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A noninvasive genetic technique using guano for identification of *Corynorhinus townsendii* (Townsend's big-eared bat) maternity roosts

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Abstract.—The use of DNA from fecal samples can allow for a better understanding of the ecology of a species without capturing and handling the animals. This is particularly useful for cryptic and elusive animals, such as bats. Being able to identify critical habitat, such as maternity roosts, for bat species in areas where they depend on abandoned mines that could be slated for closure is necessary to enact appropriate protections for such roosts. In particular, *Corynorhinus townsendii* commonly uses abandoned mines for maternity roosts. Further, maternity roosts are difficult to identify through visual surveys when only one or a few surveys are performed before mine closures. We have developed a method for identification of *C. townsendii* maternity roosts that uses fecal DNA extracted from fresh guano collected from plastic sheeting placed at a mine entrance. We provide a multiplex PCR assay to amplify a control region fragment found only in *C. townsendii,* as well as a Y-linked protein (DBY) to detect male *C. townsendii* DNA. The purpose of this study was to identify a temporal shift in the presence of male *C. townsendii* bats, which can be useful to identify a maternity roost. This method allows for noninvasive identification of critical habitat for this species and reduces the effort and safety risk of entering mines on the part of biologists.

Resumen.—El uso de ADN de muestras fecales permite comprender mejor la ecología de una especie en particular, sin tener que capturar y/o manejar a los animales. Este método es especialmente útil, cuando se trata de animales crípticos y esquivos, tales como los murciélagos. Es necesario identificar hábitats críticos para las especies de murciélagos, tales como refugios de maternidad construidos en áreas de minas abandonadas que podrían cerrarse, con el fin de adoptar medidas apropiadas que protejan los refugios. Específicamente, se descubrió que el *Corynorhinus townsendii* suele utilizar minas abandonadas como refugios de maternidad. Debido a que, es difícil identificar refugios a través de métodos visuales cuando únicamente se llevan a cabo uno o pocos estudios antes del cierre de una mina, desarrollamos un método que utiliza ADN fecal para identificar refugios de maternidad de *C. townsendii* mediante la recolección del guano fresco que queda en las láminas de plástico al ingreso de una mina. Llevamos a cabo un ensayo de Cadena de Polimerasa múltiple (PCR multiplex, por sus siglas en inglés) para amplificar un fragmento de la región control mitocondrial de *C. townsendii.* De igual forma, amplificamos una proteína de unión al cromosoma Y (DBY) para detectar el ADN de *C. townsendii* machos. El objetivo de este estudio fue identificar tendencias temporales ante la presencia de murciélagos *C. townsendii* machos, que podrían ser útiles para identificar refugios de maternidad. Este método permite identificar de manera no invasiva el hábitat crítico de esta especie, a la vez que, reduce el esfuerzo y el riesgo de los biólogos que ingresan a las minas.

Bats are cryptic and elusive mammals that face many challenges to their population viability. Most of these challenges are anthropogenic and include impacts from wind energy, abandoned-mine closures, pesticides, and pathogens transported by humans into naïve populations (e.g., white-nose syndrome) (Cryan and Barclay 2009, O'Shea et al. 2016). In a quest to understand the impacts of these challenges on bat populations, biologists strive to locate and identify critical bat habitat (e.g., maternity roosts or breeding sites) to monitor for viability and protect from elimination. However, it may be difficult to characterize critical habitat given the elusive and cryptic nature of bat species. Further, some bat species may be negatively impacted by human visitation to their roosts (McCracken 1989). Thus, the utility of noninvasive approaches for identification of critical bat habitat is appealing. The amplification of

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bat DNA from guano samples has been widely used to detect bat presence, identify individuals, estimate population size, and model population structure (Puechmaille et al. 2007, Boston et al. 2012, Korstian et al. 2015, Walker et al. 2016, Afonso et al. 2017, Oyler-McCance et al. 2018, Zarzoso-Lacoste et al. 2018) as well as to understand diet and feeding behaviors across habitats (Clare et al. 2009, 2011, Maslo et al. 2017).

In the western United States, there are few bat species that are considered to be of conservation concern. However, Townsend's big-eared bat (*Corynorhinus townsendii*) is listed as a vulnerable or threatened species by many western states. In much of this region, *C. townsendii* is considered dependent on caves and abandoned mines for roosting (Twente 1960, Ports and Bradley 1996, Pierson et al. 1999, Sherwin et al. 2000). Sherwin et al. (2009) categorized *C. townsendii* as a Guild 1 (cavernicolous) species, indicating that ultimate survival is dependent on availability of subterranean habitat. In many parts of its western range, this species has become increasingly associated with abandoned mines, which tend to be located more broadly and in greater local densities than caves (Sherwin et al. 2009). Its increasing dependence on abandoned mines is notable because abandoned mines in the United States have been targeted for large-scale closure programs due to their inherent environmental hazards and the risk they pose to the public (Sherwin et al. 2003).

A noninvasive genetic technique to determine whether *C. townsendii* bats are or have recently been roosting in abandoned mines, and whether or not there is a maternity roost, could be a nonintrusive and efficient method of surveillance. Species-specific fecal DNA assays can be inexpensive relative to DNA barcoding approaches. When particular research or management questions only require detection of a single target species in areas where multiple species may occupy (e.g., bat roosts), species-specific assays can be more sensitive. Other studies have developed species-specific assays and included markers to determine sex (Afonso et al. 2017, Oyler-McCance et al. 2018, Zarzoso-Lacoste et al. 2018); however, none have been developed for our target species. Some previous studies sought to obtain individual identification with microsatellites, and thus, they did not pool samples (Zarzoso-Lacoste et al. 2018). Barcoding samples can be costly; our aim was to minimize cost through sample pooling and a simple mtDNA test that could be rapidly assessed through qPCR, which can be accomplished in most standard PCR labs. This study serves as a proof-of-concept for identifying critical habitat for bats. We sampled a maternity roost of *C. townsendii* through collection of guano from plastic sheeting placed at roost entrances, thus minimizing disturbance to the bats themselves. We aim to show that this species-specific, noninvasive sampling can give insight into whether *C. townsendii* inhabits the mine and whether the mine is a maternity roost.

METHODS

Sample Collection

Guano for this study was collected at a large mine complex located on the eastern side of the Schell Mountains, roughly 40 miles northeast of Ely, White Pine County, Nevada, USA. This maternity roost of Townsend's big-eared bats was first documented during preclosure abandoned-mine surveys conducted by the Nevada Department of Wildlife in 2010; monitoring and research have been conducted and maintained at the mine since that time (Afonso et al. 2017, Oyler-McCance et al. 2018, Zarzoso-Lacoste et al. 2018). An aggregation of ≥200 mature females (i.e., a maternity roost) spend April through September birthing and rearing pups in the upper levels of this mine. All openings to this mine have been protected with bat-compatible closures, and the maternity portion of the mine is immediately accessible through a gated opening (3.5 m high and 9 m wide). This large opening is the primary entry/exit used by individual Townsend's big-eared bats throughout the maternity season (McTheny 2016). Drop cloths were placed immediately outside of this opening to capture guano excreted by individuals as they passed through the bat gate. Drop cloths were placed on at least 2 nights of each week between 1 June and 20 August 2014. Technicians wore latex gloves and used sterilized forceps to collect guano pellets the morning after each deployment. Guano from each collection date was placed into individual sterilized vials for storage.

Additional guano (1–2 pellets) was collected from males of 4 other species (*Myotis californicus*, *Eptesicus fuscus*, *Parastrellus hesperus*,

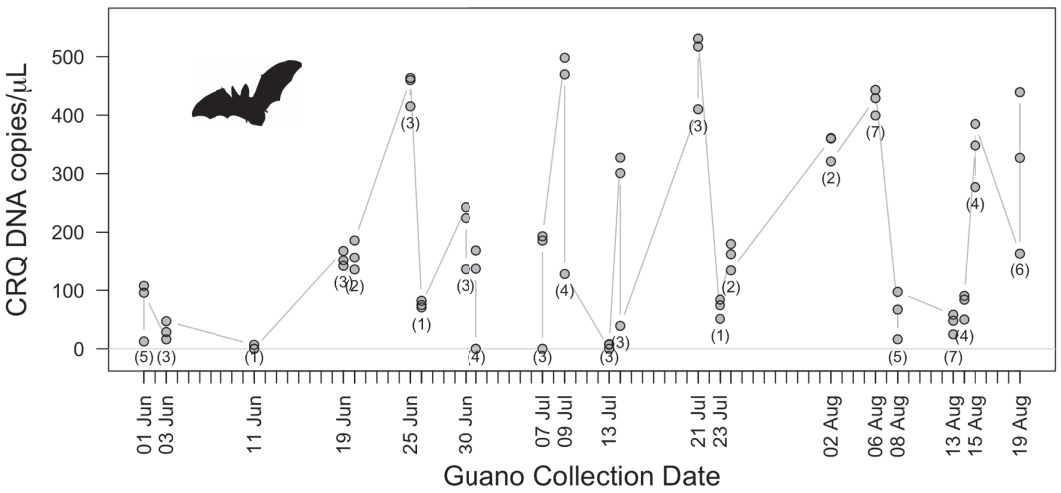

Fig. 1. *Corynorhinus townsendii* DNA was detected throughout the study and varied over time. Each point represents a qPCR replicate of DNA extracted from pooled guano pellets (3 qPCR replicates per collection date). The number of guano pellets collected are shown in parentheses at each date. Dates of guano collected through the 2014 field season are represented on the *x*-axis, and DNA concentration of species-specific CRQ *Corynorhinus townsendii* DNA measured in copies/µL is shown on the *y*-axis. The bat silhouette was obtained from PhyloPic (image by Yan Wong, Public Domain Dedication 1.0; http://phylopic.org/name/51ebafcf-6577-4399-bc79-23981ac5533b).

and *Myotis ciliolabrum*—specific identification confirmed using acoustic detectors) that were captured during netting surveys as part of a broader study in the area to test for cross-amplification of our designed primers, which were aimed to be *C. townsendii* specific. All guano was immediately placed into a field cooler and then transferred to a −80 °C freezer for long-term storage. Due to the remoteness of the field site, guano was often stored in the field cooler (between −2 °C and −17 °C based on ambient conditions) for 5–7 d prior to placement in long-term storage pending DNA extractions.

DNA Extraction

Guano pellets from plastic sheets placed at the mine entrances on each collection date were pooled into a 2-mL tube (range 1 to 7 pellets; Fig. 1). The pooled samples were then homogenized using the TissueLyser LT (Qiagen) set at 30 Hertz for 6 min (Smith et al. 2011). For nontarget species samples, we extracted DNA from one guano pellet to test for assay specificity. After disruption, DNA was extracted from the pulverized guano following the 200-mg DNeasy *mericon* Food Kit® protocol (Qiagen). We chose this kit for DNA extraction because it performed well with DNA extraction from bird guano to identify prey (Zarzoso-Lacoste et al. 2013) as well as eDNA extraction from pelleted turbid water samples (Williams et al. 2017). Extractions were stored in a −80 °C freezer. To monitor for contamination, a negative control was included for each extraction round using only kit reagents.

Amplification

Two sets of primers and probes were designed using AlleleID for species (CRQ) and sex (DBY) identification (Kearse et al. 2012). For species detection of *C. townsendii*, primers and a probe were designed to target a species-specific fragment of the mitochondrial control region (CRQF 59 TCC CAG ATT AGC ACT AAC; CRQR 59 GAG GAT CAT GTG TCT TTA G; CRQ Probe 5HEX/ ATC TGC TCC AAT CAC ATC CTT CCA /3BHQ_1). These primers were designed using control region (CRQ) sequences initially from 236 individuals of *C. townsendii* from across the range (Piaggio and Perkins 2005). Due to extensive genetic variability, regions for primers and probes that worked across *C. townsendii* subspecies could not be identified. Our assay targets the broadest taxonomic range that could be covered with one set of primers and probes across *C. t. townsendii*, which is the most broadly ranging subspecies (Piaggio and Perkins 2005). For sex identification of male *C. townsendii*,

primers and a probe were designed to target a fragment of the Y-linked protein (DBY) gene (DBYQF 59 AGG TCT TTG ATT CTA ATT TAC A; DBYQR 59 AGG AGA TTG GAT ATT GTT TAC; DBYQ Probe 56FAM/ CGT CCA TAC CTC CCA GTT TCC T /3BHQ_1) by generating DBY sequences from multiple *C. townsendii* using previously published primers HDBY7R (Hellborg and Ellegren 2004) and HDBY7F (Lim et al. 2008) and then aligning with *M. daubentonii* DBY sequences (Genbank accession HM596899.1) to identify regions unique to *C. townsendii*. Designed primers and probe were tested in silico through the U.S. National Center for Biotechnology Information (NCBI) GenBank using Primer-Blast.

Each qPCR reaction multiplexed the designed primers and probes and consisted of a 25-μL reaction containing 15 μL of Taqman Multiplex Mastermix (Life Technologies, Applied Biosystems), 1 μL of each CRQ primer (10 μM), 1 μL CRQ probe (2.5 μM), 0.5 μL of each DBYQ primer (10 μM), 0.5 μL of DBYQ probe (2.5 μM), 5.5 μL of distilled water, and 5 μL of DNA extract. Samples were run on a Bio-Rad real-time PCR thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA). The qPCR program involved 10 min at 95 °C followed by 55 cycles of 95 °C for 15 sec and 1 min at 56.5 °C. We ran standard curves of synthetic gene sequences of our target amplicons for *C. townsendii* COTO CRQ (5′TCCCAGATTAGCACTAACTTTACCACGGACCTGTACACTCAAACCAAAATCTGCTCCAATCACATCCTTCCACTCTAAAGACACATGATCCTC3′) and DBY (5′AGGTCTTTGATTCTAATTTACAAAATTGTACTTATTTTTTAGGAAACTGGGAGGTATGGACGCCGTAAACAATATCCAATCTCCT3′) (each at 4 nmole Ultramer® DNA Oligo, IDT). We developed a standard curve from 1:10 serial dilutions of the standard (1×10^5 DNA copies/μL to 1 DNA copy/μL) to evaluate our qPCR assay. We determined our limit of quantification (LOQ) to be the lowest dilution in which 8 qPCR replicates amplified and our limit of detection (LOD) as the standard dilution that was 10-fold below the LOQ (CRQ: E = 90.2%, R^2 = 0.997, Slope = −3.583; DBY: E = 90.0%, R^2 = 0.996, Slope = −3.588). We determined the LOD and LOQ using a method by Klymus et al. (2015) adapted from the MIQE guidelines (Taberlet et al. 1996, Bustin et al. 2009, Klymus et al. 2015). We determined that the LOD for CRQ was 1 copy/μL and for DBY was 10 copies/μL.

Each qPCR run included a no-template control of PCR reagents and extraction negative controls to monitor for amplification, which would demonstrate contamination. Further, positive controls of DNA extracted from fecal samples of *C. townsendii* males and females were included on each qPCR run to ensure that our PCR assay performed accurately. Each extracted guano slurry sample from the field was subsampled for 3 replicate qPCR reactions to account for stochasticity in DNA amplification due to low-quality/low-quantity DNA (Taberlet et al. 1996). We ran 5 qPCR replicates on guano extractions from the nontarget species for in vitro primer specificity testing. A sample was considered positive if at least 1 out of 3 replicates was positive (above LOD).

Analysis

The DNA concentration for CRQ (species identification) and species-specific DBY (male) for each qPCR replicate was plotted against the date the samples were collected. Because DBY only amplifies with CRQ in *C. townsendii* individuals that have a Y chromosome (males), if the mine was used as a *C. townsendii* maternity roost, we would expect to lack DBY amplification but have positive CRQ amplification in the temporal period before pups are volant. This would imply that we had detected primarily females. However, we would expect that after early August some samples with CRQ amplifications would also have positive DBY amplification representing the volant male juveniles entering and exiting the roost. We performed a 2-sample *t* test (Welsh's) to determine whether DBY concentrations before and after 8 August were significantly different.

RESULTS

In silico testing of primers demonstrated that the forward and reverse primers of the control region amplify exclusively in *C. townsendii*. In silico analysis of the DBY forward and reverse primers suggests that these primers might be able to amplify DBY from *Saccopteryx bilineata* with 3 mismatches to the forward primer and 1 to the reverse primer. However, this species is not found in the United States, and the probe provides further specificity. In fact, we did not amplify DNA (CRQ or DBY) extracted from guano samples

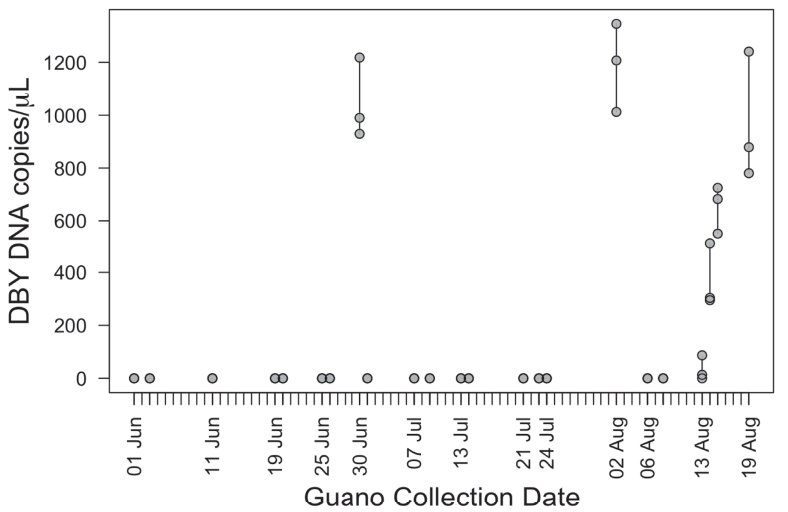

Fig. 2. *Corynorhinus townsendii* male-specific DNA (DBY) was detected rarely in early summer and occurred primarily in late summer, when pups became volant. Three qPCR replicates of DBY DNA extracted from pooled guano pellets are plotted at each 2014 collection date. Dates of guano collection are represented on the *x*-axis, and DNA concentration of male-specific (Y chromosome) DNA copies/µL is represented on the *y*-axis.

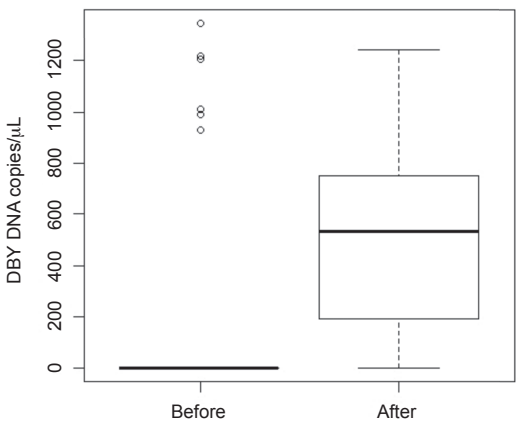

Fig. 3. *Corynorhinus townsendii* male-specific DNA (DBY) concentration was significantly higher after 8 August 2014. Male-specific DNA copies/µL amplified from DNA extracted from pooled guano collected at the entrance of the mine are represented before and after the biologically significant date when juvenile bats begin to fly.

of nontarget bat species (*Myotis californicus*, *Eptesicus fuscus*, *Parastrellus hesperus*, and *Myotis ciliolabrum*), thus demonstrating lack of cross-amplification of some species commonly found in roosts with *C. townsendii*.

Throughout the duration of the field trial (June 2014–August 2014), we successfully detected the target fragment for *C. townsendii* DNA (CRQ). Concentration of *C. townsendii* DNA in fecal samples varied throughout the sampling period (Fig. 1). We detected male *C. townsendii* DNA in late June (positive DBY) and then did not detect it again until 2 August 2014, in one single sample, but then not again until 13 August 2014, from which time we continued to detect male DNA throughout until termination of sample collection (Fig. 2). The concentration of DBY PCR product was significantly different after 8 August compared to before ($t = -3.2556$, $P = 0.00526$, $\alpha = 0.05$) (Fig. 3).

DISCUSSION

We conclude that noninvasive sampling of *C. townsendii* roosts can provide valuable insight into the nature of the roost use (i.e., maternity roost). In the early months of sampling, it was expected that the roost would be primarily adult females because juveniles would not be flying until late summer (Oyler-McCance et al. 2018). We predicted an increase of DBY DNA as male pups began to fly in and out of the mine. We did detect male bat DNA in 2 individual collection samples earlier than we had predicted pups to be flying (late June and early August), but it is common for a single adult male to join a maternity roost for a short period of time, so the source of this male bat DNA (early volant juvenile or bachelor

male) is not entirely certain (Dechmann and Kerth 2008).

Our goal was to develop a species-specific test while minimizing the cost and time impact to make it a viable approach for abandoned-mine surveying. It was not our goal to develop a method for estimating population size or identifying individuals but rather to have an index of relative detection of the *C. townsendii* species identification and male *C. townsendii* presence. Therefore, although our test may have missed some male juveniles due to fecal composition or frequency of deposition of juvenile feces (Zarzoso-Lacoste et al. 2018), the test accomplishes the goal of demonstrating a shift in demography from primarily females (maternity roosts before volant young) to both males and females (maternity roost with volant young).

Many mine closure programs will provide a bat gate opening that provides closure to humans but access for bats if it can be demonstrated that *C. townsendii* uses the site as a roost, particularly if it is a maternity roost (Pierson et al. 1999). Thus, when used in conjunction with on-site surveys, this method may help identify such roosts, and it can also strengthen the chance of detecting an important roost when bats are not present. This is particularly useful when bats are not present at the time of the survey but evidence of use (i.e., guano, moth wings) is present. This method helps minimize the likelihood of confusing maternity roosts with other roost types where a more equal ratio of males to females is typically expected (e.g., night roosts) or where a skew toward males might be anticipated (e.g., bachelor roosts). The ability to parse reproductive sites from other sites is foundational to the conservation of Townsend's big-eared bats as closure of abandoned mines increases and funds for gating sites are limited.

SUPPLEMENTARY MATERIAL

One online-only supplementary file accompanies this article (https://scholarsarchive.byu.edu/wnan/vol80/iss4/4).

SUPPLEMENTARY MATERIAL 1. *Corynorhinus townsendii* guano pellets collected outside of Piermont Mine, Nevada, and pooled for DNA extraction and amplification of species-specific CRQ DNA and DBY (male) DNA at each collection date. DBY and CRQ DNA copies/µL are reported for each of the 3 qPCR replicates performed on guano extraction.

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AUTHOR CONTRIBUTIONS

AJP and RES conceived the concept of study. RES and JCR collected guano samples. KKV and KEW optimized the lab protocol for species/sex detection and processed samples. AJP and KEW analyzed data. AJP, RES, and KEW prepared the manuscript. All authors read and approved the final manuscript.

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