Original Article

Sensory ecology of the frog-eating bat, *Trachops cirrhosus*, from DNA metabarcoding and behavior

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Metabarcoding of prey DNA from fecal samples can be used to design behavioral experiments to study the foraging behavior and sensory ecology of predators. The frog-eating bat, *Trachops cirrhosus*, eavesdrops on the mating calls of its anuran prey. We captured wild *T. cirrhosus* and identified prey remains in the bats' fecal samples using DNA metabarcoding of two gene regions (CO1 and 16S). Bats were preying on frogs previously unknown in their diet, such as species in the genus *Pristimantis*, which occurred in 29% of *T. cirrhosus* samples. Twenty-three percent of samples also contained DNA of *Anolis* lizards. We additionally report apparently rare predation events on hummingbirds and heterospecific bats. We used results from metabarcoding to design acoustic and 3D model stimuli to present to bats in behavioral experiments. We show predatory responses by *T. cirrhosus* to the calls of the frog *Pristimantis taeniatus* and to the rustling sounds of anoles moving through leaf-litter, as well as attacks on a stuffed hummingbird and a plastic anole model. The combination of species-specific dietary information from metabarcoding analyses with behavioral responses to prey cues provides a unique window into the foraging ecology of predators that are difficult to observe in the wild.

Key words: **Chiroptera, diet, fecal sample, foraging, metabarcoding, *Trachops cirrhosus*.**

INTRODUCTION

Developments in DNA metabarcoding have enabled new insights into animal diets (Deagle et al. 2005, 2009; De Barba et al. 2014), foraging flexibility (Kowalczyk et al. 2019), trophic positions (Chronopoulou et al. 2019), niche partitioning (Kartzinel et al. 2015), and population structure (Bohmann et al. 2018). Metabarcoding is particularly useful as a tool to understand the diets and foraging behaviors of nocturnal (Zeale et al. 2011) or rare (Galan et al. 2018) animals for which observing foraging behavior in the field is difficult. In addition, unlike any other type of diet analysis, metabarcoding provides information on precise

prey species, rather than general groups (Belwood 1988) or trophic levels (Rex et al. 2011). This information on prey species can then be used to design behavioral experiments to examine foraging behavior of predators.

The Neotropical frog-eating bat, *Trachops cirrhosus*, hunts frogs by eavesdropping on their mating calls (Tuttle and Ryan 1981). Wild-caught adult bats respond strongly (flying toward and attacking speakers) to playback of the calls of local palatable frogs (Tuttle and Ryan 1981), and extend these responses to similar-sounding calls of allopatric prey (Ryan and Tuttle 1983). In contrast, bats do not respond to the calls of poisonous toads or prey that are too large for bats to eat (Tuttle and Ryan 1981). Bats caught at the same site across different seasons differ in their responses to seasonally variable prey cues (Jones et al. 2014), indicating flexibility in this

eavesdropping response to species-specific prey cues in wild-caught bats. Additionally, captive *T. cirrhosus* can be trained to reverse their behavioral responses for palatable and nonpalatable prey cues (Page and Ryan 2005), and to respond to entirely novel cues such as cellphone ringtones (Jones, Ryan, et al. 2013). Experiments with social learning have shown that naive bats can learn these novel prey cue associations from exposure to trained conspecifics (Page and Ryan 2006; Jones, Ryan, et al. 2013), and that likelihood of social learning of novel cues is impacted by a bats' current foraging success with known prey cues (Jones, Ryan, et al. 2013). For at least one prey species, the túngara frog, *Engystomops pustulosus*, bats are sensitive to the temporal order of the components of the frog's complex calls (Jones, Farris, et al. 2013). These experiments conducted in captivity with wild-caught *T. cirrhosus* highlight the selectivity with which bats respond to species-specific prey cues, and indicate a potentially important role of learning in bat acquisition of response to species-specific prey cues. The role of learning is also supported by behavioral experiments showing that at a site where túngara frogs are absent bats do not respond to túngara calls (Jones et al. 2014). Understanding of the foraging ecology of this predator therefore requires identification of prey in bat diets at the species level.

Although extensive research has been conducted on captive *T. cirrhosus* behavioral responses to acoustic prey cues, the nocturnality of this species and the thick vegetation in which it forages has resulted in limited information on the diet of wild *T. cirrhosus*. The behavioral studies that have been conducted have focused predominantly on bats foraging on a few anuran species (particularly the túngara frog, *E. pustulosus*; Tuttle and Ryan 1981) which they hunt by eavesdropping on frog calls. There is physiological evidence that *T. cirrhosus* may have adaptations to specialize on anurans including auditory adaptations for low-frequency sound, perhaps particularly for the mating calls of anurans (Bruns et al. 1989), and unique salivary glands that may function to neutralize the toxins in anuran skin (Tandler et al. 1997). Previous diet studies, however, have indicated *T. cirrhosus* is an omnivore, consuming large numbers of arthropods (Fleming et al. 1972; Belwood 1988) as well as reptiles (Valdez and LaVal 1971; Humphrey et al. 1983), birds (Rodrigues et al. 2014), and other species of bats (Bonato and Facure 2000). However, it is largely unknown how bats are capturing these nonanuran prey. They could be using prey calls (Tuttle and Ryan 1981), sounds of prey motion (Geipel et al. 2020; Jones et al. 2011), relying on echolocation to catch silent stationary prey which has not been shown for *T. cirrhosus* but has been shown for other species (Geipel et al. 2013), or some combination of cues (Halfwerk, Dixon, et al. 2014; Gomes et al. 2016).

Studies that have examined the diet of *T. cirrhosus* using fecal samples (Giannini and Kalko 2004) or stomach contents (Pine and Anderson 1979; Bonato et al. 2004), have only been able to assign prey remains to phylum or order (reviewed in Leal et al. 2018). Our interest in the cognitive ecology of these bats, particularly their response to species-specific prey calls, and ability to learn those prey calls requires the analysis of prey remains to the species level. We therefore developed methods to conduct DNA metabarcoding of prey remains from *T. cirrhosus* fecal samples collected over a 10-year period. We wished to understand how well behavioral responses to prey cues recorded for wild-caught bats in previous research correlated with the dietary composition of *T. cirrhosus* at our study site in Soberanía National Park, Panamá. Given the high responsiveness of *T. cirrhosus* to the calls of the túngara frog, *E. pustulosus* (Jones et al. 2014), we predicted that many bats would have *E. pustulosus* DNA in their fecal samples. As bats have been shown to seasonally

shift their behavioral response to prey cues (Jones et al. 2014), we also expected to see corresponding seasonal shifts in the prey species DNA present in bat feces. As so much of our understanding of the species in the diet of *T. cirrhosus* is based on behavioral experiments where we have chosen what stimuli to present to bats, we also hoped that the DNA metabarcoding would identify previously unknown prey species, opening new avenues for behavioral research.

As we are particularly interested in eavesdropping behavior on anurans, we used vertebrate primers from targeted regions of two genes: 16S and CO1. For comparison to *T. cirrhosus*, we metabarcoded DNA from feces of *Lophostoma silvicolum*, a closely related bat species that has some niche overlap with *T. cirrhosus* in the consumption of katydids (Orthoptera: Tettigoniidae) (Tuttle et al. 1985; Kalko et al. 1999; Falk et al. 2015), but are believed to not consume anurans. We predicted that *L. silvicolum* samples would not contain anuran DNA, but would inform us if our primers amplified insect, particularly katydid, DNA. We then used the results from our metabarcoding of prey remains in *T. cirrhosus* feces to design behavioral experiments to understand how bats might be capturing novel prey we found in diet analysis. Other studies have shown that metabarcoding can reveal foraging behaviors that are missed during field observations, even for diurnal animals (Pornon et al. 2016). To our knowledge, our study in the first to combine metabarcoding results with behavioral experiments to confirm and further investigate those metabarcoding results, an approach that opens new research opportunities for many organisms. This methodology highlights the potential of DNA metabarcoding to be used to design ecologically relevant behavioral experiments.

METHODS

DNA metabarcoding

Fecal sample analysis was conducted on a total of 147 fecal samples: 143 samples from 136 different *T. cirrhosus* and 4 samples from different *Lophostoma silvicolum*. *Trachops cirrhosus* were captured in mist nets and hand nets at multiple sites within Panama (Barro Colorado Island, Gamboa and Pipeline Road, Plantation Road, and the Darien) between 2003 and 2013 and at La Selva Biological Station in Costa Rica in 2010. *Lophostoma silvicolum* were captured on Barro Colorado Island in 2012. All components of this project were reviewed and approved by the Smithsonian Tropical Research Institute (IACUC # 2007-14-06-15-07; 20100816-1012-16; 2017-0102-2020), University of Texas (IACUC # 04113002; 07113001; AUP-2017-00292), Panama's Ministry of the Environment (Autoridad Nacional del Ambiente (ANAM) and Ministerio de Ambiente (MiAmbiente): SE/A-43-07; SE/A-91-09; SE/A-91-09; SE/A-95-10; SE/A-6–11; SE/A-46-11; SE/A-94-11; SE/A-58-12; SE/A-19-13; SE/AP-13–18; SE/AP-22-19), and the Costa Rican environmental authorities (Sistema Nacional de Áreas de Conservación: 027-2010-SINAC).

After capture, bats were placed in clean cloth bags for 1–2 h. When a bat was removed, we collected the fecal sample from the cloth bag and placed it in a glassine envelope, in which it was frozen at −20 °C at the Smithsonian Tropical Research Institute in Gamboa. Cloth bat capture bags were washed between nights of mistnetting to reduce potential contamination of samples. Captured *T. cirrhosus* were individually marked with RFID PIT tags (Trovan Ltd., Santa Barbara, CA) and released at their capture sites. In 2013, all of the fecal samples were transferred into

2 mL tubes with RNA*later* (Qiagen, Valencia, CA) and transported to the United States for sequencing (Export Permit: Autoridad Nacional del Ambiente, Republica de Panama, Permiso Científico No. SEX/A-29-13).

All extraction, PCR, DNA library preparation, and sequencing were performed by MR DNA (www.mrdnalab.com, Shallowater, TX). Extraction of DNA from each fecal sample was conducted using the PowerSoil Kit® (MO BIO Laboratories, Inc., Carlsbad, CA) following the manufacturers' instructions. Extracted DNA from each fecal sample was divided and separately combined with 16S and CO1 primers and Roche 454 (for 16S) and Illumina MiSeq (for CO1) indexes and adapters so that reads from each bat fecal sample could later be traced back to their host samples. To best determine the vertebrate diet of *T. cirrhosus*, metabarcoding was conducted with a set of universal 16S rRNA primers (*16SA-L/16SB-H*) (Palumbi et al. 1991) that amplifies a 550-bp region in amphibians (Vences et al. 2005), and a CO1 universal fish primer set (*FF2d/FR1d*) that amplifies a 650-bp region (Ivanova et al. 2007). As anecdotal reports have described *T. cirrhosus* preying on other bat species (Bonato and Facure 2000), we did not add a bat blocking primer (Piñol et al. 2014). Given the limitations on amplicon size when sequencing DNA with Illumina MiSeq, paired-end reads for CO1 sequencing were nonoverlapping and covered the first 270 bp each direction. Cycling parameters for PCR were the same for both gene regions. The HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) was used to conduct a single-step 30-cycle PCR under the conditions of 94 °C for 3 min, then 28 cycles of 94 °C for 30 s, followed by 53 °C for 40 s and 72 °C for 1 min. The final elongation step was at 72 °C for 5 min. After amplification, PCR products were checked on 2% agarose gels to determine the success of amplification and the relative intensity of bands. The amplicon products were combined from all the different samples in equimolar concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, Massachusetts). The DNA library for 16S sequencing was prepared and sequenced using Roche 454 FLX titanium instruments and reagents according to the manufacturer's guidelines. The DNA library for the CO1 sequencing was prepared with the Illumina TruSeq DNA library preparation protocol and sequenced on an Illumina MiSeq following the manufacturer's guidelines.

The resulting sequences were analyzed using a modification of the QIIME (Quantitative Insights into Microbial Ecology) (Caporaso et al. 2010) pipeline (QIIME version 1.9.1). We used the QIIME tools to demultiplex and quality filter the 1,559,417 16S sequence reads, removing any reads with Phred quality scores under 25 and reads shorter than 200 bp. This resulted in 1,326,077 reads. Sequences were then clustered into operational taxonomic units (OTUs) using the SWARM method allowing a 2-bp difference (Mahé et al. 2014), creating 81,043 OTUs. We filtered OTUs to remove those that did not occur at least 10 times in each sample, resulting in 955 OTUs. Taxonomic assignment was conducted using remote blastn from the NCBI blast+ package (Camacho et al. 2009) at a 90% similarity threshold, that is, amplified sequences had to be ≥90% similar to a reference sequence to be included in the results. We used 90% as there is poor species-level coverage of many tropical taxa in GenBank, and we hoped that a 90% similarity threshold would allow to identify some prey taxa to the family or order level (Holovachov 2016), which would still be informative.

The CO1 sequences were analyzed with the same protocol described for 454 (16S) above. After initial quality filtering, the resulting 3,909,051 reads were clustered into 1,077,390 OTUs. After further filtering by a copy number of 10, 3,890 OTUs remained. Taxonomic assignment was conducted using the "bold" R package (Chamberlain 2020) and results were filtered to remove matches less than 90% similarity and those that matched reference sequences from taxa that do not occur in the Americas and Caribbean Islands. Following filtering we recovered taxonomic information for 147 OTUs, which we collapsed to 58 taxa. We combined the data from both gene regions to assess all of the prey DNA amplified from bat samples. We compared the number of samples from bats captured in the dry and wet season containing the most common prey species with a Fisher's Exact Test in R version 3.6.1.

Response to acoustic stimuli

Preliminary DNA sequencing results indicated that bats were consuming previously unreported prey species. To assess how bats were locating those prey, we captured 10 adult *T. cirrhosus* in the field (May–September 2019) and tested their responses to prey stimuli broadcast from speakers in a large 5 × 5 × 2.5 m (l, w, and h) flight cage under ambient conditions. Flight cages contained a roost for bats in one corner, playback speakers under a 1 × 1 m screen covered in leaf litter in a second corner, and the experimenters were in a third corner of the cage with a laptop for playback and infrared lights and infrared cameras for recording bat responses.

The flight cage was lit by a 25 W red lightbulb and five infrared lights. Bats were given at least 1 h to acclimate to the flight cage before testing began. Each bat was presented with 10 acoustic stimuli including calls of the frog species found in bat fecal samples (*Craugastor fitzingeri*, *Dendropsophus ebraccatus*, *Diasporus diastema*, *E. pustulosus*, *Pristimantis taeniatus*, and *Scinax boulengeri*), recordings of the rustles of anoles moving through both wet and dry leaf litter, calls of the hummingbird *Florisuga mellivora*, and a 5 kHz pure tone as a control. The 10 acoustic stimuli were 2 min long with 2 min of silence between each test. All calls were broadcast at their natural call rates and approximately natural call amplitudes. All sonic stimuli (the frog calls and the pure tone) were broadcast with Fostex FE103En speakers (Fostex Co., Akishima, Tokyo, Japan). All stimuli with higher frequency components (hummingbird calls and the anolis rustling noises) were broadcast with Avisoft Scanspeak speakers (Avisoft Bioacoustics, Berlin, Germany). We randomized the order of stimulus presentation for each bat. During testing, one Sony Handycam DCR-SR45 camcorder was focused on the speakers to determine how close bats flew to the speakers, and the other was on a tripod to be manipulated by the experimenters to follow the flight paths of the bats.

Upon video analysis, we scored bat response on a scale from 0 to 4: 0) no response, 1) movement of the ears in time with the stimulus, 2) orientation of the head toward the stimulus, 3) hovering within 0.5 m of the speaker, and 4) landing on the speaker. We used cumulative link mixed models in R to model the responses (ordinal package; Agresti 2010; Christensen 2015). The ordinal response score was our response variable, playback type was our explanatory variable, and individual bat was our random effect. We compared this model to a null model with only the random effect using an analysis of variance (Anova). We used the emmeans package (Lenth 2020) to generate average estimates of the probability distribution of each score for each treatment, as well as 95% confidence intervals and significant differences for the model estimates. *P* values were adjusted using the Tukey method for a family of 10 estimates. Because the default for this method plots on a 1–5 scale, we subtracted 1 from the mean class and the upper and lower bounds of

the confidence intervals to put the output on our response scale, 0–4 (Lenth 2020).

Response to 3D models

Previous research has shown that *T. cirrhosus* predominantly does not appear to hunt silent, motionless prey (Page and Ryan 2008; Halfwerk, Jones, et al. 2014). However, DNA from a hummingbird, *F. mellivora*, was found in one of our bat fecal samples. As hummingbirds are diurnal and are likely asleep during much of the time that *T. cirrhosus* hunts, we were interested in whether bats might be hunting silent and motionless sleeping hummingbirds. Additionally, anoles were a common prey item in samples from *T. cirrhosus*, but they are also diurnal and do not call, so it is not clear how *T. cirrhosus* is detecting them. We were interested in whether *T. cirrhosus* could be capturing silent, motionless anoles as well. To test bat response to silent and motionless prey, we placed *T. cirrhosus* individuals ($N = 4$) in a ($130 \times 220 \times 110$ cm) tent with three potted plants, a hummingbird mount (*Amazilia tzacatl*) attached to a branch, and three plastic anole models on the rachises of leaves, where they can be found sleeping in nature. In the tent, we also included two model frogs: a mostly plaster *E. pustulosus* model (Klein et al. 2012) and a plastic generic brown frog. We also presented bats with two control stimuli: a plastic ball and a small doll, unnatural objects of similar size to the models, to compare whether the bats responded to the plastic models as prey or simply as novel objects (Figure 1). Bats were placed singly in the tent for at least 1 h, and the trials were videotaped and scored for attacks on the models.

RESULTS

Metabarcoding

The two gene regions differed in the number of samples from which sequences were amplified and the types of prey organisms identified (Table 1). The 16S region identified amphibian DNA from more fecal samples and of more anuran species than the CO1 region, and CO1 identified more reptilian (*Anolis* lizards) and insect prey from more fecal samples (Figure 2). In some samples, the same prey species was amplified by both gene regions. A combined total of 65 bat fecal samples amplified DNA from prey species; 26

samples (40%) only amplified DNA from the 16S region, 23 samples (35%) only amplified DNA from the CO1 region, and 16 samples (25%) amplified DNA from both regions. These samples came from two *L. silvicolum* individuals (both captured in the wet season), and 62 different *T. cirrhosus* individuals (two samples came from one *T. cirrhosus* individual recaptured in the same year). The *T. cirrhosus* samples came from bats captured from all years (2003–2013) except 2004 and 2009, with the most samples from 2010. Samples with amplified prey sequences came from all study sites including three samples from bats captured at La Selva in Costa Rica and one from the Darien region of Panamá. An individual bat sample typically contained DNA from one to three different prey species, with one prey species being the most common within each sample.

The prey species that occurred with the greatest frequency (i.e., in the most samples) at similarities of 99–100% were frogs in the genus *Pristimantis* (17 samples or 27% of the samples), followed by *Anolis* lizards (14 samples, 22%), the frog *C. fitzingeri* (9 samples, 14%), and the túngara frog, *E. pustulosus* (6 samples, 10%). In total, the two genes identified prey DNA from 11 amphibian species, with *P. taeniatus* and *C. fitzingeri* occurring in the most samples. There were slightly more *Anolis* and *Pristimantis* from dry season-captured bats and more *E. pustulosus* from wet season-captured bats (Table 2), but the prey distribution across species was not statistically significant (Fisher's Exact test, $P = 0.19$).

In addition to the frogs and *Anolis* lizards, one bat sample from an adult male *T. cirrhosus* captured in Gamboa in 2013 contained DNA from a white-necked jacobin hummingbird, *F. mellivora*, at 100% similarity. We also found evidence for heterospecific bat predation by *T. cirrhosus*. One adult male *T. cirrhosus* from Gamboa in 2013 contained DNA from the nectarivorous bat *Glossophaga soricina* at 100% similarity, and a juvenile female *T. cirrhosus* from Gamboa in 2010 contained the DNA of the frugivorous bat *Carollia perspicillata* at 99–100% similarity. Finally, the sample from an adult female from Gamboa in 2010 contained DNA from the big-eared bat *Micronycteris megalotis* at 99% similarity (phylogenetic revisions indicate this is in fact *M. microtis*; Simmons 1996). These three samples likely reflect rare predation events on larger vertebrate prey.

The sample from an adult female *T. cirrhosus* captured in the Darien region of Panama was the only sample to amplify DNA from the frog *D. diastema*. The three *T. cirrhosus* samples from the La Selva biological station in Costa Rica contained DNA from an *Anolis* lizard, as well as a scarab beetle, *Cyclocephala amazona*, at 99% similarity, and a lepidopteran at <97% similarity. The two *L. silvicolum* samples that amplified prey DNA both contained DNA that matched the cricket *Anurogryllus muticus* at 93 or 94% similarities, indicating both bats likely consumed another Orthopteran species.

Figure 1
Testing setup with 3D models. Illustration by Damond Kyllo.

Table 1

Details on bat fecal samples that amplified DNA in each gene region

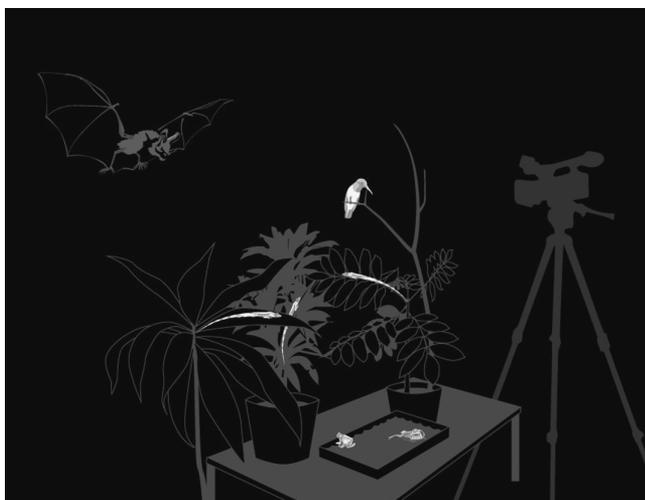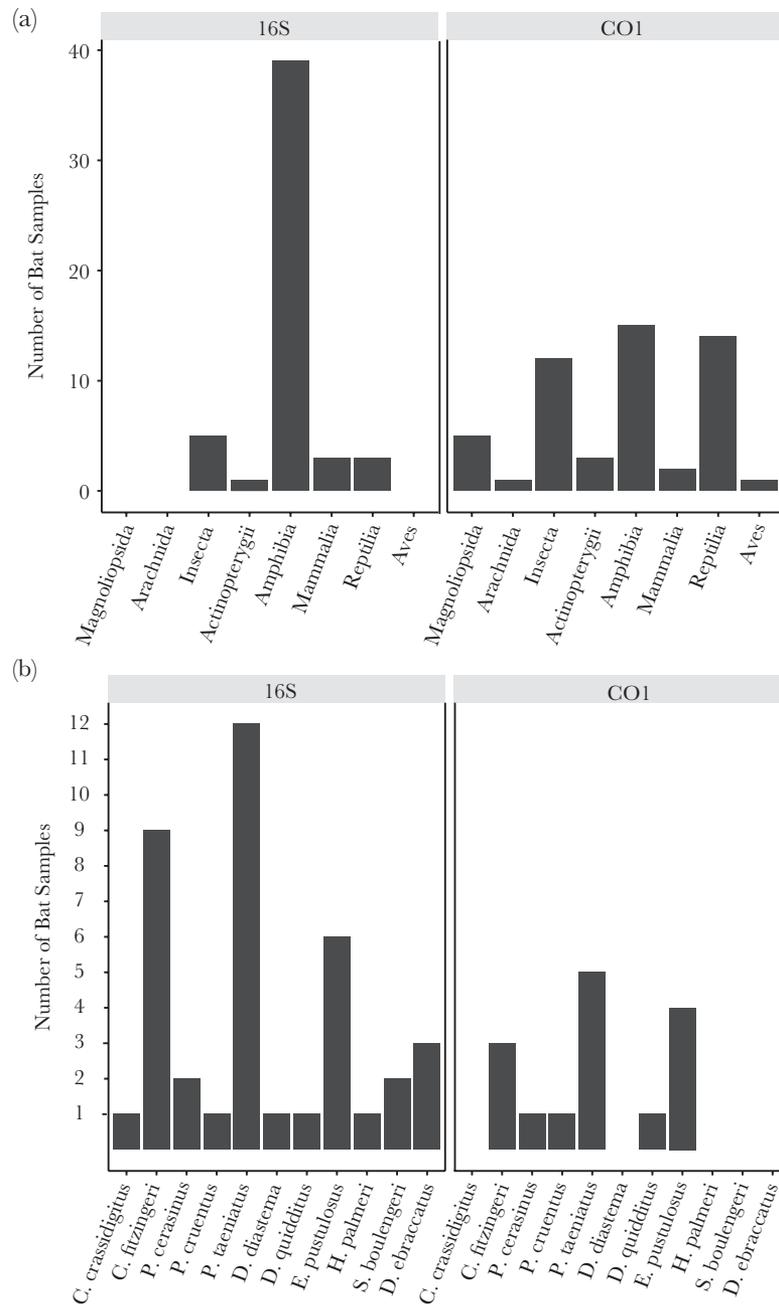|	16S	CO1
Number of samples that amplified DNA (of initial 147)	147	82
Samples that amplified prey (not *T. cirrhosus* or *L. silvicolum* DNA)	48	39
Mode % similarity match to a reference sequence	99%	100%
Most commonly identified OTUs	Anura and *T. cirrhosus*	Prokaryota

Figure 2
(A) The number of bat samples within each gene region that amplified prey DNA from diverse taxonomic groups. The 16S region worked better for amplifying amphibian prey DNA and the CO1 region was better for amplifying reptile and insect prey DNA. (B) The number of bat samples that contained DNA from anuran (Amphibia) prey DNA for both gene regions. The 16S region amplified anuran DNA from more fecal samples total, and also from more anuran species than the CO1 region.

Response to acoustic and 3D stimuli

We tested the responses of 10 *T. cirrhosus* to acoustic prey stimuli Table 3. One of the bats did not respond at all to any of the stimuli; therefore, we removed it from the dataset, resulting in a final sample size of $N = 9$. Bats responded to the different playbacks with varying levels of intensity (Anova; df = 9, $P < 0.001$; Figure 3). They responded most strongly to the mating calls of the túngara frog, *E. pustulosus* (Table 2), although their response scores to túngara were not significantly different from their responses to

the mating calls of the frogs *C. fitzingeri*, *S. boulingeri*, and *P. taeniatus*. Bats responded least to the mating calls of the tink frog, *D. diastema*, although the mean estimate was not significantly different from the responses to the *F. mellivora* hummingbird vocalizations, the pure tone, the sound of *Anolis* lizard *sp.* moving through wet leaves, and mating calls of the hourglass treefrog, *D. ebraccatus*. The *Anolis sp.* moving through dry leaves had an intermediate mean response score. We additionally tested four of these *T. cirrhosus* individuals for their responses to the 3D models. Three of the bats did
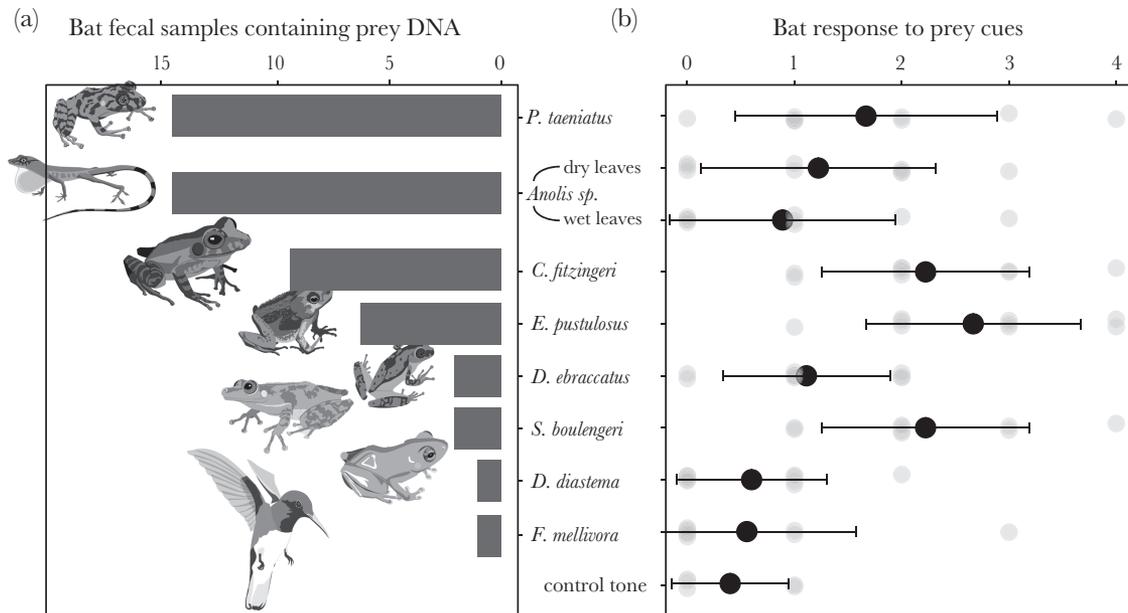
Figure 3

(A) The number of the *T. cirrhosus* fecal samples (*N* = 62) that contained DNA from each prey species using data from both gene regions. Illustrations of prey species by Damond Kyllo. (B) Mean responses of wild-caught *T. cirrhosus* (*N* = 9) to acoustic prey cues. Lines indicate the standard deviation of the mean, and grey points are the raw responses of each bat to a given stimulus, jittered for visibility. 0 = no response, 1 = ears twitched in response to call, 2 = oriented body to stimulus, 3 = hovered within 50 cm of speaker, and 4 = landed on speaker.

Table 2

The number (and percent) of *T. cirrhosus* faecal samples containing each prey species separated by bats captured in the Panamanian dry season (January–April) and wet season (May–December)

Prey species	Dry season *T. cirrhosus* (40)	Wet season *T. cirrhosus* (23)
Anolis sp.	11 (28%)	3 (13%)
Craugastor fitzingeri	5 (13%)	4 (17%)
Dendropsophus ebraccatus	1 (3%)	1 (4%)
Diasporus diastema	0 (0%)	1 (4%)
Diasporus quidditus	0 (0%)	1 (4%)
Engystomops pustulosus	2 (5%)	4 (17%)
Pristimantis cerasinus	1 (3%)	1 (4%)
Pristimantis cruentus	1 (3%)	0 (0%)
Pristimantis taeniatus	10 (25%)	4 (17%)
Scinax boulengeri	0 (0%)	2 (8%)
Hyloscirtus palmeri	1 (3%)	0 (0%)

not demonstrate any response to the 3D models, but one bat made vigorous attacks on both the hummingbird mount and one of the anole models (see Supplementary Videos). No bats attacked the model frogs or control objects (Table 3).

DISCUSSION

The use of DNA metabarcoding enables us to understand the diet of the frog-eating bat, *T. cirrhosus*, at a taxonomic resolution previously not possible. Because *T. cirrhosus* is able to eavesdrop on species-specific mating calls of some of their prey to locate food, detailed knowledge of exact prey species is necessary to fully understand the foraging behavior of this predator. We used our metabarcoding data on prey diet to design a behavioral experiment to examine foraging behavior. Although the sample size for our behavioral experiments is small at 10 bats, we show predatory responses to the calls of prey species previously unreported, such as *Pristimantis* sp., and predatory attacks on a motionless hummingbird

mount and a plastic anole model, neither of which had ever been tested with *T. cirrhosus*. This predatory behavior highlights the discovery that predation by *T. cirrhosus* is likely an agent of selection on a wider range of anuran mating calls than previously studied, and provides evidence that *T. cirrhosus* is likely more capable of locating silent, motionless prey by echolocation than previously believed (Tuttle and Ryan 1981; Page and Ryan 2008; Halfwerk, Jones, et al. 2014).

We used two gene regions for metabarcoding, 16S and CO1. The 16S amplified more anuran DNA from samples, but the CO1 amplified more anole DNA. Using both of these primers gave us a more complete picture of the vertebrate prey consumed by *T. cirrhosus*, but these primers were designed for vertebrates, and thus neither primer amplified substantial amounts of insect DNA. Previous research has indicated that fecal samples from *T. cirrhosus* contain over 80% arthropods (Kalko et al. 1999). This is likely an overestimate because the chitinous exoskeleton of arthropods is less easily digested than vertebrate tissue, resulting in a higher proportion

Table 3

The acoustic and 3D models presented to bats in behavioral tests and the responses of bats to those stimuli

Prey species	Number of stimulus recordings used (each from a different individual)	Broadcast amplitude	Number of *T. cirrhosus* that hovered over the stimulus	Number of *T. cirrhosus* that attacked the stimulus
Aves: *Florisuga mellivora* vocalization	2	75 dB at 1 m	1	0
Anura: *Dendropsophus ebraccatus*	5	75 dB at 1 m	0	0
Anura: *Diasporus diastema*	9	75 dB at 1m	0	0
Anura: *Scinax boulengeri*	10	75 dB at 1 m	2	1
Anura: *Craugastor fitzingeri*	3	75 dB at 1 m	2	1
Anura: *Pristimantis taeniatus*	3	75 dB at 1 m	1	1
Anura: *Engystymops pustulosus*	9	75 dB at 1 m	3	2
Reptilia: *Anolis* rustle on dry leaves	9	63 dB at 10 cm	1	0
Reptilia: *Anolis* rustle on wet leaves	8	58 dB at 10 cm	1	0
Control: 5 kHz tone	NA	75 dB at 1 m	0	0
Hummingbird mount	NA	NA	0	1
Plastic anole model	NA	NA	0	1
Model *E. pustulosus*	NA	NA	0	0
Generic model frog	NA	NA	0	0
Control model ball	NA	NA	0	0
Control model doll	NA	NA	0	0

of arthropod remains in fecal samples than the bat actually consumed (Giannini and Kalko 2005). Stable isotope data, however, have shown extensive overlap in the composition of the diets of *T. cirrhosus* and insectivorous species (Oelbaum et al. 2019). Despite using vertebrate primers, we did find beetles in samples from five *T. cirrhosus* individuals, and orthopterans in three *T. cirrhosus* samples as well as two *L. silvicolum* samples. *L. silvicolum* is often considered a specialist on katydids (Orthoptera: Tettigoniidae), and has been studied for its predatory response to katydid calling songs (Tuttle et al. 1985; Falk et al. 2015). We did not find any evidence of anuran DNA in *L. silvicolum* samples, despite their similarities in physiology and foraging niche to *T. cirrhosus* (Tuttle et al. 1985). *Trachops cirrhosus* also eavesdrops on katydid songs (Tuttle et al. 1985; Jones et al. 2014; Falk et al. 2015), and katydids may be an important part of the diet of *T. cirrhosus* that we are missing in this study. Tropical insects remain poorly covered in reference databases, but with increases in DNA barcoding, a future study should analyze the insect prey of *T. cirrhosus* using insect-specific primers (Zeale et al. 2011).

Even within the taxa our primers amplified well, such as anurans, both primers are likely to have biases (Alberdi et al. 2018) and amplify some prey species better than others. Some of the discrepancies we observe between our behavioral responses to prey cues and sequence data could therefore be due to primer biases. Inconsistencies between diet and predatory response to prey cues may also, however, provide insights into *T. cirrhosus* foraging behavior. In behavioral testing, more *T. cirrhosus* attacked the speaker in response to the calls of the túngara frog, *E. pustulosus*, than any other prey species, and this is the species for which *T. cirrhosus* predation has been studied the most extensively (Barclay et al. 1981; Ryan and Tuttle 1983; Ryan et al. 1983; Tuttle et al. 1985; Bruns et al. 1989; Page and Ryan 2005, 2006; Akre et al. 2011; Halfwerk, Jones, et al. 2014; Hemingway et al. 2017). It is rare for a wild bat brought into captivity to be unresponsive to the calls of *E. pustulosus* (R.A.P., personal communication). *Engystomops pustulosus* was therefore surprisingly uncommon in the diet, occurring in only 10% of samples, less common than other anurans such as *P. taeniatus* and *C. fitzingeri*. It is possible that *E. pustulosus* is a highly preferred prey but less accessible to *T. cirrhosus* than previously believed, perhaps because *E. pustulosus* are generally more common and call more around urban areas and open areas (Halfwerk et al. 2019) than in the forest where the majority of these bats were captured.

A similar phenomenon could be occurring for *S. boulengeri*, where bats were very responsive to frog calls, as noted in previous research as well (Tuttle and Ryan 1981), but only a few bats had consumed *S. boulengeri*. This may be because *S. boulengeri* frequently call from deep in vegetation where it may be difficult for bats to access them (M.J.R., personal communication).

The most common prey species found in fecal samples of *T. cirrhosus* were frogs in the genus *Pristimantis*, particularly *P. taeniatus* (Craugastoridae, formerly *Eleutherodactylus taeniatus*). *Pristimantis taeniatus* is a small leaf-litter frog that calls from trees with high-pitched trilled tinks (Ibáñez et al. 1999). Very little is known about this species, and the behavioral response of *T. cirrhosus* to the calls of *P. taeniatus* had never before been tested. We observed an attack on the speaker in response to this frog call, and the bat response to these species was not significantly different from that to the túngara frog, indicating that it is likely a species that bats are hunting by call. This opens a new area for investigation in how predation by *T. cirrhosus* may be an agent of selection on the calling behavior of *P. taeniatus*.

We found DNA from *Anolis* lizards in almost a quarter of our bat samples, but bats showed a fairly low response to the rustling sounds of anoles moving through leaf-litter. A previous anecdotal report from Honduras described finding a dead anole in the mistnet pocket with a female *T. cirrhosus* (Valdez and LaVal 1971). Anoles do make alarm sounds, but do not call, therefore we anticipate that the rustling sounds made by anoles moving through leaf-litter is the most obvious cue they present to *T. cirrhosus*. We had hypothesized that we would find greater responses to anole rustling sounds in dry season conditions (lizards moving through dry leaves) than wet because movement through dry leaves produces louder, more conspicuous rustling sounds. While we did find anoles in the diet of more *T. cirrhosus* in the dry season than in the wet season, and we did find higher response to the sounds of anoles moving through dry versus wet leaf litter, these differences were not significant. Why bats did not show more response in general to anole rustles is unclear. One possibility is that since the speaker was in a fixed location, the rustling sound does not move in space as a real moving animal would. Additionally, rustles are relatively low amplitude compared to the mating calls we presented to the bats, which could account for the lower responses. Also, anoles are diurnal, therefore we might not predict them to be moving around at night, and rustling sounds

could be indicative of many different potential prey, some more palatable than others. Bats may thus be locating anole prey by some other mechanism than rustling sounds, and one bat did attack a silent, motionless plastic anole model, indicating that *T. cirrhosus* may be able to locate sleeping lizards using echolocation alone. Anoles are diurnal, so unless one was scared off of its perch, they are unlikely to be moving through the leaf litter at night.

Diet samples indicated some predation events that appear to be rare, including predation on the hummingbird *F. mellivora*, and on the bats *Glossophaga soricina*, *C. perspicillata*, and *Micronycteris microtis*. Previous research with *T. cirrhosus* has demonstrated predation on birds (Rodrigues et al. 2014), and other species of bats have been videotaped attacking nesting birds (Perrella et al. 2019). We tested bat response to both hummingbird calls and stationary, silent hummingbird mounts to mimic roosting asleep birds. We saw fairly low responsiveness to hummingbird calls, with only one bat hovering over the speaker. We would not expect bats and hummingbirds to both be active during the same times of the day/night, except brief periods at dusk and dawn. We recorded a strong predatory attack on the hummingbird mount, indicating that while we generally do not consider *T. cirrhosus* to be adept at catching silent, motionless prey (Tuttle and Ryan 1981), they may be able to hunt roosting birds better than we might expect. This is supported by previous research reporting manakin predation by *T. cirrhosus* (Rodrigues et al. 2014), although this previous study could not determine how *T. cirrhosus* were capturing manakins. *Trachops cirrhosus* has also previously been shown to predate on heterospecific bats (e.g., Arias et al. 1999; Bonato and Facure 2000; Rodrigues et al. 2014). *Trachops cirrhosus* frequently roosts with all three of the bat species we found in the diet (Jones et al. 2017), and therefore, it is easy to imagine scenarios in which bats have access to these species as prey. The possibility must be raised that some of these rare prey found in our data represent contamination of our samples. We feel this unlikely, as we consistently washed bat capture bags between mistnetting nights and with the exception of *C. perspicillata*, we rarely capture these other taxa, and it is highly unlikely they would be placed in a bat capture bag.

Trachops cirrhosus is becoming a model system for the cognitive ecology of predators (Page and Ryan 2005, 2006, 2008; Jones, Ryan, et al. 2013; Hemingway et al. 2017, 2018; Hemingway, Lea, et al. 2019; Hemingway, Ryan, et al. 2019), but its diet has remained poorly understood. Our link of sensory ecology with fine-scale dietary information reveals new prey species and opens a wide range of new questions in *T. cirrhosus* sensory ecology and cognition. In particular need of further investigation are how bats are locating and capturing frogs in the genus *Pristimantis* that call with short inconspicuous calls from branches in the tree canopy, and how often bats are hunting stationary roosting prey such as hummingbirds and *Anolis* lizards, given that it had previously been believed that *T. cirrhosus* did not hunt by echolocation alone (Tuttle and Ryan 1981). Inconsistencies we report between bat response to prey cues and presence of prey in bat diets may also point to insights into prey ecology, such as prey that call from protected perches resulting in lower than expected occurrences in diet samples than might be expected from bat response to prey calls. This methodology of combining dietary metabarcoding with behavioral experiments is applicable across any animal taxa for which behavioral experiments can be performed, and is an ideal approach for studying the sensory ecology of foraging.

SUPPLEMENTAL MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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