A DIETARY ANALYSIS ON PANAMANIAN BAT SPECIES

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2019

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A DIETARY ANALYSIS ON PANAMANIAN BAT SPECIES

A THESIS SUBMITTED TO THE

HONORS COLLEGE

IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR HONORS IN THE DEGREE OF

BACHELOR OF SCIENCE

DEPARTMENT OF BIOLOGY

COLLEGE OF LETTERS AND SCIENCES

BY

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COLUMBUS, GEORGIA

2019

Abstract

Bats are found all over the world, and they are the most diverse group of mammals in the tropics. They are a key component in all ecosystems as predators, pollinators, or seed dispersers. As a conservation tool, dietary studies show what different bat species need to survive. Through metabarcoding, the diet can be accurately assessed. This method involves the DNA extraction of material from the feces of the individual being studied. PCR is used to amplify the DNA and next generation sequencing is used to identify, separate, and align the DNA that was extracted. By comparing the results from different seasons, we are able to track changes in diet based on seasonal variance and eventually anthropogenic sources. Due to unforeseen problems with sampling technique, the results of this study were not significant. A larger data set and improved sterilization is needed to confirm any changes within species and between seasons.

Acknowledgments

John D. Hanson for mentoring me through this project and providing the samples

RTLgenomics for sequencing the samples

Jeremy E. Wilkinson for helping to calculate the dietary diversity

Kevin Burgess for guiding me through working with barcode data sets

This research was funded in part by a Student Research and Creative Endeavors Grant from Columbus State University

Table of Contents

List of Figures

Introduction

Bats belong to the mammalian order Chiroptera and play an important ecological role as predators for arthropods (Long, Kurta, & Clemans 2013). They also act as pollinators, and seed dispersers for plants (Garcia-Estrada et al. 2012). Studying their diet helps us gain insight into the best conservation efforts, ecological benefits, and intraspecific interactions (Moosman, Thomas, & Veilleux 2012; Gregory, Whitaker, & Hartman 2014; Long, Kurta, & Clemans 2013).

Effective bat conservation requires a clear understanding of the dietary habits of the species in question. This understanding can be gained through observation and identification of arthropod fragments or plant material found in bat feces. Most commonly, visual identification methods are conducted to identify food species, however this method is usually accurate only to the order level (Kervyn et al. 2012; Graclik & Wasielewski 2012; Rolfe, Kurta, & Clemans 2014). Recent advances in PCR and DNA barcoding allow for the prey to be identified to the species level (Rolfe, Kurta, & Clemans 2014). With this level of identification, conservation can be focused on specific arthropod prey or plant species in an effort to boost the bat population. For example, efforts to remove the threatened classification from the Rafinesque's big-eared bat (*Corynorhinus rafinesquii*) has focused on learning more about the bat's diet and behavior (Gregory, Whitaker, & Hartman 2014). Similarly, a study on bat diet sought to reduce the deaths for migratory bats as they encounter wind turbines. In an effort to reduce bat mortality, it is important to understand what draws them close to the turbine. One strong hypothesis states that their food is drawn to the turbines (Valdez & Cryan 2013).

Economically bats are important for pest control as they eat a majority of their body weight in insects each night. Some of these insects are pests to human agricultural products, and

an increase in the bat population can be a natural alternative to the commonly used chemical pesticides. Research has been conducted to analyze the most hunted insects within an apple orchard. This research sought to prove the need of more roosting habitats for bats near agricultural fields (Long, Kurta, & Clemans 2013).

Studying bat diet is also one way to quantify the effects of human disturbance in an environment. Herbivorous bats are affected by the layout of a coffee plantation (Garcia-Estrada et al. 2012). With, more modification to the environment, there is a negative trend in the diversity of bats in the area. A dietary analysis in this instance will show the preferred plants of each species and an effort can be made to reintroduce or preserve these plants (Garcia-Estrada et al. 2012).

The ability to switch between a high protein (insect filled) diet and one largely consisting of carbohydrates (nectar) is not seen in many mammals. This type of diet switching has the potential to upset an animal's digestion and cause massive physiological complications (Frick et al. 2014). The ability to survive on a diet made entirely from nectar is also very uncommon. A few bat species exhibit feeding habits like these. By studying their diets, we can learn more about how they are able to survive and make proteins when their food source has very few essential amino acids and a very low nitrogen concentration (Viogt et al. 2011).

Another question to be studied is the effect of seasonal variations in available prey, and what happens when the preferred prey migrates or is in a developmental stage inaccessible to the bat (Graclik & Wasielewski 2012; Hope et al. 2014). To add another layer of complexity to the dietary analysis of a bat, we can learn how the life stages of the prey affect the predator (Wollerskar et al. 2015). In herbivorous bats, it is important to understand the relationship between land use and bat diversity (Garcia-Estrada et al. 2012). Conservation of bat species

through the retention of their food can only be done after a strong knowledge of species interactions had been identified. Many bat species live very closely to one another and compete for the same food sources. By studying their diets, it is possible to learn more about how they compete and the preferred prey of each bat species (Moosman, Thomas, & Veilleux 2012).

This study focuses on bats native to Panama. It seeks to identify a difference between both herbivorous and insectivorous bats when comparing dietary diversity during the wet and dry seasons. Polymerase chain reaction (PCR) of the Cytochrome 1 gene (COI) was used to identify insect prey species. In plants the trnL gene is used in place of COI. While, Simpsons and Shannon's diversity indices were used to calculate the diversity of species found in bat fecal samples. The wet season was expected to have a greater diversity of food available to the bats and therefore should show a greater in fecal samples collected during that time.

Methods

Sampling

Samples forming the basis of this research were collected in Panama July 2018 and January 2019. Following IACUC approved protocol, bats were caught in mist nets and sacrificed. Lower intestine and any available fecal pellets were preserved in 95% ethanol. Each sample was assigned an individual "TK" number, which corresponds to a voucher specimen and meta-data associated with the sample.

Molecular

In the lab, dissections were performed to remove fecal material for analysis. To limit carry over and cross contamination, DNAaway (Thermo Fisher Scientific, Wilmington, MA) was used to clean the workbench, tools, and gloves before and between dissections. Forceps were used to remove the intestine samples from their vials and scissors for the dissection. Specimens

were excluded if no fecal matter could be recovered during dissection. The FastPrep DNA kit (MP Biomedicals, Solon, OH) for soil/stool was used to extract the DNA from the samples according to the manufacturer instructions. After the extraction, the DNA samples were stored at -80^{0}C until they were sent to RTLGenomics (Lubbock, TX) for PCR (using COI primers) and next gen sequencing with an Illumina MiSeq (Illumina, San Diego, CA).

Samples were amplified for sequencing at RTLGenomics (Lubbock, TX) in a two-step process. The forward primer was constructed with (5'-3') the Illumina i5 sequencing primer (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG) (Gaiero et al. 2018). The reverse primer was constructed with (5'-3') the Illumina i7 sequencing primer (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) (Gobbi et al. 2019). Amplifications were performed in 25 ul reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1ul of each 5uM primer, and 1ul of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, California) under the following thermal profile: 95°C for 5 min, then 35 cycles of 94°C for 30 sec, 54°C for 40 sec, 72°C for 1 min, followed by one cycle of 72°C for 10 min and 4°C hold.

Products from the first stage amplification were added to a second PCR based on qualitatively determine concentrations. Primers for the second PCR were designed based on the Illumina Nextera PCR primers as follows: Forward - AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC and Reverse - CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG. The second stage amplification was run the same as the first stage except for 10 cycles.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was size selected in two rounds

using SPRIselect (BeckmanCoulter, Indianapolis, Indiana) in a 0.7 ratio for both rounds. Size selected pools were then run on a Fragment Analyzer (Advanced Analytical, Ankeny, Iowa) to assess the size distribution, quantified using the Qubit 3.0 fluorometer (Life Technologies), and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California) 2x300 flow cell at 10pM and sequenced at RTLGenomics.

Bioinformatics

Post sequencing, all DNA results were taken in FASTQ format and paired end reads were merged (Zhang et al. 2013). They were then converted to FASTA format. reads are trimmed then clustered at 4% divergence according to the USEARCH clustering algorithm (Edgar 2010). Single reads that could not be clustered are removed from the data set. Operational taxonomic units (OTUs) were derived by OTU selection algorithm before the removal of chirmera clusters as defined by UCHIME chimera detection software (Edgar 2013; Edgar 2010; Edgar et al. 2011). These OTUs were compared to GenBank and a database maintained by RTLGenomics. This allowed for the accurate identification of bat species and prey species retrieved from the fecal samples.

Diversity was determined by Shannon's and Simpson's diversity indices. Each sample was treated as the "local" scale to allow for alpha diversity to be calculated. Beta diversity was calculated as the comparison between samples in the same season (wet or dry) and with the same diet (insectivorous or herbivorous). An analysis of variance (ANOVA) was used to compare the seasonal differences of the herbivorous and insectivorous groups.

Results

This study included 34 bat samples, but only 26 of the samples could be amplified successfully. Of the 26 samples that amplified, six samples came from herbivorous bats.

Chloroplast DNA was used to identify consumed plants based on the trnL gene; the COI gene, from the mitochondria, was used to identify the bat. Insectivorous bats made 20 of the 26 samples that amplified. COI was the only gene tested for in this group. The wet season was represented by 10 samples; six of those 10 were insectivorous, while the other four were herbivorous. The dry season was represented by 16 samples; two of the 16 were herbivorous, while 14 were insectivorous.

Alpha diversity of prey was calculated within individuals and compared through ANOVAs. For herbivorous bats, Shannon's diversity showed no significant seasonal variance in diet (1-way ANOVA, $F_{1,3}$=0.81, P=0.44). Similarly, among insectivorous bats, Shannon's diversity index shows no seasonal variance between seasons (2-way ANOVA, $F_{1,9}$=1.30, P=0.28). Simpson's diversity was calculated and compared as well. No difference was found between the wet or dry seasons in either the insectivorous (2-way ANOVA, $F_{1,9}$=0.80, P=0.40) or herbivorous (1-way ANOVA, $F_{1,3}$=0.88, P=0.42) samples.

Obscurities

Some of the samples showed results that were not expected. Homo sapien DNA was found in three samples. Multiple bat species were found in six samples, and one sample shows two species of rodent along with the bat DNA. Some samples failed to get any bat DNA; this includes five herbivorous samples and one insectivorous sample. *Artibeus glaucus* was found in a sample but does not have a range that includes Panama. Chrysomelidae is a family of beetles that was found in 19 samples including two herbivorous samples from the wet season. In herbivorous species, Poaceae, a family that includes grasses worldwide, was the most prevalent, found in four samples.

Discussion

The eight samples that did not amplify likely failed due to an insufficient amount of fecal material used during DNA extraction. The amount of fecal material may also account for weak samples showing low diversity. Alternatively, the bats may have been caught before they had a chance to digest the fruit or insects from that night. The four samples that showed high diversity are not enough to distinguish a difference in diet between the wet and dry seasons.

These samples were collected and processed in the field by students that were learning the techniques. The fecal samples were not collected in the most sterile manner and carry over contamination may be the cause of the odd results. Samples with sequential TK numbers seemed to show results of the specimen collected before (Figure 1). This may explain the samples with multiple mammal results but not the samples failing to show mammal results.

To determine the source of error for samples that showed multiple results or results differing from the original identification (ID), another visual ID was made. The initial field ID was wrong for two bats, and they were corrected. The six samples with multiple bats found were re-examined to determine the correct ID. Re-examining the bats by visual ID solved the conflicting results from carry over contamination.

Errors resulting in no mammalian DNA being present may be due to a bioinformatics error within the algorithm used to analyze the samples. RTLgenomics has had trouble in which the computer fails to recognize sequences in samples that deviate from the expected. This can occur if the DNA present is longer or shorter than the length of the DNA expected to be found.

Conclusion

Identification of dietary components are more accurate now based on advances in PCR and barcoding databases (Rolfe, Kurta, & Clemans 2014). The COI gene has been standardized

in animals, and the trnL gene in chloroplasts has been standardized in plant species to identify organisms on a molecular level. By using metabarcoding with next gen sequencing answers to dietary questions can be answered for hundreds of organisms in a relatively short amount of time.

Bats, worldwide, are important ecological contributors. Some groups, like Myotis, reduce insect populations through predation (Long, Kurta, & Clemans 2013; Kervyn, Godwin, & Libois 2012). Others are necessary for seed dispersal; Artibeus (Figure 2), in particular, is important for the spread of fig seeds (Salana-Vazquez 2014; Heer, Albrecht, & Kalko 2010). Still more are important pollinators (Garcia-Estrada et al. 2012). For this reason, it is important to find efficient ways to protect them and the roles they play in their habitats. A dietary analysis can be an effective place to start. By protecting the preferred food of bat populations, they can be bolstered without breeding programs (Valdez & Cryan 2013).

As we learn more about the food species we need to protect, the human impact will become more apparent (Garcia-Estrada et al. 2012; Valdez & Cryan 2013). Analyzing diet can also show species interactions that may not have an obvious connection (Wollerskar et al. 2015). Additionally, we may start to find drawbacks to advancements previously thought to be the way of the future (Valdez & Cryan 2013).

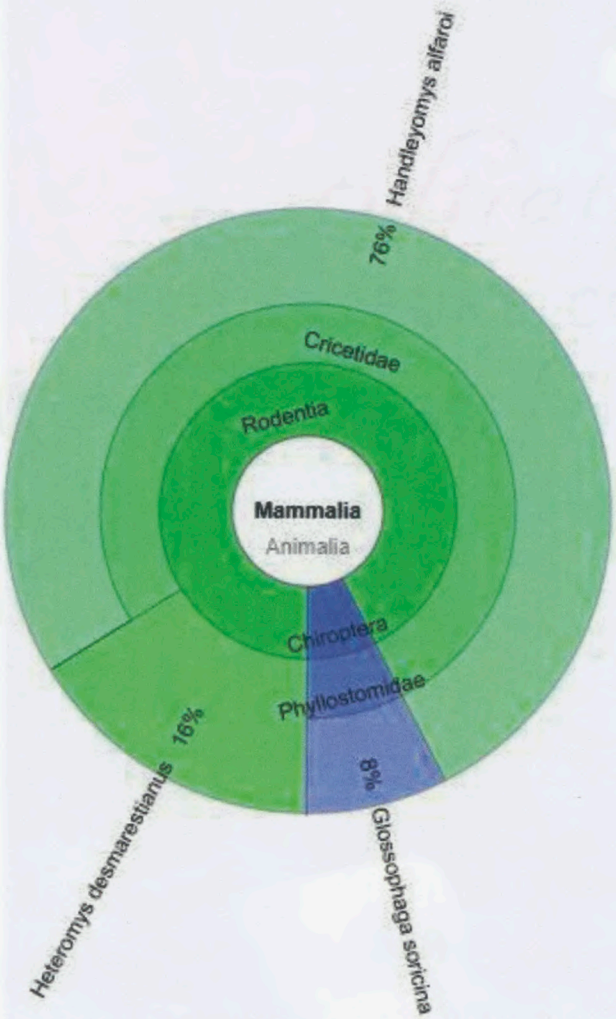

Figure 1. Krona graph showing multiple mammals were discovered in the same sample.

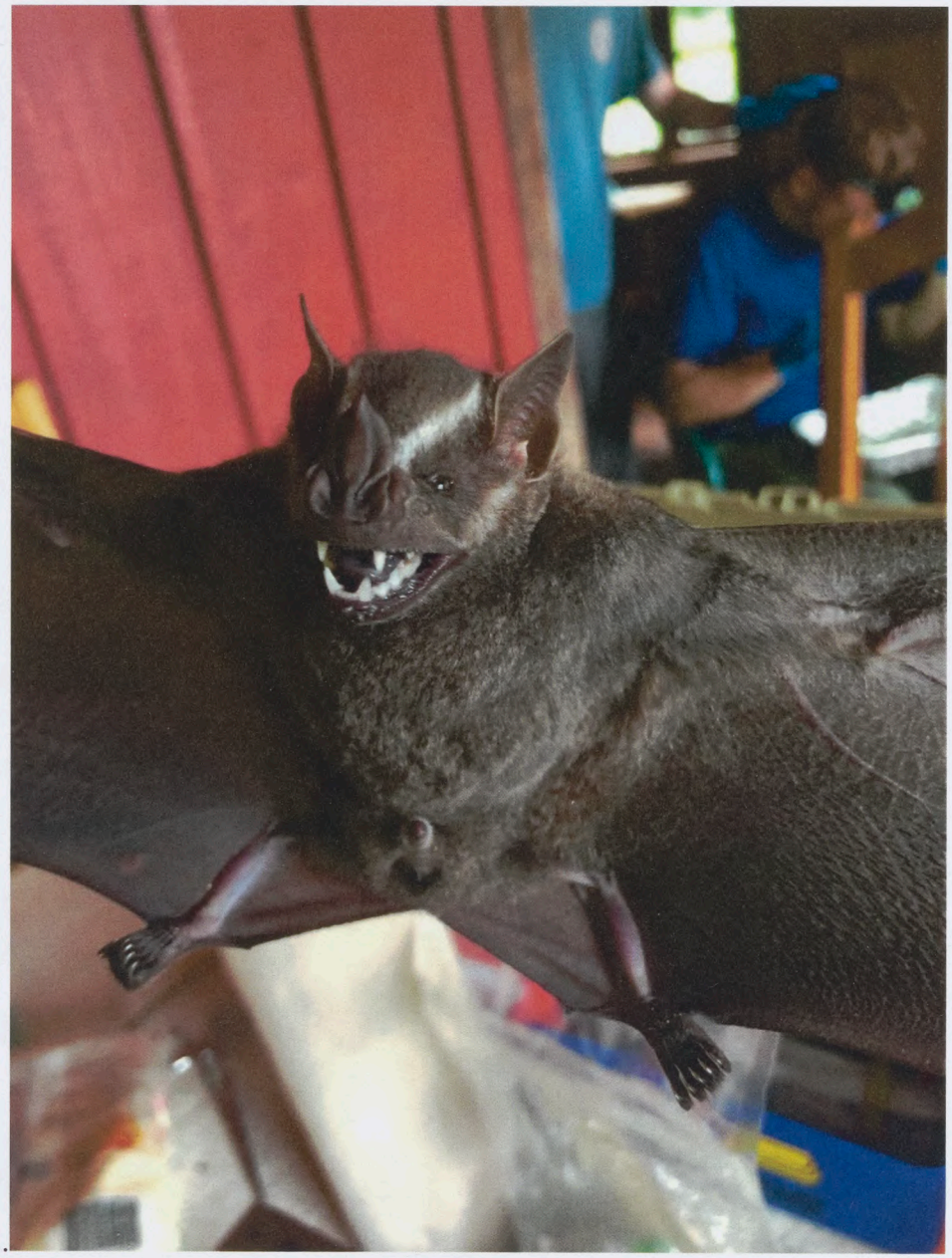

Figure 2. Image of a bat from the important seed dispersing genus, Artibeus.

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