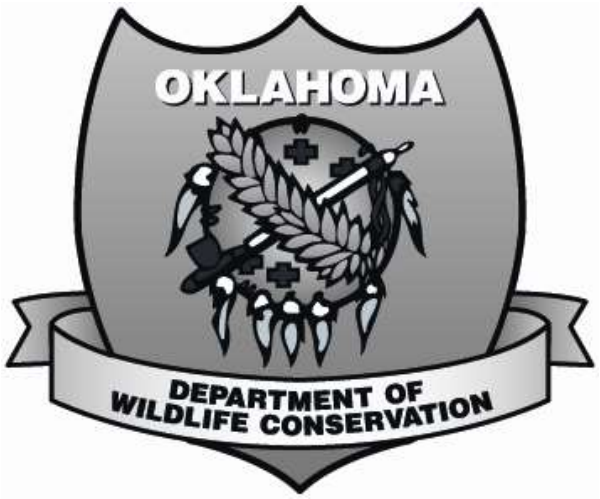
FINAL PERFORMANCE REPORT



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DNA Analysis of Fecal Material to Identify Prey Selection of Ozark Big-eared Bats

Oklahoma Department of Wildlife Conservation

October 1, 2015 through September 30, 2016

FINAL PERFORMANCE REPORT

STATE: OKLAHOMA **GRANT NUMBER**: F15AP00924 (E-83-R-1)

GRANT PROGRAM: Endangered Species Act Section 6 - Traditional

TITLE: DNA Analysis of Fecal Material to Identify Prey Selection of Ozark Big-eared Bats

GRANT PERIOD: 1 October 2015 – 30 September 2016

REPORT PERIOD: 1 October 2015 – 30 September 2016

PRINCIPAL INVESTIGATOR: Ronald Van den Bussche, Oklahoma State University

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I. ABSTRACT:

The Ozark big-eared bat (*Corynorhinus townsendii ingens*) is a federally listed endangered subspecies restricted to the Ozark Highlands and Boston Mountain Ecoregions of northeastern Oklahoma and north-central and northwestern Arkansas (USFWS 2008). In addition to protecting essential Ozark Big-eared Bat populations, there is a need in understanding the foraging ecology of the species. Direct observations of predation events are difficult because they are night-time aerial foragers. Through this project, we analyzed Ozark Big-eared Bat guano from two essential use caves for the purposes of identifying insect prey items down to the species level.

II. BACKGROUND:

Accurate determination of the dietary habits of Ozark big-eared bats is central to understanding their trophic relationships within ecosystems. In addition, it is integral to elucidating their role in regulating prey populations and how prey availability potentially affects density and distribution. This information is also a key component to the development of biologically meaningful management/conservation plans for the species. Leslie and Clark (2002) collected guano from Ozark big-eared bat maternity colonies in Adair County, Oklahoma and identified prey items using key morphological characteristics of the wings, legs, elytra, antennae, and other chitinous remains. Because Ozark big-eared bats often remove the wings and legs (typically those appendages with the most diagnostic traits to identify arthropods), they were not able to identify insect remains below the Ordinal-level. Morphologically identifying prey species from fecal material is difficult even when dealing with insectivorous species that do not remove wings or legs of insects prior to ingestion because key features may be damaged by digestion and typically under-represent soft-bodied prey. Because of these limitations, Dodd and Lacki (2007) examined prey of Ozark big-eared bats by collecting and identifying culled moth body parts from the floor of caves. Using this approach, they were able to identify 49 species from eight families of moths.

Although these morphological approaches have provided insight into the foraging behavior of Ozark big-eared bats, the list of prey items is likely an underestimation of the actual number of species ingested because body parts could have been damaged during chewing or digestion or damaged while remaining on the cave floor (Dodd and Lacki 2007). Molecular techniques provide an alternative approach for identifying prey items and can be used with highly degraded DNA typically found in fecal material. Amplifying and sequencing a specific portion of the insect genome can serve as a species-specific barcode and has been successful in identifying prey items from a variety of bat species (Alberdi et al. 2012, Bohmann et al. 2011, Clare et al. 2009, Dodd et al. 2012, Razgour et al. 2011).

To evaluate the ability of the DNA based approach for determining foraging behavior of Ozark big-eared bats, we conducted a preliminary study of feces collected at an Ozark big-eared bat maternity cave (AD-10) in Adair County, Oklahoma. During the spring of 2012, we placed a guano collecting apparatus (mesh screening attached to a 2' X 3' wooden frame) in the cave under a roost site used by Ozark big-eared bats and within the flyway of the cave. Screens were placed in the cave on 4 April 2012 and removed after young were reared on 30 July 2012. Individual pellets were collected and placed collectively in 30-ml collection tubes and transferred back to our laboratory at Oklahoma State University where lids were removed to allow the guano pellets to air dry. Twenty individual guano pellets were selected for DNA isolation in December 2012 and DNA was extracted.

Because northern long-eared myotis (*Myotis septentrionalis*), tri-colored bats *(Perimyotis subflavus*) and big brown bats (*Eptesicus fuscus*) are known to use this cave, we first sequenced 190 bp for the 16S rRNA gene to identify that bat species to which the guano pellet belonged. Because all 20 guano pellets belonged to Ozark big-eared bats, we then amplified the mitochondrial Cytochrome Oxidase I (COI) gene via the Polymerase Chain Reaction (PCR), cloned the gene products, and then sequenced 108 clones. Results of this analysis identified 20 species from 9 families and 2 orders of insects. Moreover, using this approach we detected four families and 15 species not previously detected in the diets of Ozark big-eared bats.

III. OBJECTIVES

To use a PCR-based approach and Next Generation Sequencing (NGS) technologies to obtain COI sequences from prey items recovered from the guano of Ozark big-eared bats and match these sequences against a reference library to identify their origin.

IV. SUMMARY OF PROGRESS

A. APPROACH

We began the analysis with guano pellets collected in 2013 and 2014 from caves AD-10 and AD-125 in eastern Oklahoma. These caves serve as essential maternity caves and winter hibernacula (caves considered necessary for the continuing existence of Ozark big-eared bats as per the Recovery Plan; USFWS 1995). At the time of collection, all guano collected was placed in 50 ml tubes or plastic Ziploc® bags (separate tube or plastic bag for each cave) and then shipped to Oklahoma State University. Once in the lab, DNA was isolated as described in Van

Den Bussche et al. (2016), and all samples of DNA were sent to RTLGenomics in Lubbock, TX to perform Next Generation Sequencing on these samples. Because pellets may have originated from other bat species known to use these caves, RTLGenomics first sequenced a portion of the mitochondrial genome to determine species identification. Upon determining which pellets originated from Ozark big-eared bats, these pellets were then sequenced for a portion of the mitochondrial COI gene for prey species identification. RTLGenomics performed initial genetic analyses, and some of these results are summarized in this report. Due to the large amount of data retrieved through this approach, the results described below are our initial results. We are continuing to examine these results, validate the species identification made by RTLGenomics and perform more thorough analyses. After all identifications are verified and statistical analyses complete, the results will be written for publication in a peer-reviewed journal and that manuscript will be submitted as a follow up to this report.

B. RESULTS

We sent to RTLGenomics isolated DNA from fecal pellets collected for this study and were able to include 129 and 102 fecal pellets from AD-10 and AD-125, respectively, that originated from Ozark big-eared bats. **Table 1 (**Appendix) provides the distribution of pellets across the sampling period and caves as well as the total number of DNA sequences obtained for each cave and month of collection. Pellets that were not included in this study either originated from another species of bat (*Eptesicus fuscus*, *Myotis septentrionalis*, or *Perimyotis subfl*avus) or were sufficiently contaminated with DNA originating from these species that we did not perform further analysis on the samples.

After we identified the species of bat that the fecal pellet originated, the next step was to amplify and sequence a portion of the mitochondrial COI gene. This gene was chosen as it has been shown to accurately identify to species-level a wide variety of animals. Moreover, entomologists have been using the COI as a universal barcoding gene and Genbank has a rich supply of insects represented for this mitochondrial gene. We were able to generate 693,711 COI DNA sequences (355,234 from pellets originating from Ozark big-eared bats in AD-10 and 338,477 from pellets originating from Ozark big-eared bats in AD-125), and as expected, we received a large number of hits to sequences in Genbank that came back as Lepidopteran. However, we also received a large number of hits that came back as Diptera, Coleoptera, Hemiptera, and even a couple for crayfish and rotifers. Thus, initial analyses were performed on the 30 taxa that occurred most frequently in our collections. Due to the large variance in number of DNA reads per sampling (Table 1), our ranking was based on the number of times a species was detected in either cave. Thus, *Mythimna unipuncta* (Appendix, **Table 2**) was detected a total of 10 times as occurring either in AD-10 and/or AD-125 over this study period. As can be seen from this table, the majority (22 of 30) of taxa in this list are lepidopterans. However, 4 are dipterans, 3 coleopterans, and 1 hemipteran. We are continuing to evaluate these data, but it is likely that many, if not all of these represent other taxa in the cave system and not food sources.

Our first step was to evaluate for multivariate differences among caves and sampling period using the Permutational Multivariate Analysis of Variance Using Distance Matrices. Distances among samples first were calculated using un-weighted (presence and absences of OTUs) or weighted (abundance of OTUs) and then an ANOVA-like simulation was conducted. Regardless

of the approach used to analyze the data there were significant differences among caves and sampling period (un-weighted analysis all $P < 0.001$; weighted analysis all $P < 0.001$). We next performed similar analyses but across sampling periods for AD-10 and across sampling period for AD-125). Again, regardless of whether we used a weighted or un-weighted approach, all *P*-values were < 0.001. Thus, not only did the diet of Ozark big-eared bats differ across months of the study, but they also differed significantly between the two caves for the same months. Although difficult to visualize due to the large number of taxa in this study, this pattern of differences across months and between caves can be seen in the Appendix, **Table 3**. Table 3 contains only lepidopteran samples detected in this study (along with their initial identification). For sampling period in which a taxon was identified, the box is shaded red if found in only AD-10, yellow if detected only in AD-125 and blue if detected in both caves during that month. Taxon names that are in bold font represent the 14 taxa that we previously identified as prey items of Ozark big-eared bats through a molecular analysis (Van Den Bussche et al. 2016).

As can be seen from the data in Table 3, for November, we detected two lepidopterans, *Bleptina caradrinalis* and *Lochmaeus manteo*, both at AD125. During the December sampling period, we detected *Erannia tiliaria* only at AD-10, and then in January and February, we detected a single taxon (*Paleacrita vernata*) only at AD-125.

While we have provided considerable new information regarding the prey items eaten by Ozark big-eared bats at two essential use caves in eastern Oklahoma, we have not finished verifying or analyzing the data. The reason for this is the large amount of data generated through Next Generation Sequencing approaches. To put this in perspective, in our previous study (Van Den Bussche et al., 2016), from 33 pellets (24 from AD-10, 9 from AD-125) that we identified as originating from Ozark big-eared bats, we generated 398 COI fragments for analysis. In contrast, for this study, we examined prey DNA from 231 Ozark big-eared bat fecal pellets (129 from AD-10, 102 from AD-125) and generated 693,711 potential prey sequences. Documenting the sensitivity of the Next Generation Sequencing approach is the larger number of sequences and greater diversity of lepidopterans detected. However, this increased sensitivity adds additional complications in that it is able to detect environmental DNA, even in low quantity. As an example, we identified DNA from two species of crayfish and well as rotifers and spiders. They are not prey species of Ozark big-eared bats and thus represent extraneous environmental DNA we detected. One of the other issues we are currently sorting out is the large number of dipterans, hemipterans, and coleopterans that we detected in our analysis. Thus, we are currently re-evaluating all species identifications for lepidopterans, dipterans, hemipterans, and coleopterans. After we have re-evaluated the identifications, we will evaluate their natural history (did they occur in eastern Oklahoma during the survey period, are they in flight during the period they were detected, etc.) to finalize our list of prey items of Ozark big-eared bats. After these aspects are finalized, we will finish our data analysis and submit a manuscript for review by the ODWC and publication in a peer-reviewed journal. Although this report serves as our final report, after we have these identifications and analyses finalized, the submitted manuscript will be provided as an update to this final report.

V. RECOMMENDATIONS

The project is completed for the current segment. Of interest and importance is the detection of lepidopterans in Ozark big-eared bat fecal material in November – March. White-Nose Syndrome has claimed the lives of millions of bats. Part of this destruction is due to the bats using their fat reserves during a time of the year that prey items are not abundant. The fact that we detected fresh pellets (although substantially reduced in number relative to other times of the year) and that lepidopteran DNA was detected in this fecal matter is encouraging for the possibility that if White-Nose Syndrome should impact these caves, bats may not see the devastating effects of this disease due to the presence of prey items. We recommend that research continue into the foraging ecology of the Ozark Big-eared Bat so that such information can assist conservation agencies in both management and recovery of the species.

VI. SIGNIFICANT DEVIATIONS

No significant deviations.

VII. EQUIPMENT

No equipment was purchased during this period.

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DATE: 9 February 2017

ARRPOVED BY:

Wildlife Division Administration
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APPENDIX

Table 1. Summary of months sampled, the number of fecal pellets identified as originating from Ozark Big-eared bats (OBEB) for AD-10 and AD-125 and the number of insect COI sequences obtained from the fecal DNA.

Sampling Date	AD-10 OBEB Pellets	Reads	AD-125 OBEB Pellets	Reads
May, 2013	17	13,427	18	113,475
June, 2013	13	29,937	14	49,917
July, 2013	4	2,783	4	12,891
August, 2013	6	16,483	11	65,035
September, 2013	13	41,916	2	86
November, 2013	2	280	2	8,587
December, 2013	2	10,432		
January, 2014			2	1,531
February, 2014			2	5,313
March, 2014	16	104,295	7	15,454
April, 2014	16	57,684	11	10,851
May, 2014	11	35,508	6	1,022
June 2014	13	6,492	12	32,240
July, 2014	16	40,997	11	22,075
Total	129	355,234	102	338,477
Mean	10.75	29,603	7.85	26,037

Table 2. Top 30 taxa detected in our study. Taxa denoted by * were detected in a previous molecular dietary analysis of Ozark big-eared bats (Van Den Bussche et al. 2016). In some cases, identification could not be made with confidence to the specific level.

Family	Order	Genus-Species	Count
Lepidoptera			18
Lepidoptera	Noctuidae	*Mythimna unipuncta**	10
Lepidoptera	Erebidae	*Bleptina caradrinalis**	9
Lepidoptera	Notodontidae	*Lochmaeus manteo**	9
Lepidoptera	Noctuidae	*Phoberia atomaris**	7
Lepidoptera	Noctuidae		7
Lepidoptera	Notodontidae	*Nadata gibbosa**	7
Lepidoptera	Geometridae	*Paleacrita vernata*	6
Lepidoptera	Noctuidae	*Achatia distincta**	6
Diptera			5
Lepidoptera	Erebidae	*Grammia arge*	5
Lepidoptera	Noctuidae	*Galgula partita**	5
Lepidoptera	Noctuidae	*Orthodes detracta**	5
Diptera	Psychodidae	*Psychoda* sp.	4
Lepidoptera	Elachistidae		4
Lepidoptera	Erebidae	*Chytolita morbidalis**	4
Lepidoptera	Erebidae	*Spilosoma congrua*	4
Diptera	Sphaeroceridae	*Spelobia semioculata*	3
Hemiptera	Pentatomidae	*Acrosternum hilare*	3
Lepidoptera	Blastobasidae	*Hypatopa simplicella*	3
Lepidoptera	Erebidae	*Apantesis nais**	3
Lepidoptera	Erebidae	*Grammia phyllira*	3
Lepidoptera	Erebidae	*Zanclognathat dentata*	3
Lepidoptera	Noctuidae	*Caenurgina erechtea*	3
Lepidoptera	Noctuidae	*Cissusa spadix**	3
Lepidoptera	Pyralidae	*Salebriaria engeli*	3
Coleoptera	Carabidae	*Galerita janus*	2
Coleoptera	Elateridae	*Anthous bicolor*	2
Coleoptera	Silphidae	*Necrodes surinamensis*	2
Diptera	Fanniidae	*Fannia*	2

Table 3. Detection of lepidopteran DNA infecal pellets of Ozark Big-eared bats. Colored cells reflect the detection of a given taxon. Red = AD-10, Yellow = AD-125 and Blue = Both caves.

Taxon	May-13	Jun-13	Jul-13	Aug-13	Sep-13	Nov-13	Dec-13	Jan-14	Feb-14	Mar-14	Apr-14	May-14	Jun-14	Jul-14
Achatia distincta														
Achyra nigrirenalis														
Acrotomodes sp.														
Agrotis subterranea														
Anicla infecta														
Apantesis nais														
Blastobasis glandulella														
Bleptina caradrinalis														
Brigrammia triviata														
Caenurgina crassiuscula														
Caenurgina erechtea														
Castaniidae														
Choristoneura occidentalis														
Chrysauginae sp.														
Chytolita morbidalis														
Cissusa spadix														
Dichomeris georgiella														
Elaphria chalcedonia														
Elaphria grata														
Epinotia nemorivaga														
Erannia tiliaria														
Eupithecia so.														
Eutrapela clemataria														
Euxoa sp.														
Galgula partita														
Grammia arge														
Grammia phyllira														
Grammia sp.														
Heterocampa subrotata														
Hypagyrtis piniata														
Hypatopa simplicella														
Hypsoropha hormos														
Ida rotundalis														
Lochmaeus manteo														
Macaria pustularia														
Malacosoma disstria														
Melanolophia signataria														
Melese sp.														
Metalectra discalis														
Mythimna unipuncta														
Nadata gibbosa														
Ogdoconta cinereola														
Orthodes detracta														
Paleacrita vernata														
Parapedesia teterrellus														
Phoberia atomaris														
Polychrysia morigera														
Polypogon jacchusalis														
Probole sp.														
Salebriaria engeli														
Scolecocampa liburna														
Sodoptera frugiperda														
Spilosoma congrua														
Spilosoma virginica														
Synemon maja														
Telphusa latifaciella														
Zanclognatha dentata														

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