal ID of the bridge. "Bridge type," "underdeck material," "bat use," "bird use," comments, and "bat species seen roosting" were all determined during 2019 bridge inspections or occasional observations during monthly detector battery changes. # S refers to the total collected at each bridge, # PCR refers to the number of samples that successfully amplified, and # Guano ID refers to the number of samples matched with each respective species (including unassembled samples with the same reverse and forward sequence species match). Species codes used throughout this table: EPFU refers to big brown bats (*Eptesicus fuscus*), MYGR refers to gray bats (*Myotis grisescens*), MYSE refers to northern long-eared bats (*Myotis septentrionalis*), MYLE refers to small-footed bats (*Myotis leibii*), and PESU refers to tricolored bats (*Perimyotis subflavus*). Not included is any bridge-specific information that was acquired from VDOT's website (i.e., latitude and longitude of the bridge location, bridge length, width, and average daily traffic).

Table A4. Guano Sample DNA Sequencing and Species Identification Details

Sample ID	Bridge FedID	Forward Seq ID	Reverse Seq ID	Assembled Seq ID	% ID Forward Seq	% ID Reverse Seq	% ID Assembled	% HQ Forward Seq	%HQ Reverse Seq	%HQ Assembled Seq	% Pairwise	Max Length
BS610	5826	MYSE	MYNI	MYSE	94.87	97.40	87.34	16.20	22.30	12.20	100.00	164

(Table A4 Cont.)

Sample ID	Bridge FedID	Forward Seq ID	Reverse Seq ID	Assembled Seq ID	% ID Forward Seq	% ID Reverse Seq	% ID Assembled	% HQ Forward Seq	%HQ Reverse Seq	%HQ Assembled Seq	% Pairwise	Max Length
BS612	5826	MYSE	N/A	N/A	88.33	N/A	N/A	6.00	0.00	N/A	N/A	151
BS613	5826	MYYU	MYNI	N/A	91.86	98.61	N/A	2.60	14.60	N/A	N/A	155
BS614	5826	MYSE	MYSE	N/A	86.72	94.53	N/A	3.90	9.90	N/A	N/A	168
BS615	5826	MYYU	EPFU	N/A	97.87	84.62	N/A	4.90	6.80	N/A	N/A	157
BS616	5826	N/A	MYLU	N/A	N/A	98.53	N/A	22.50	N/A	N/A	N/A	153
H0101	16880	EPFU	MYGR	N/A	86.78	97.55	N/A	0.60	53.80	N/A	N/A	160
H0102	16880	MYGR	MYGR	MYGR	100.00	97.53	98.84	50.80	45.10	52.50	98.70	163
H0104	16880	MYGR	MYGR	MYGR	98.58	97.55	94.15	17.60	15.20	37.40	100.00	162
H0105	16880	MYGR	MYGR	MYGR	100.00	97.55	95.93	17.90	24.50	39.40	97.80	162
H0106	16880	MYGR	MYGR	MYGR	100.00	97.55	99.42	51.10	51.00	57.10	97.80	161
H0107	16880	MYGR	MYGR	MYGR	100.00	97.55	100.00	63.10	61.90	60.10	97.80	162
H0108	16880	N/A	MYGR	N/A	N/A	97.55	N/A	0.00	49.30	N/A	N/A	161
H0110	16880	MYGR	MYGR	MYGR	100.00	97.55	98.84	32.10	37.00	50.00	95.90	163
H0111	16880	MYGR	MYGR	MYGR	100.00	97.55	98.26	32.90	38.80	49.00	97.80	161
H0112	16880	N/A	RHTU	N/A	N/A	91.80	N/A	6.20	11.50	N/A	N/A	252
H0304	19088	EPFU	EPFU	EPFU	99.19	98.55	100.00	89.90	92.20	92.00	100.00	230
H0305	19088	MYGR	N/A	N/A	95.04	N/A	N/A	7.90	5.00	N/A	N/A	315
H0401	18980	MYAU	MYSE	N/A	91.46	94.53	N/A	1.60	10.80	N/A	N/A	165
H0402	18980	MYLU	MYSE	N/A	95.51	96.85	N/A	2.40	12.10	15.10	86.60	161
H0403	18980	MYLU	N/A	N/A	94.38	N/A	N/A	3.10	0.00	N/A	N/A	162
H0405	18980	MYSE	MYSE	N/A	93.80	94.49	N/A	5.50	6.70	N/A	N/A	158
H0406	18980	MYLU	MYSE	MYSE	95.51	97.64	84.57	3.10	14.30	15.20	89.20	169
H0407	18980	MYLU	MYSE	N/A	94.38	95.93	N/A	0.90	13.10	N/A	N/A	160
H0408	18980	MYSE	MYSE	MYSE	98.29	97.66	88.51	7.40	15.90	19.80	95.50	168
H0409	18980	MYYU	MYSE	N/A	95.77	96.88	N/A	1.30	12.50	6.80	100.00	171
H0410	18980	MYSE	MYSE	N/A	91.60	98.41	N/A	1.50	14.70	N/A	N/A	167

(Table A4 Cont.)

Sample ID	Bridge FedID	Forward Seq ID	Reverse Seq ID	Assembled Seq ID	% ID Forward Seq	% ID Reverse Seq	% ID Assembled	% HQ Forward Seq	%HQ Reverse Seq	%HQ Assembled Seq	% Pairwise	Max Length
H0411	18980	MYYU	MYSE	N/A	94.37	95.31	N/A	2.90	12.20	N/A	N/A	168
H0412	18980	MYYU	MYSE	N/A	95.71	94.53	N/A	3.00	10.20	N/A	N/A	164
H0413	18980	MYSE	MYSE	MYSE	93.16	96.88	88.31	5.40	15.40	16.50	88.10	163
H0414	18980	MYSE	MYLU	N/A	91.86	98.63	N/A	3.20	11.80	N/A	N/A	158
H0415	18980	MYSE	MYNI	N/A	88.28	97.22	N/A	3.90	12.00	N/A	N/A	160
H0416	18980	MYSE	MYSE	N/A	91.45	93.75	N/A	3.90	9.40	N/A	N/A	162
H0417	18980	MYYU	MYSE	N/A	93.85	96.09	N/A	4.50	11.10	N/A	N/A	168
H0501	17426	N/A	MYSE	N/A	N/A	95.35	N/A	0.70	10.80	N/A	N/A	168
H0505	17426	MYGR	MYGR	MYGR	100.00	97.48	93.66	20.00	28.40	28.80	91.00	265
H0602	17601	MYGR	MYGR	MYGR	100.00	97.58	94.92	34.00	29.70	46.50	97.80	162
H0605	17601	MYGR	MYGR	MYGR	100.00	97.55	100.00	67.30	81.80	79.40	96.20	163
H0606	17601	N/A	MYSE	N/A	N/A	91.22	N/A	7.60	9.50	N/A	N/A	244
H0607	17601	MYGR	MYGR	MYGR	99.30	97.56	99.42	64.50	70.90	66.70	98.90	165
H0608	17601	MYGR	MYGR	MYGR	100.00	97.55	99.41	28.90	52.40	52.60	94.20	163
H0609	17601	MYGR	MYGR	MYGR	100.00	97.55	99.46	69.50	72.80	80.30	96.20	162
H0610	17601	MYGR	MYGR	MYGR	99.29	97.56	95.93	33.10	34.00	41.40	96.20	162
H0611	17601	EPFU	EPFU	EPFU	96.45	98.01	99.43	57.30	52.60	62.20	100.00	160
H0612	17601	EPFU	EPFU	EPFU	96.53	99.31	100.00	84.10	87.00	87.20	100.00	153
H0613	17601	N/A	MYSE	N/A	N/A	94.81	N/A	4.50	0.00	N/A	N/A	170
H0614	17601	MYLU	EPFU	N/A	100.00	97.87	N/A	5.60	5.10	N/A	N/A	252
H0701	17389	MYSE	MYSE	N/A	91.60	95.93	N/A	2.30	11.20	N/A	N/A	163
H0702	17389	MYAU	MYNI	N/A	91.03	98.61	N/A	2.30	11.50	N/A	N/A	164
H0703	17389	MYVE	MYSE	N/A	95.08	97.66	N/A	0.00	19.40	N/A	N/A	169
H0704	17389	MYAU	MYSE	N/A	91.46	97.66	N/A	1.60	17.50	N/A	N/A	169
H0705	17389	MYYU	N/A	N/A	97.18	N/A	N/A	0.00	5.60	N/A	N/A	168
H0706	17389	MYYU	MYVO	N/A	97.18	97.26	N/A	1.30	6.80	N/A	N/A	160

(Table A4 Cont.)

Sample ID	Bridge FedID	Forward Seq ID	Reverse Seq ID	Assembled Seq ID	% ID Forward Seq	% ID Reverse Seq	% ID Assembled	% HQ Forward Seq	%HQ Reverse Seq	%HQ Assembled Seq	% Pairwise	Max Length
H0707	17389	MYVE	MYSE	N/A	95.38	94.44	N/A	0.00	16.20	N/A	N/A	169
H0708	17389	MYYU	MYNI	N/A	93.97	95.77	N/A	3.30	6.90	N/A	N/A	166
H0709	17389	MYSE	MYSE	N/A	93.69	96.88	N/A	1.60	10.60	N/A	N/A	170
H0710	17389	MYSE	RHMI	N/A	90.08	98.11	N/A	2.30	9.20	N/A	N/A	162
H0711	17389	MYSE	MYSE	N/A	92.79	93.13	N/A	0.80	16.30	N/A	N/A	170
H0712	17389	MYSE	MYSE	N/A	92.79	97.66	N/A	3.10	13.80	N/A	N/A	169
H0713	17389	MYSE	MYSE	N/A	92.79	97.67	N/A	0.80	11.10	N/A	N/A	161
H0714	17389	MYSE	MYSE	N/A	92.31	95.31	N/A	1.50	8.40	N/A	N/A	169
H0715	17389	MYSE	MYSE	N/A	90.57	98.44	N/A	5.50	13.60	6.70	100.00	166
NR301	2993	N/A	MYGR	MYGR	N/A	90.30	98.55	8.60	15.30	18.20	87.70	163
NR302	2993	EPFU	EPFU	EPFU	95.74	99.31	100.00	66.90	62.70	74.90	97.10	156
NR303	2993	EPFU	EPFU	EPFU	97.90	99.31	100.00	87.00	85.80	86.40	100.00	221
NR304	2993	MYMY	MACA	N/A	82.96	95.24	N/A	5.30	3.20	N/A	N/A	256
NR305	2993	MYGR	MYGR	MYGR	100.00	98.18	97.08	23.00	23.10	33.20	94.10	242
NR307	2993	N/A	MYSE	N/A	N/A	91.45	N/A	7.90	11.60	N/A	N/A	246
NR308	2993	N/A	MYGR	N/A	N/A	90.74	N/A	5.00	10.40	N/A	N/A	162
NR309	2993	EPFU	EPFU	EPFU	90.74	95.59	93.46	12.00	12.90	25.30	94.00	165
NR311	2993	MYGR	MYGR	MYGR	100.00	97.55	97.67	30.00	36.60	46.50	97.80	163
NR312	3022	MYGR	MYGR	MYGR	99.29	97.55	99.42	60.30	63.90	62.60	98.90	161
NR313	3022	MYGR	MYGR	MYGR	100.00	96.93	95.74	29.10	23.60	42.40	97.80	161
NR314	3022	MYGR	MYGR	MYGR	100.00	96.93	92.91	17.90	19.90	38.40	96.70	162
NR315	3022	EPFU	EPFU	EPFU	96.45	98.03	100.00	82.00	69.60	74.90	100.00	153
NR317	3022	EPFU	EPFU	EPFU	97.90	98.01	100.00	83.20	84.90	86.80	100.00	266
NR318	3022	EPFU	EPFU	EPFU	100.00	99.31	100.00	84.70	82.60	87.20	100.00	155
NR319	3022	EPFU	EPFU	EPFU	100.00	98.04	100.00	88.30	81.20	84.50	100.00	155
NR320	3022	EPFU	EPFU	EPFU	96.48	98.03	98.85	46.70	81.80	70.10	100.00	154

(Table A4 Cont.)

Sample ID	Bridge FedID	Forward Seq ID	Reverse Seq ID	Assembled Seq ID	% ID Forward Seq	% ID Reverse Seq	% ID Assembled	% HQ Forward Seq	%HQ Reverse Seq	%HQ Assembled Seq	% Pairwise	Max Length
NR321	3022	EPFU	EPFU	EPFU	97.79	99.31	100.00	44.80	89.10	79.30	100.00	273
NR322	3022	EPFU	EPFU	EPFU	97.08	98.03	96.55	13.70	28.90	35.80	97.50	168
NR323	3022	EPFU	EPFU	EPFU	97.20	98.04	100.00	89.10	84.90	90.40	100.00	235
NR324	3022	EPFU	EPFU	EPFU	96.45	98.68	100.00	81.50	80.30	80.60	100.00	156
NR325	3023	MYGR	MYGR	N/A	97.89	92.02	N/A	10.60	16.90	24.50	90.10	222
NR326	3023	N/A	MYGR	N/A	N/A	91.51	N/A	9.90	10.90	22.20	77.70	163
NR327	3023	EPFU	EPFU	EPFU	100.00	97.42	100.00	86.90	87.70	84.50	100.00	152
NR328	3023	MYGR	MYGR	MYGR	99.29	96.96	89.36	15.00	21.60	32.80	93.80	162
NR329	3023	EPFU	EPFU	EPFU	96.77	99.31	99.43	85.00	89.10	87.20	100.00	153
NR330	3023	MYGR	MYGR	MYGR	97.87	95.36	90.21	15.00	11.50	30.20	96.80	164
NR332	3023	MYSE	MYSE	MYSE	91.43	96.03	85.06	4.20	11.70	11.50	84.70	163
NR333	3023	EPFU	EPFU	EPFU	96.53	98.69	100.00	85.90	83.60	91.30	100.00	232
NR334	3023	EPFU	EPFU	EPFU	97.90	98.03	100.00	84.10	83.30	88.80	100.00	458
NR335	3023	EPFU	EPFU	N/A	96.32	98.01	N/A	10.80	15.30	26.20	98.70	152
NR336	2992	MYLU	N/A	N/A	89.13	N/A	N/A	2.10	11.40	N/A	N/A	161
NR337	2992	EPFU	EPFU	N/A	95.93	97.14	N/A	11.40	20.30	25.80	84.50	162
NR338	2992	MYSE	MYSE	MYSE	98.64	98.68	100.00	74.80	80.70	89.30	97.90	155
NR339	2992	PTNE	EPFU	N/A	100.00	88.03	N/A	4.20	8.50	N/A	N/A	166
NR340	2992	N/A	MYSE	N/A	N/A	85.06	N/A	3.80	7.50	N/A	N/A	166
NR341	3023	N/A	MYLU	N/A	N/A	92.86	N/A	8.30	15.00	N/A	N/A	246
NR701	8775	N/A	MYSE	N/A	N/A	91.78	N/A	8.70	7.10	N/A	N/A	259
NR703	8775	EPFU	EPFU	N/A	100.00	99.28	N/A	41.70	47.70	54.70	98.50	260
P0301	10709	EPFU	EPFU	EPFU	95.71	98.03	100.00	85.30	86.20	86.10	98.80	154
P0302	10709	MYSE	MYSE	N/A	86.01	95.42	N/A	4.80	8.30	N/A	N/A	166
P0303	10709	MYYU	MYAU	N/A	93.44	96.35	N/A	5.00	10.80	N/A	N/A	171
P0501	10893	MYYU	MYSE	N/A	93.44	90.77	N/A	15.40	27.00	14.70	55.40	170

(Table A4 Cont.)

Sample ID	Bridge FedID	Forward Seq ID	Reverse Seq ID	Assembled Seq ID	% ID Forward Seq	% ID Reverse Seq	% ID Assembled	% HQ Forward Seq	%HQ Reverse Seq	%HQ Assembled Seq	% Pairwise	Max Length
S0303	16604	N/A	MYSE	N/A	N/A	84.42	N/A	9.70	9.30	N/A	N/A	257
S0304	16604	N/A	MYSE	N/A	N/A	86.96	N/A	6.10	6.50	N/A	N/A	296
S0306	16604	N/A	MYGR	N/A	N/A	83.64	N/A	5.60	11.30	N/A	N/A	251
S0307	16604	MYGR	MYGR	MYGR	99.29	99.30	97.18	17.20	44.60	25.20	97.50	379
S0311	16604	MYGR	MYGR	MYGR	99.29	100.00	98.82	79.60	74.80	83.70	97.30	248
S0312	16604	N/A	MYVE	N/A	N/A	92.42	N/A	5.10	7.40	N/A	N/A	247
S0402	16612	MYSE	MYSE	N/A	90.41	93.15	N/A	1.40	7.90	N/A	N/A	166
S0404	16612	MYGR	N/A	N/A	95.74	N/A	N/A	12.10	17.90	21.00	85.40	269
S0406	16612	MYSE	MYSE	N/A	94.96	92.86	N/A	2.10	10.60	N/A	N/A	250
S0408	16612	MYGR	MYGR	MYGR	100.00	98.15	100.00	90.80	88.00	93.80	98.90	161
S0501	16612	MYGR	MYGR	MYGR	93.62	91.02	95.00	15.00	17.60	23.90	88.00	165
S0503	16612	MYGR	MYGR	MYGR	99.30	97.55	100.00	81.50	82.40	88.40	97.20	164
S0504	16612	MYGR	MYGR	MYGR	100.00	97.59	99.42	57.60	56.00	62.60	96.30	164
S0718	25321	MYGR	N/A	N/A	91.49	N/A	N/A	10.80	0.00	N/A	N/A	170
S0722	16323	MYGR	N/A	N/A	88.99	N/A	N/A	0.00	0.00	N/A	N/A	172
S0723	16323	N/A	NYHU	N/A	N/A	93.48	N/A	8.70	4.80	N/A	N/A	187
S0903	25687	MYVE	MYSE	N/A	92.42	95.45	N/A	5.50	9.50	N/A	N/A	175
S0905	25687	MYGR	MYGR	MYGR	100.00	100.00	100.00	85.20	85.70	88.40	98.80	246
S0906	25687	N/A	MYSE	N/A	N/A	93.23	N/A	7.30	15.60	N/A	N/A	256
S0907	25687	EPFU	EPFU	EPFU	99.16	100.00	100.00	84.30	95.20	90.70	98.60	160
S0909	25687	EPFU	EPFU	EPFU	94.03	97.89	96.28	17.30	24.10	24.90	91.20	245
S1102	18486	MYYU	MYSE	N/A	98.21	96.60	N/A	4.30	12.80	N/A	N/A	249
S1104	18486	MYGR	MYGR	MYGR	100.00	100.00	100.00	75.80	80.70	81.50	96.80	253
S1202	18519	N/A	MYSE	N/A	N/A	94.74	N/A	4.20	9.20	N/A	N/A	171
S1203	18519	N/A	MYLU	N/A	N/A	86.36	N/A	5.80	9.90	N/A	N/A	169
S1204	18519	MYYU	MYSE	N/A	92.42	94.57	N/A	5.50	11.00	N/A	N/A	172

(Table A4 Cont.)

Sample ID	Bridge FedID	Forward Seq ID	Reverse Seq ID	Assembled Seq ID	% ID Forward Seq	% ID Reverse Seq	% ID Assembled	% HQ Forward Seq	%HQ Reverse Seq	%HQ Assembled Seq	% Pairwise	Max Length
S1206	18519	N/A	MYSE	N/A	N/A	93.80	N/A	5.80	7.30	N/A	N/A	169
S1207	18519	N/A	RHMI	N/A	N/A	96.23	N/A	6.40	5.40	N/A	N/A	250
S1208	18519	MYSE	N/A	N/A	91.54	N/A	N/A	5.00	10.00	N/A	N/A	248
S1209	18519	MYSE	N/A	N/A	86.36	N/A	N/A	5.80	9.90	N/A	N/A	272
UC101	10697	N/A	MYLU	N/A	N/A	89.09	N/A	9.60	5.60	N/A	N/A	326
UC105	10697	MYGR	MYGR	MYGR	100.00	97.93	100.00	83.30	74.10	86.90	96.30	166
UC107	10697	N/A	MYRI	N/A	N/A	94.12	N/A	3.20	2.60	N/A	N/A	166
UC108	10697	N/A	MYMU	N/A	N/A	94.12	N/A	0.00	6.30	N/A	N/A	165

Note. Species code names: NYHU, evening bat (*Nycticeius humeralis*); MYSE, northern long-eared bat (*Myotis septentrionalis*); MYLU, little brown bat (*Myotis lucifugus*); EPFU, big brown bat (*Eptesicus fuscus*); MYGR, gray bat (*Myotis grisescens*) Codes for unlikely species matches from low-quality sequences: PTNE, great flying fox (*Pteropus neohibernicus*); MYMY, Greater mouse-eared bat (*Myotis myotis*), MYYU, Yuma myotis (*Myotis yumanesis*); MYVO, long-legged myotis (*Myotis volans*); MYNI, black myotis (*Myotis nigricans*); MYVE, cave myotis (*Myotis velifer*); MYAU, southeastern myotis (*Myotis austoriparius*); MYRI, Ridley's bat (*Myotis ridleyi*); MYMU, whiskered myotis (*Myotis muricola*); RHTU, black-winged little yellow bat (*Rhogeessa tumida*); RHMI, least yellow bat (*Rhogeessa mira*); MACA, California leaf-nosed bat (*Macotus californicus*) The Sequence ID fields show the species with the highest percent match in GenBank. The % ID fields refer to the percentage of species identity match from GenBank, the % HQ fields show the percentage of high-quality bases, % Pairwise is the average percent identity of bases over the alignment (if the N/A sample did not assemble), and Max Length refers to the maximum number of base pairs in a sequence. Unassembled samples that had no ID match for both the reverse and forward sequences were excluded from this table (n = 99). Seq, sequence.

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Table A5. AICc Table of Models for Big Brown Bat (*Eptesicus fuscus*) Predicting Occupancy Based on Species Identification From Guano Sampled in VDOT's Bristol District, Virginia, in 2019 via DNA Barcoding Methods

Detection Prediction Parameter(s)	Occupancy Prediction Parameter(s)	K	AICc	Δ AICc	AICc Wt
% HQ	% Karst	4	83.41	0	0.3573
% HQ	1	3	86.45	3.038	0.07824
% HQ	Minimum LFI	4	86.6	3.188	0.07257
% HQ	Gray Bat Occupancy	4	86.81	3.4	0.06527
% HQ	Latitude	4	86.87	3.461	0.06331
% HQ	Maximum LFI	4	87.1	3.69	0.05646
% HQ	% Developed Landcover	4	87.52	4.109	0.04579
% HQ	% Karst + % Developed + Gray Bat Occupancy	6	88.06	4.645	0.03503
% HQ	Minimum LFI + % Karst + % Developed	6	88.76	5.348	0.02465
% HQ	% Low Vegetation	4	88.86	5.45	0.02342
% HQ	Bridge Width (ft)	4	89.06	5.645	0.02124
% HQ	Maximum Elevation	4	89.07	5.658	0.02111
% HQ	% Forest	4	89.12	5.712	0.02055
% HQ	Bridge Length (ft)	4	89.23	5.817	0.0195
% HQ	Year Bridge Was Built	4	89.23	5.814	0.01953
% HQ	Minimum Elevation	4	89.26	5.843	0.01925
% HQ	Minimum LFI + % Karst + Gray Bat Occupancy	6	89.28	5.868	0.019
% HQ	Mean Elevation	4	89.4	5.985	0.01792
% HQ	Underdeck Material	5	91.22	7.812	0.007188
% HQ	Bridge Type	5	91.25	7.838	0.007098
% HQ	Minimum LFI + % Karst + % Developed + Gray Bat Occupancy	7	92.38	8.965	0.004039
% HQ	Avg Daily Traffic	4	95.07	11.66	0.001052
% HQ	Stream Order	7	96.74	13.33	0.0004559
1	Latitude	3	133.4	49.96	5.057E-12
1	Minimum LFI	3	134.2	50.8	3.324E-12
1	Maximum LFI	3	134.3	50.87	3.219E-12
1	1	2	134.4	50.98	3.037E-12
1	Maximum Elevation	3	135.6	52.21	1.645E-12
1	Bridge Width (ft)	3	135.8	52.41	1.49E-12
1	Mean Elevation	3	135.9	52.45	1.461E-12
1	% Karst	3	136.2	52.76	1.249E-12
1	Gray Bat Roosting	3	136.4	52.97	1.123E-12
1	Minimum Elevation	3	136.5	53.05	1.077E-12
1	% Forest Landcover	3	136.6	53.22	9.924E-13
1	Bridge Length (ft)	3	136.7	53.32	9.45E-13
1	% Developed Landcover	3	136.7	53.26	9.739E-13
1	% Low Vegetation Landcover	3	136.8	53.36	9.272E-13
1	Year Bridge Was Built	3	137	53.57	8.338E-13
1	Bridge Type	4	137.7	54.27	5.877E-13
1	Avg Daily Traffic	3	138	54.61	4.953E-13
1	Underdeck Material	4	138.5	55.05	3.975E-13
1	Stream Order	6	140.4	56.96	1.531E-13

Note. AIC, Akaike information criterion; Avg, average; LFI, landform index; Max, maximum; Min, minimum

Table A6. AICc Table of Models for Gray Bat (*Myotis grisescens*) Predicting Occupancy Based on Species Identification From Guano Sampled in VDOT's Bristol District, Virginia, in 2019 via DNA Barcoding Methods

Detection Prediction Parameter	Occupancy Prediction Parameter(s)	K	AICc	Δ AICc	AICc Wt
% HQ	% Karst	4	147.6	0	0.2347
% HQ	Bridge Width (m)	4	148.3	0.7069	0.1648
% HQ	MCD Within 2 km	4	149.3	1.757	0.09753
% HQ	MCD Within 2 km	4	149.3	1.757	0.09753
% HQ	MCD Within 2 km + Big Brown Bat Occupancy	5	149.8	2.288	0.07479
% HQ	Bridge Length (m)	4	149.8	2.234	0.07683
% HQ	MCD Within 2 km + % Karst	5	150.1	2.568	0.06502
% HQ	1	3	150.3	2.781	0.05845
% HQ	% Karst + Big Brown Bat Occupancy	5	150.5	2.948	0.05375
% HQ	% Karst + Stream Order	8	151.1	3.53	0.04018
% HQ	Big Brown Bat Occupancy	4	153.1	5.555	0.0146
% HQ	Underdeck Material	5	153.6	6.021	0.01157
% HQ	Bridge Type	5	155	7.442	0.005682
% HQ	Stream Order + MCD Within 2 km	8	155.9	8.318	0.003668
% HQ	Stream Order	7	158.9	11.33	0.0008126
1	% Karst	3	170.7	23.12	2.239E-06
1	% Karst + Mean Elevation	4	171.2	23.65	1.715E-06
1	Bridge Width (m) + % Karst	4	173.4	25.82	5.818E-07
1	MCD Within 2 km	3	174.1	26.5	4.14E-07
1	Min Elevation	3	174.7	27.15	2.99E-07
1	Mean Elevation	3	174.8	27.25	2.843E-07
1	Max Elevation	3	175	27.47	2.544E-07
1	1	2	175.3	27.77	2.189E-07
1	Bridge Length (m)	3	175.9	28.36	1.63E-07
1	Latitude	3	176.2	28.6	1.445E-07
1	Bridge Width (m)	3	176.6	29	1.184E-07
1	% Developed Landcover	3	177.1	29.59	8.82E-08
1	Underdeck Material	4	177.3	29.76	8.115E-08
1	Big Brown Bat Occupancy	3	177.6	30.05	7.015E-08
1	% Forest Landcover	3	177.7	30.11	6.795E-08
1	Max LFI	3	177.8	30.21	6.48E-08
1	Min LFI	3	177.8	30.26	6.291E-08
1	% Low Veg Landcover	3	177.9	30.31	6.138E-08
1	Year Bridge Was Built	3	177.9	30.33	6.092E-08
1	Bridge Type	4	179.5	31.94	2.72E-08

Detection Prediction Parameter	Occupancy Prediction Parameter(s)	K	AICc	Δ AICc	AICc Wt
1	Stream Order	6	179.9	32.35	2.215E-08
% HQ	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy	12	183	35.49	4.623E-09
1	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy	11	205.6	58.08	5.745E-14
1	Avg Daily Traffic	3	207.3	59.77	2.46E-14
1	Distance to Hibernaculum (m)	3	207.3	59.77	2.469E-14
1	Distance to Maternity Colony (m)	3	207.3	59.77	2.46E-14
% HQ	Distance to Hibernaculum (m)	4	210.1	62.54	6.166E-15
% HQ	Distance to Maternity Colony (m)	4	210.1	62.55	6.143E-15
% HQ	Distance to Hibernaculum (m) + MCD Within 2 km	5	213.1	65.58	1.349E-15
1	Latitude + Underdeck Material + Bridge Type + Avg Daily Traffic + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy	12	245.7	98.18	1.127E-22
1	MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst	12	246.5	98.97	7.578E-23
% HQ	Latitude + Underdeck Material + Bridge Type + Avg Daily Traffic + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy	13	253.4	105.9	2.381E-24
1	MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Mean Elevation	13	254.2	106.7	1.595E-24
% HQ	MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst	13	254.2	106.7	1.601E-24
% HQ	MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Mean Elevation	14	263.2	115.7	1.772E-26
1	MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Min LFI + Max Elevation	14	263.3	115.7	1.755E-26
1	MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Max LFI + Min Elevation	14	263.3	115.7	1.766E-26
% HQ	MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Min LFI + Max Elevation	15	273.9	126.3	8.602E-29
% HQ	MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Max LFI + Min Elevation	15	273.9	126.3	8.658E-29

Detection Prediction Parameter	Occupancy Prediction Parameter(s)	K	AICc	Δ AICc	AICc Wt
1	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy + MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst	21	427	279.5	4.81E-62
% HQ	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy + MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst	22	497.2	349.7	2.744E-77
1	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy + MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Mean Elevation	22	497.2	349.7	2.744E-77
1	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy + MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Min LFI + Max Elevation	23	614.2	466.7	1.08E-102
1	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy + MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Max LFI + Min Elevation	23	614.2	466.7	1.08E-102
% HQ	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy + MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Mean Elevation	23	614.2	466.7	1.08E-102
% HQ	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy + MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Min LFI + Max Elevation	24	848.2	700.7	1.66E-153
% HQ	Latitude + Underdeck Material + Bridge Type + Bridge Width (m) + Bridge Length (m) + Year Bridge Was Built + Big Brown Bat Occupancy + MCD Within 2 km + Stream Order + Distance to Hibernaculum (m) + Distance to Maternity Colony (m) + % Forest Landcover + % Developed Landcover + % Karst + Max LFI + Min Elevation	24	848.2	700.7	1.66E-153

Note. AIC, Akaike information criterion; Avg, average; LFI, landform index; MCD, mean cave density within a 2-km radius; Max, maximum; Min, minimum