RESEARCH ARTICLE

Exploratory analysis reveals arthropod consumption in 10 lemur species using DNA metabarcoding

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Abstract

Arthropods (insects, spiders, etc.) can fulfill major nutritional requirements for primates, particularly in terms of proteins, fats, vitamins, and minerals. Yet, for many primate species we know very little about the frequency and importance of arthropod consumption. Traditional methods for arthropod prey identification, such as behavioral observations and fecal dissections, offer limited taxonomic resolution and, as a result, underestimate true diversity. Metabarcoding arthropod DNA from primate fecal samples provides a promising but underused alternative. Here, we inventoried arthropod prey diversity in wild lemurs by sequencing two regions of the CO1 gene. Samples were collected opportunistically from 10 species of lemurs inhabiting three national parks in southern Madagascar using a combination of focal animal follows and live trapping. In total, we detected arthropod DNA in 98 of the 170 fecal samples analyzed. Although all lemur species included in these analyses showed evidence of arthropod consumption, those within the family Cheirogaleidae appeared to consume the highest frequency and diversity of arthropods. To our knowledge, this study presents the first evidence of arthropod consumption in *Phaner pallescens*, *Avahi peyrierasi*, and *Propithecus verreauxi*, and identifies 32 families of arthropods as probable food items that have not been published as lemur dietary items to date. Our study emphasizes the importance of arthropods as a nutritional source and the role DNA metabarcoding can play in elucidating an animal's diet.

KEYWORDS
Cheirogaleidae, CO1 gene, Indriidae, insectivory, Lemuridae, Madagascar

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1 | INTRODUCTION

Arthropod consumption can be an important nutritional strategy in primates, with nearly every species studied showing some incorporation of arthropods in their diet (McGrew, 2001, 2014). This reliance on arthropods has been attributed to their high nutritional content (Janiak, 2016; Janiak et al., 2017; McBeath & McGrew, 1982). Arthropods constitute one of the most protein and fat-rich food sources available in the environment (Oliveira et al., 1976; Raubenheimer & Rothman, 2013; Redford & Dorea, 1984). In addition, arthropods are a vital source of essential vitamins and minerals, such as B_{12} and sodium, acting as a potential selective factor for arthropod consumption in many primate species (Raubenheimer & Rothman, 2013). The high protein, fat, vitamin, and mineral content found in arthropods is equal to that of most vertebrate prey and far exceeds that of fruit and leaves (DeFoliart, 1989; McGrew, 2001; Raubenheimer & Rothman, 2013), suggesting that this resource can be utilized to fulfill essential nutritional requirements for primates.

While arthropods are an important nutritional source for many primates, taxa vary in their nutritional content (Raubenheimer & Rothman, 2013; Rothman et al., 2014). For example, lipid content has been shown to vary from 7.9% in spiders to 58.5% in dragonflies (% dry matter aside from moisture; Rothman et al., 2014). Therefore, precise identification of arthropod prey items permits a more complete understanding of the importance and diversity of arthropods in primate diets. However, performing in-depth studies of arthropod consumption in primates remains difficult. Traditional feeding observations typically provide coarse arthropod taxonomic resolution or missed records of arthropod consumption altogether because of difficulties in visually discerning the prey items being consumed, particularly for arboreal and/or nocturnal species. Additionally, attempts to study arthropod consumption by identifying arthropod fragments in fecal samples, which relies on morphological taxonomy, are hindered by the degraded nature of arthropod specimens in feces resulting from mastication and digestion (Zeale et al., 2011). Gut content analyses are often highly invasive or require euthanasia and are therefore not suitable for many primate studies, with 65% of primate species being classified as Vulnerable, Endangered, Critically Endangered, or Data Deficient by the International Union for Conservation of Nature (IUCN, 2020). Alternative molecular methods, such as DNA metabarcoding of feces, overcome these limitations, and can improve taxonomic resolution of dietary prey items, serving as a powerful and noninvasive tool for dietary analyses.

DNA metabarcoding supplements traditional taxonomic identification, using short, standardized, and species-specific DNA sequences to classify even trace amounts of genetic material. Metabarcoding can return higher sensitivity and taxonomic resolution than traditional morphological assessments of arthropod parts in feces (Hajibabaei et al., 2006; Meusnier et al., 2008; Zeale et al., 2011). As such, it has become common to use metabarcoding in ecological studies to gain a more complete understanding of prey consumption in mammals (Pompanon et al., 2012). In primatology, these methods are gaining traction, and are beginning to reveal the importance of arthropod consumption in primates (e.g., great apes, Hamad et al., 2014; *Cercopithecus*

spp., Lyke, 2018; *Saguinus weddelli*, Mallott et al., 2015; *Cebus capucinus*, Mallott et al., 2017; six New World monkeys, Pickett et al., 2012).

The lemurs of Madagascar represent one such group that could benefit from metabarcoding studies to understand the role of arthropods as a nutritional source in their diets, as these methods have to our knowledge not yet been applied to this group of primates. Most lemur species are classified as either frugivores or folivores, with lemurs of the family Cheirogaleidae displaying the highest levels of arthropod consumption (Mittermeier et al., 2010). While frugivorous lemur species consume a high quantity and variety of fruit, Malagasy fruit contains insufficient levels of nitrogen to meet lemur protein needs (Donati et al., 2017; Ganzhorn et al., 2009). Moreover, some lemurs may experience more extreme resource scarcity and stochasticity than other primate groups (Wright & Randrimanantena, 1989; Wright, 1999, but see Federman et al., 2017). These highly variable environments may necessitate the supplementation of fruit by either leaves or arthropods during periods of resource scarcity, leading to high levels of arthropod consumption in many species (Ganzhorn et al., 2009). Despite this, the utilization of arthropods as a nutritional source for lemurs remains highly understudied.

While most dietary studies of lemurs neglect arthropod consumption altogether, those that have included information provide missing or low taxonomic resolution of prey items, typically categorizing insects as general animal prey, insects, or spiders (e.g., Arrigo-Nelson, 2006; Charles-Dominique, 1990; Fietz & Ganzhorn, 1999; Ganzhorn et al., 1999; Hladik et al., 1980), or by simply reporting common name groups (e.g., Lahann, 2007; Overdorff, 1993; Pages, 1980; Sauther, 1993; Simmen et al., 2003). Metabarcoding can address this gap in our knowledge of lemur dietary strategies by providing high-resolution taxonomic information that traditional methods fail to capture.

To aid in a more complete understanding of the importance of arthropods as a nutritional source in lemurs, and to drive future research directions surrounding this topic, we conducted an exploratory analysis using DNA metabarcoding to document arthropod consumption in 10 lemur species (Supporting Information 1) inhabiting three national parks in Madagascar. While this study is exploratory in nature, we expected that general trends of arthropod consumption would follow patterns described in the literature. Specifically, we expected that lemurs in the family Cheirogaleidae would consume arthropods more frequently and have a higher arthropod dietary richness than the other lemur families studied (Kay, 1984; Mittermeier et al., 2010). Additionally, we expected that lemurs in rainforest environments would display higher arthropod dietary richness than dry forest environments as a byproduct of environmental richness discrepancies (Currie, 1991; Hawkin et al., 2003).

2 | METHODS

2.1 | Ethical statement

All research adhered to the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Non-Human Primates and the Code of Best Practices for Field Primatology and was conducted in

compliance with animal care regulations and applicable national laws of Australia, Madagascar, and the United States. All research protocols were approved by the appropriate Animal Care and Use committees of Australia (The Australian National University #A2018/61), Madagascar (Ministere de l'Environment et des Eaux et Forets, MINEEF), and the United States (Stony Brook University #1177457-3 and #11323621-2, University of Kentucky #2018-2919).

2.2 | Study sites and subjects

Lemur fecal samples were collected from three national parks within Madagascar: Zombitse-Vohibasia, Ranomafana, and Isalo (Figure 1). Zombitse-Vohibasia National Park (ZVNP) in southwestern Madagascar comprises 36,850 ha of succulent woodland habitat ranging from 300 to 825 m in elevation (UNEP-WCMC & IUCN, 2019). ZVNP represents an important remnant of dry deciduous forest, due to its position as one of the only remaining transitional zones between Madagascar's dry and humid forests and eastern and southern floristic domains (Zinner et al., 2001). At this site, we collected fecal samples from three lemur species (*Cheirogaleus medius* (n = 16), *Mirza coquereli* (n = 10), *Phaner pallescens* (n = 6)) from a community of lemurs from December 30, 2018 to January 16, 2019.

Ranomafana National Park (RNP) in southeastern Madagascar consists of 43,000 ha of lowland to montane ecosystems ranging from 500 to 1500 m in elevation (Arrigo-Nelson, 2006). Samples from RNP were collected from three subsites: Vatoharanana (June 1–June 13, 2018), Amboasary (June 21–June 25, 2018), and Sakaroa (June 22–July 21, 2018). Samples from five lemur species were collected in RNP (*Microcebus rufus* (n = 49), *Avahi peyrierasi* (n = 9), *Eulemur rubriventer* (n = 16), *Eulemur rufifrons* (n = 16), *Propithecus edwardsi* (n = 14)).

Isalo National Park (INP) is comprised of 81,500 ha of varying terrain ranging from 510 to 1268 m in elevation in tropical dry climate located in south-central Madagascar (Jones et al., 2008). We collected samples from three lemur species at this site from the Lemur/Rat Canyon area of the park (*E. rufifrons* (n = 9), *Pro. verreauxi* (n = 10), *Lemur catta* (n = 15)) from July 5, 2018 to July 10, 2018.

2.3 | Fecal sample collection

Small-bodied lemur species (*C. medius, Mir. coquereli, Mic. rufus*) were live captured using Sherman live traps and tomahawk traps. Sherman live traps were placed 5 m apart and 2 m off the ground (Atsalis, 1999; Zohdy et al., 2012, 2014). Tomahawk traps were placed 5 m apart and 6–10 m off the ground (Fietz & Ganzhorn, 1999). At dusk each night, traps were opened and baited with banana or pineapple (Atsalis, 1999). Traps were checked each morning at dawn, and fecal samples were collected using sterile tweezers from inside the trap or opportunistically from the animal during handling if defecation occurred (Atsalis, 1999; Zohdy et al., 2017).

We conducted follows of the large-bodied lemur species (*Pha. pallescens, A. peyrierasi, Pro. edwardsi, E. rubriventer, E. rufifrons, Pro. verreauxi, L. catta*) and opportunistically collected fecal samples. Fecal samples were collected from the ground with sterile tweezers no longer than 1 min after defecation. All fecal samples were stored in 100% ethanol in 2 ml sterile screw cap vials and transported back to the United States for DNA extractions.

2.4 | Laboratory analysis

Arthropod DNA from approximately 0.20 g of each lemur fecal sample was extracted using the QIAamp Powerfecal DNA Kit (Qiagen) following the manufacturer's protocol with lengthened bead beating and centrifuge time. DNA was dried and stored in preservation plates (Ivanova & Kuzmina, 2013) and shipped to Queen Mary University of London for polymerase chain reaction (PCR) and sequencing. PCR was conducted using two different primer sets, targeting separate regions of the
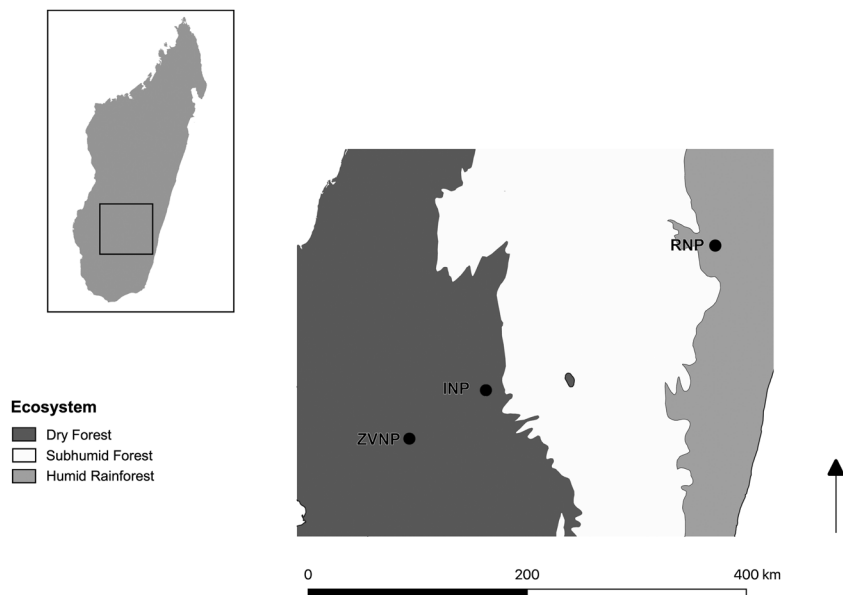

FIGURE 1 Location of sample collection sites in Madagascar

Ecosystem
- Dry Forest
- Subhumid Forest
- Humid Rainforest

RNP

INP

ZVNP

0   200   400 km

mitochondrial cytochrome *c* oxidase subunit 1 gene (CO1), one of 157 bp (Zeale et al., 2011) and the other 312 bp (Cuff et al., 2020). The mitochondrial cytochrome *c* oxidase subunit 1 gene has been recognized as a particularly useful metabarcoding gene, as it is nearly universal across animals, but substitutions allow for species-level differentiation (Hebert et al., 2003).

PCR amplifications were conducted in 22.5 μl reactions that included 15 μl of Multiplex Master Mix (Qiagen), 0.25 μl of both the forward and reverse primers (10 μM), 5 μl of nuclease-free water, and 2 μl of DNA template. The thermocycler conditions were 95°C for 15 min, followed by 35 cycles of 94°C for 40 s, 46°C for 1 min, 72°C for 30 s, and a final extension of 72°C for 5 min.

DNA quantification was conducted using a Qubit dsDNA HS Assay Kit (Invitrogen, Life Technologies), and further quality control was performed using the TapeStation D1000 (Agilent Technologies). Sequencing was performed using 10-bp Fluidigm indexes and MiSeq2 chemistry (Illumina) for 2 × 250 bp target length, at Bart's and the London Genome Centre, London, UK.

2.5 | Bioinformatics

Primers were first trimmed from all the sequences and paired-end reads were merged using USEARCH (Edgar, 2010) before the merged reads were uploaded to mBRAVE (www.mbrave.net). This bioinformatics platform is integrated with the Barcode of Life Data System (Ratnasingham & Hebert, 2007). The mBRAVE platform performs filtering and quality control before taxonomic assignment. The parameters we set were: Trim Length = 600 bp, Min QV = 0 qv, Min Length = 100 bp, Max Bases with Low QV (<20) = 75%, Max Bases with Ultra Low QV (<10) = 75%, ID Distance Threshold = 1.5%, Exclude from OTU Threshold = 3%, Minimum OTU Size = 1, and OTU Threshold = 2%. We used the following system reference databases: (1) SYS-CRLINSECTA = INSECTS, (2) SYS-CRNNONARTINVERT = NON-ARTHROPOD INVERTEBRATE, (3) SYS-NONINSECTARTH = NON_INSECT ARTHROPOD, and (4) SYS-CRLCHORDATA = CHORDATES. Reads from both primer sets were combined into one data set and all hits that had less than 200 sequences assigned were removed from the subsequent analyses based on comparisons with control mock community samples (data unpublished) used to establish optimal parameters for minimizing false-positive assignments while maximizing good taxonomic recoveries.

2.6 | Statistical analyses

All statistical analyses were performed using R statistical software (R Core Team, 2015). Data were collapsed into presence/absence data and bootstrapped 100,000 times to calculate the probability of arthropods being present in a single sample, and the probability of a given arthropod family being present in a sample of 10. These analyses were repeated for each lemur species and results are presented as percentages. To ensure that arthropod dietary richness was being compared across comparable sequencing depths, we generated rarefaction curves across species and used the species with the lowest sampling effort as the point at which richness values were compared. We estimated average arthropod dietary richness for each species by rarifying estimates to 1231 sequences, the point of lowest sampling effort (Donohue et al., 2019; Lyke, 2018). Due to the exploratory nature of this study, and the limited periods of collection, we did not apply statistics to explain the patterns observed in arthropod consumption frequency and dietary richness. As such, the presented results are descriptive in nature, displaying the general trends, directions, and magnitudes of patterns.

3 | RESULTS

We detected arthropod DNA in 98 of the 170 lemur fecal samples analyzed. These 98 samples contained a total of 4,495,081 sequences, with an average of 45,868 sequences per sample (*SD* = 27,982). Of these sequences, 26.43% (1,188,271) were assigned to arthropod taxa (average per sample = 12,125.21, *SD* per sample = 12,097.01). The remaining sequences were either lemur DNA or unassigned. In all, 100% of the arthropod sequences found were assigned to a class, 97.60% were assigned an order, 82.03% a family, 66.82% a genus, and 56.65% a species. In total, we recovered 119 unique sequences (barcode index numbers). These sequences comprised three classes, 11 orders, and 32 families of identified arthropods (Table 1 and Supporting Information 2).

Although all lemur species analyzed showed evidence of arthropod consumption, cheirogaleids (*C. medius, Mir. coquereli, Pha. pallescens, Mic. rufus*) displayed the highest relative frequency (Figure 2; 80%–100% probability of finding arthropod DNA in a single sample) and greatest species richness (Figure 3; 17–48 arthropod species per 1231 sequences). Cheirogaleids most commonly consumed Lepidoptera (moths and butterflies). Additionally, *C. medius* consumed Diptera (flies), *Mir. coquereli* consumed Diptera, Hymenoptera (bees, wasps, and ants), and Hemiptera (cicadas), *Mic. rufus* consumed Diptera and Blattodea (cockroaches), and *Pha. pallescens* consumed Hymenoptera and Araneae (spiders). While Lepidoptera were found in *E. rufifrons* and *L. catta* samples, Hemipterans (true bugs) were more commonly consumed in non-cheirogaleid species (*A. peyrierasi, Pro. edwardsi, E. rubriventer, E. rufifrons, Pro. verreauxi*), with the exception of *L. catta*. Additionally, Hymenopterans were common in *A. peyrierasi* and *Pro. edwardsi* samples, while Diptera were common in *E. rufifrons* samples (Figure 4; Table 1; Supporting Information 2).

ZVNP displayed the highest site-wide probability of arthropod DNA being detected (Figure 2) and the highest site-wide dietary richness (Figure 3). RNP and INP had similar site-wide probability of arthropod DNA being detected (Figure 2) and site-wide dietary richness (Figure 3), with a few notable exceptions. *Microcebus rufus* of RNP had a similar probability of arthropod DNA being detected as the other members of the family Cheirogaleidae from ZVNP but had the highest dietary richness. *Eulemur rufifrons* were tested in both RNP and INP. This species displayed a higher probability of arthropod DNA being present in INP but displayed a higher dietary richness in RNP.

TABLE 1 Arthropod orders and families found in lemur diets

Number[a]	Arthropod Order	Arthropod Family	Lemurs
1	Lepidoptera	Blastobasidae	*E. rufifrons*
2		Crambidae	*C. medius, Pha. pallescens*
3		Erebidae	*C. medius, Mir. coquereli, Pha. pallescens*
4		Eutellidae	*C. medius, Mir. coquereli, Pha. pallescens*
5		Gelechiidae	*Pha. pallescens, Mic. rufus*
6		Geometridae	*C. medius, Mir. coquereli, Pha. pallescens, Mic. rufus*
7		Hesperiidae	*Mic. rufus*
8		Lecithoceridae	*Pha. pallescens*
9		Noctuidae	*Mir. coquereli, Pha. pallescens, Mic. rufus*
10		Notodontidae	*C. medius*
11		Nymphalidae	*Mic. rufus*
12		Papilionidae	*C. medius, Mir. coquereli, Mic. rufus*
13		Saturniidae	*Pha. pallescens*
14		Scythrididae	*Mic. rufus*
15		Sphingidae	*C. medius, Mic. rufus*
16		Stathmopodidae	*L. catta*
17		Tortricidae	*Mic. rufus, E. rufifrons*
18	Diptera	Cecidomyiidae	*C. medius, Mir. coquereli, Mic. rufus, E. rufifrons*
19		Chironomidae	*Mic. rufus*
20		Drosophilidae	*C. medius, Mir. coquereli, Mic. rufus, A. peyrierasi, E. rufifrons*
21		Psychodidae	*Mic. rufus, E. rufifrons*
22	Hemiptera	Aphrophoridae	*Mic. rufus, E. rufifrons*
23		Cicadellidae	*Mic. rufus, A. peyrierasi, Pro. edwardsi, E. rubriventer, E. rufifrons, Pro. verreauxi*
24		Cicadidae	*Mir. coquereli*
25	Hymenoptera	Formicidae	*Mir. coqureli, Pha. pallescens, Mic. rufus, A. peyrierasi, Pro. edwardsi*
26		Vespidae	*Mir. coqureli*
27	Orthoptera	Gryllidae	*Mic. rufus*
28		Tetrigidae	*Mic. rufus*
29	Coleoptera	Carabidae	*Mic. rufus*
30	Araneae	Areaneidae	*Pha. pallescens, Mic. rufus*
31		Trachelidae	*Mic. rufus*
32	Sarcoptiformes	Peloppidae	*Mic. rufus, L. catta*
33	Scorpiones	N/A	*Mic. rufus*
34	Blattodea	N/A	*Mic. rufus*

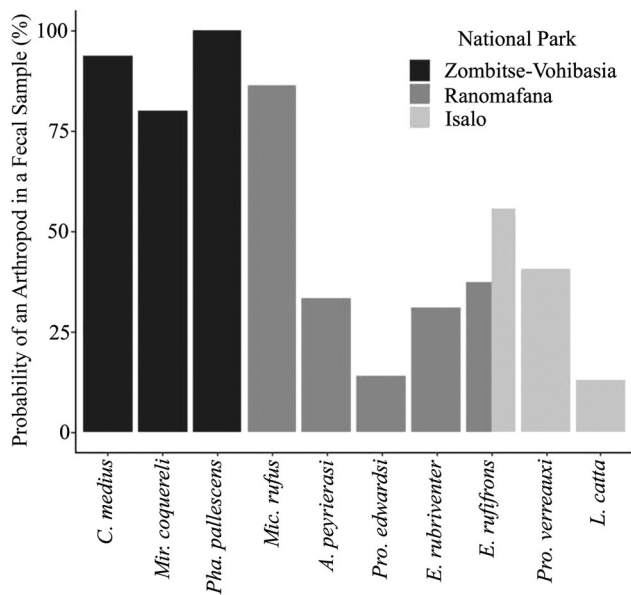[a]See Figure 4 for associated probabilities of being in the diet.

FIGURE 2 Bootstrapped calculations of the probability of arthropod DNA being present in a single fecal sample of each of 10 lemur species from three national parks presented as percentages
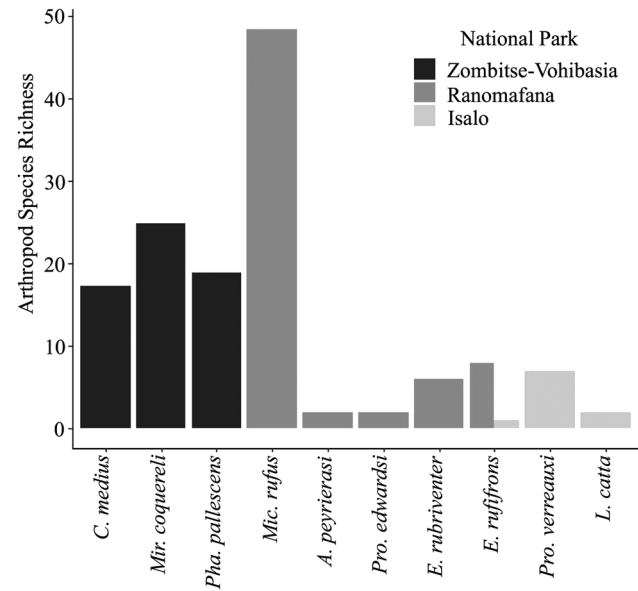

FIGURE 3 Arthropod dietary richness for each of the 10 lemur species from three national parks, rarified to 1231 sequences

4 | DISCUSSION

To our knowledge, this is the first study to publish evidence of arthropod consumption in *Pha. pallescens*, *A. peyrierasi*, and *Pro. verreauxi*. *Phaner pallescens* proves difficult to study due to its nocturnal habit and tendency to occupy the highest portions of the canopy (Kappeler, 2003; Schülke, 2003). Previous studies have pointed to insect foraging as a strategy in *Phaner furcifer* (now classified as *Pha. pallescens*; Groves & Tattersall, 1991) only when the protein content of plant exudates in the forest was low (Hladik et al., 1980). While *Pha. furcifer* (*Pha. pallescens*) have been seen consuming arthropods, fork-marked lemurs of the Marosalaza forest are thought to gain most of their protein needs from gums of *Terminalia* spp. trees (Hladik et al., 1980). Our study overcomes traditional observational difficulties and reports the first specific arthropod dietary items in *Pha. pallescens*, suggesting arthropods may be an important nutritional source for *Pha. pallescens*, warranting further study.

Avahi spp. are medium-sized nocturnal lemurs that primarily consume leaves, supplemented by a small amount of flowers and fruit (Faulkner & Lehman, 2006; Ganzhorn et al., 1985; Harcourt, 1991; Norscia et al., 2011). The nocturnal and folivorous nature of these lemurs may have led to the underestimation of arthropods as a potential nutritional source in past studies. However, as *Avahi* spp. have a relatively small body mass for a folivore at 700–1400 g (Mittermeier et al., 2010), and gut differentiation more similar to frugivores leading to inefficient leaf digestion (Chivers & Hladik, 1980), arthropods may provide an important and easily accessible form of nutrients which act as a necessary supplement during times when leaves do not meet nutritional needs.

The diet of *Pro. verreauxi* seasonally varies between leaves, fruits, and flowers (Koch et al., 2016; Richard, 1977). While the ability to rely on

leaves and fruits may have led to arthropods being overlooked as a potential supplement in the past, arthropods could prove to be an important nutritional source for this species, particularly during periods of resource scarcity and when fruit provides insufficient levels of nitrogen to meet lemur protein needs (Donati et al., 2017; Ganzhorn et al., 2009). Additionally, arthropod consumption may have previously been underestimated in this species due to unintentional consumption of arthropods with leaves and fruits that would formerly be difficult to observe/record using traditional observational methods yet provide important nutritional content.

Where information regarding arthropod consumption has been published for the remaining study species, taxonomic resolution of arthropod prey has been lacking, with most studies reporting the arthropod consumption as general animal prey, insects, or spiders (e.g., Arrigo-Nelson, 2006; Fietz & Ganzhorn, 1999; Ganzhorn, 1988; Hladik et al., 1980) or reporting broad common name groups (e.g., Lahann, 2007; Overdorff, 1993; Pages, 1980; Sauther, 1993; Simmen et al., 2003). Two studies have reported arthropod consumption to species level through observation of feeding, naming three millipede species consumed by *E. rubriventer* and *E. rufifrons* (Overdorff, 1993) and one spider species by *E. rufifrons* (Simmen et al., 2003).

Possibly the most comprehensive studies to date reported arthropod consumption to the order level using microscopy in *Mic. rufus* (Atsalis, 1999; Pages, 1980), with orders Coleoptera, Orthoptera, Hymenoptera, and Hemiptera dominating the diet. Our results supported the consumption of these orders by *Mic. rufus*, validating the ability of metabarcoding to identify hard-bodied, chitinous arthropods. However, we also found the additional presence of Blattodea, Scorpiones, and Sarcoptiformes in the diet, and a high diversity of soft-bodied Lepidoptera.
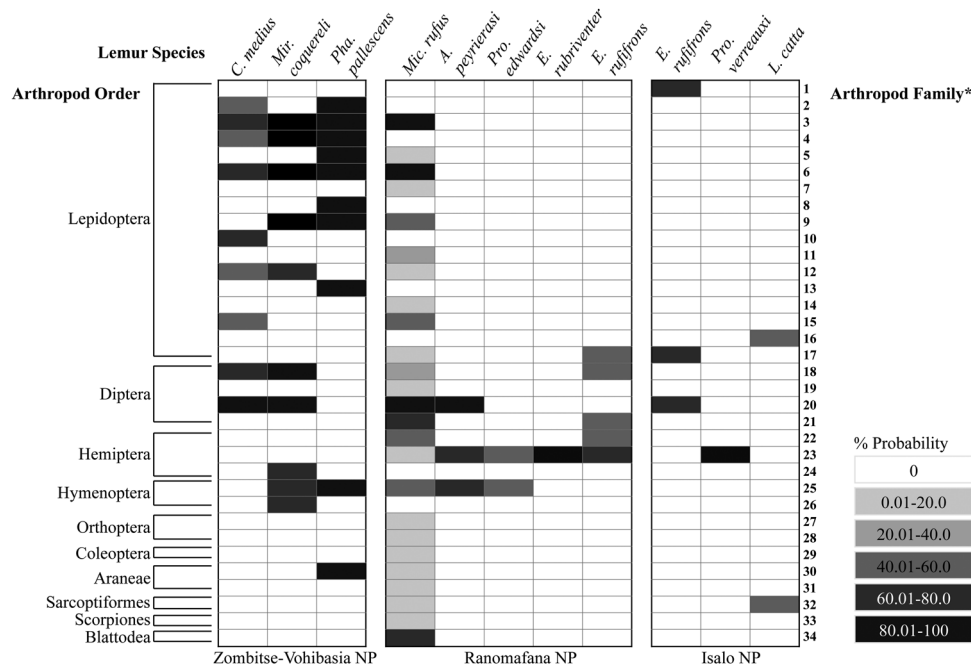
FIGURE 4 Bootstrapped calculations of the probability of each arthropod family being present in a subset of 10 samples from each of the 10 lemur species from three national parks. *See Table 1 for a list of associated arthropod families

Cheirogaleus medius has also been reported to prefer Coleoptera as prey, followed by consumption of Blattodea, Hymenoptera, and Hemiptera (Hladik et al., 1980); this contrasts with our results, which show a high diversity of Lepidoptera in the diet. However, both of these previous studies showing a high presence of Coleoptera in the diet used traditional morphologic assessment, which are biased toward the detection of prey with more chitin, such as beetles (Zeale et al., 2011). This may explain the discrepancy between these studies' findings and ours, as molecular methods are now able to identify not only hard-bodied arthropods with more chitin, such as Coleoptera, but soft-bodied arthropods such as Lepidoptera as well. Our study highlights this discrepancy and identifies 32 families of arthropods that to our knowledge have not been published as lemur dietary items, many of which are cryptic and difficult to identify via traditional methods. However, current technology does not allow the quantification of relative abundances of a prey item in an individual's diet, or indicate the developmental stage of the arthropod consumed (King et al., 2008; Mallott et al., 2015, 2017; Pompanon et al., 2012). To gain a more complete understanding of arthropod consumption in lemurs, molecular methods are optimally combined with more traditional methods to both increase taxonomic coverage and clarify the proportions of arthropods being consumed.

In line with our expectations and the literature, we found that although all lemurs species analyzed showed evidence of arthropod consumption, lemurs of the family Cheirogaleidae displayed the highest relative frequency and greatest species richness. This pattern may be driven by the tendency for smaller primates to incorporate more arthropods in their diet to meet nutritional requirements (Kay, 1984). In addition to taxon-based variation in arthropod

consumption, we found site-based differences in the frequency and richness of arthropods in the diet. While ZVNP showed the highest site-wide probability of arthropod DNA being detected (Figure 2) and the highest site-wide dietary richness (Figure 3), this pattern may be driven by taxonomic bias, as the study species at ZVNP were primarily lemurs of the family Cheirogaleidae. In comparison with ZVNP lemurs in the family Cheirogaleidae, RNP *Mic. rufus* displayed a similar frequency of arthropod consumption but a higher dietary richness. This may be due to higher environmental arthropod richness in a humid forest versus dry forest ecosystem (Currie, 1991; Hawkin et al., 2003). Additionally, *E. rufifrons* displayed a higher probability of arthropod DNA being present in INP compared to RNP, but the *E. rufifrons* of RNP displayed a higher dietary richness. This trend also supports the notion that the potentially higher environmental richness of a humid forest habitat may lead to higher dietary richness.

While this exploratory analysis demonstrates the utility of metabarcoding as a powerful tool in identifying arthropod consumption in lemurs, it is important to comment on the limitations of relying solely on molecular analyses to study arthropod consumption. By using only molecular methods when studying diet, there is a potential for environmental contamination if the fecal sample contacts soil or leaf litter. For example, it is unclear whether Dipteran sequences are a result of intentional consumption, or are due to contamination from fly eggs being laid on the surface of the fecal sample before collection (Hofreiter et al., 2010; Mallott et al., 2015, 2017; Pompanon et al., 2012). While the collection of fecal samples quickly after defecation minimized this bias in our study, we cannot exclude potential contamination. Future studies will attempt to experimentally isolate this variable. The occurrence of a mite family (Peloppidae) in some

samples of *Mic. rufus* and *L. catta* may be the product of environmental contamination or may have occurred due to these mites parasitizing consumed arthropods.

Metabarcoding can be further limited by primer biases and degradation of DNA, leading to amplification failure (King et al., 2008; Mallott et al., 2015, 2017; Pompanon et al., 2012), and should always remain a consideration of analysis. We strongly suggest future studies employ multiple primer sets, as we did, to mitigate amplification and binding bias. Despite these limitations, this exploratory analysis showed that arthropods are eaten by all study species, and that our limited knowledge of arthropod consumption in lemurs could be greatly augmented by the use of metabarcoding methods.

CONFLICT OF INTERESTS

Patricia C. Wright is on the advisory board of Primate Conservation, Inc. (PCI), one of the funders of this project. She did not advise PCI on funding this project.

DATA AVAILABILITY STATEMENT

Raw sequence data that support the findings of this study are publicly accessible at https://doi.org/10.6084/m9.figshare.c.5272589.v1. Full taxonomic information of the sequenced arthropods is summarized in the supporting information. All additional data are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

Amanda K. Rowe: Conceptualization (lead); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing-original draft (lead); writing—review and editing (equal). **Mariah Donohue**: Conceptualization (supporting); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing—review and editing (equal). **Elizabeth L. Clare**: Conceptualization (supporting); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing—review and editing (equal). **Rosie Drinkwater**: Data curation (equal); formal analysis (equal); investigation (equal); project administration (equal); software (equal); supervision (equal); validation (equal); visualization (equal); writing—review and editing (equal). **Andreas Koenig**: Conceptualization (supporting); formal analysis (supporting); funding acquisition (supporting); methodology (supporting); supervision (equal); visualization (equal); writing—review and editing (equal). **Zachary M. Ridgway**: Conceptualization (supporting); investigation (supporting); project administration (supporting); visualization (supporting); writing—review and editing (equal). **Luke D. Martin**: Conceptualization (supporting); funding acquisition (supporting); investigation (supporting); project administration (supporting); resources (supporting); writing—review and editing (equal). **Eva S. Nomenjanahary**: Investigation (supporting); project administration (supporting); writing—review and editing (supporting). **Lovasoa J. Randriamanandaza**: Investigation (supporting); project administration (supporting); writing—review and editing (supporting). **Thierry E. Rakotonirina**: Investigation (supporting); project administration (supporting); writing—review and editing (supporting). **Patricia C. Wright**: Conceptualization (supporting); funding acquisition (supporting); methodology (supporting); resources (equal); supervision (equal); writing—review and editing (equal).

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