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Research Article

A metabarcoding assessment of the diet of the insectivorous bats of Madeira Island, Macaronesia

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Abstract

Understanding the trophic structure of species assemblages is crucial in order to comprehend how syntropic species coexist in space and time. Bats are the second most taxonomically diverse group of mammals and display a wide range of dietary strategies. Due to their ability to disperse over water, ca. 60% of all extant bat species occur on islands and for the most part their interspecific ecological interactions are poorly known. Using DNA metabarcoding, this study offers the first insights into the diet of Macaronesian bats by providing a holistic overview of prey consumed by all 3 bat species found on Madeira Island (*Pipistrellus maderensis*, *Nyctalus leisleri verrucosus*, and *Plecotus austriacus*) and investigating both interspecific (between *P. maderensis* and *N. l. verrucosus*) and intraspecific (between female and male *N. l. verrucosus*) dietary differences. We identified a total of 110 species of arthropod prey in the diet of the 3 bat species, including multiple agriculture and forestry pest species, a human disease-relevant species, and numerous taxa not previously recorded on the island. Lepidoptera was the primary prey order for all 3 bat species. The diet composition of *P. maderensis* and *N. l. verrucosus* differed significantly, with *P. maderensis* consuming more Diptera and multiple prey taxa not found in the diet of *N. l. verrucosus*. Moreover, male *N. l. verrucosus* exhibited a broader niche breadth than females. This study is among the first to use DNA metabarcoding to evaluate the diet of insular bats and thus greatly advances knowledge regarding the trophic ecology and pest suppression services of these poorly-known mammals.

Key words: agricultural pests, ecosystem services, insular bats, resource partition, syntropic species, trophic ecology.

Predator–prey interactions play a crucial role in shaping ecosystem dynamics, and understanding the trophic structure of biological communities is essential for effective species and ecosystem management (Jia et al. 2021). Despite covering a relatively small percentage of terrestrial land area, islands are home to a significant number of unique taxa and ecological communities (Nori et al. 2022). Compared to mainland areas of similar size, latitude, and topographical complexity, islands generally have fewer species, which translates to fewer interspecific competitors for individual species (Lister 1976). As a result, insular species may have higher densities and wider niches than their mainland counterparts, since competitors often constrain resource use and abundance (Herrmann et al. 2020). Over evolutionary time, insular species are likely to adapt their diet to local conditions, which may lead to novel trophic relationships.

Insectivorous bats play an important role in suppressing arthropod populations (Kunz et al. 2011) and have great potential

as bioindicators (Kunz et al. 2011; Ramírez-Fráncel et al. 2022). Due to their ability to fly, bats tend to be good colonizers of oceanic islands, where they usually represent most (or all) of the native, nonmarine mammals (Fleming and Racey 2009). About 60% of the over 1,400 bat species occur on islands, and about 25% of all species are island endemics (Jones et al. 2009; Conenna et al. 2017). However, insular bats, which inhabit some of the most vulnerable terrestrial habitats, are relatively poorly studied (Conenna et al. 2017).

The oceanic island of Madeira is home to 3 bat species: the Macaronesian endemic Madeira Pipistrelle *Pipistrellus maderensis*, the Lesser Noctule *Nyctalus leisleri*, and the Grey Long-eared Bat *Plecotus austriacus* (Teixeira and Jesus 2009; Ferreira et al. 2022). Although classified as Vulnerable by IUCN, the Madeiran population of *P. maderensis* is very flexible regarding its habitat requirements and forages over most of the island habitats, being particularly associated with forest, agricultural, and urban

biotopes (Ferreira et al. 2022; Rocha 2023). Despite the lack of diet studies, it has been suggested that *P. maderensis* feeds mainly on mosquitoes (Diptera), caddisflies (Trichoptera), butterflies/moths (Lepidoptera), and small beetles (Coleoptera; Jesus et al. 2009; Nóbrega et al. 2023). On the other hand, *N. leisleri*, which in Madeira is represented by the endemic subspecies *N. l. verrucosus* (Palmeirim 1991), is an aerial-hawking species particularly associated with deciduous and coniferous woodlands (Boston et al. 2020; Ferreira et al. 2022). Despite insufficient information, the diet of *N. leisleri* in mainland Europe seems to consist mostly of small- to medium-sized Diptera, Trichoptera, Coleoptera, and Lepidoptera (Waters et al. 1999; Kaňuch et al. 2005)—with a particular preference for aquatic insects or with aquatic larvae (Vaughan 1997; Shiel et al. 1998). *Plecotus austriacus*, which arrived to Madeira during its relatively recent westward expansion across Europe (Juste et al. 2004), is the rarest of all 3 bat species found on the island and is mostly detected in lowland production forests (Ferreira et al. 2022). The species forages mostly on open-edge habitats (Razgour 2021) and within its non-Macaronesian distribution the diet seems to consist primarily of moths (Lepidoptera) complemented to a large extent with Diptera, Neuroptera, Hemiptera, Trichoptera, and Isopoda (Ashrafi et al. 2011; Razgour et al. 2011).

Traditionally, direct observation or microscopic examination of prey fragments in the feces were used in order to analyze trophic interactions of a specific animal. However, since most bats prey aerially at night and masticate larger prey fragments, these methods are not particularly effective for analyzing their diet. Therefore, the emergence of DNA metabarcoding has greatly expanded our understanding of bat trophic ecology, allowing for a more reliable and systematic approach to diet studies (Yoccoz 2012; Mata et al. 2016; Hemprich-Bennett et al 2021) and—relative to diet analysis using more traditional approaches—increasing the probability of identifying a higher proportion of the prey species consumed (Yoccoz 2012). Furthermore, when using this approach, the level of prey identification does not depend on the experience of the observer, and there is no hardness bias, i.e., hard prey being more easily identifiable due to presence of distinctive morphological characters (Yoccoz 2012).

The main aim of this study is to investigate the trophic ecology of the 3 bat species found on Madeira Island using DNA metabarcoding. Specifically, we address the following questions: First, which prey taxa are consumed by *P. maderensis* (endemic species to Macaronesia), *N. l. verrucosus* (subspecies endemic to Madeira), and *P. austriacus*? We anticipate that all 3 species prey on a wide diversity of prey native and non-native to Madeira Island, including potential agricultural and agroforestry pests and human disease vectors. Second, how similar is the diet of *P. maderensis* and *N. l. verrucosus* (the 2 most common species) in terms of prey composition and diversity? Due to considerable differences in habitat use and foraging ecology of *P. maderensis* and *N. l. verrucosus*, we hypothesize that the 2 species are likely to exhibit considerable differences in prey selection. Third, are there differences in prey species composition and prey species richness in the diet of female and male *N. l. verrucosus*? Considering the increased energetic demands of females associated with pregnancy and lactation, we expect sex-specific differences in the diet of *N. l. verrucosus*.

Materials and methods

Study area

The study was conducted on Madeira, a volcanic island with a maximum elevation of 1,889 m a.s.l. located in the northeastern Atlantic Ocean. The archipelago of Madeira is part of the Macaronesian biogeographic subregion, which also includes the archipelagos of Azores, Selvagens, Canary Islands, and Cabo Verde. Fieldwork took place at the Ecological Park of Funchal (Fig. 1), a municipal protected area established in 1994 (Nunes et al.
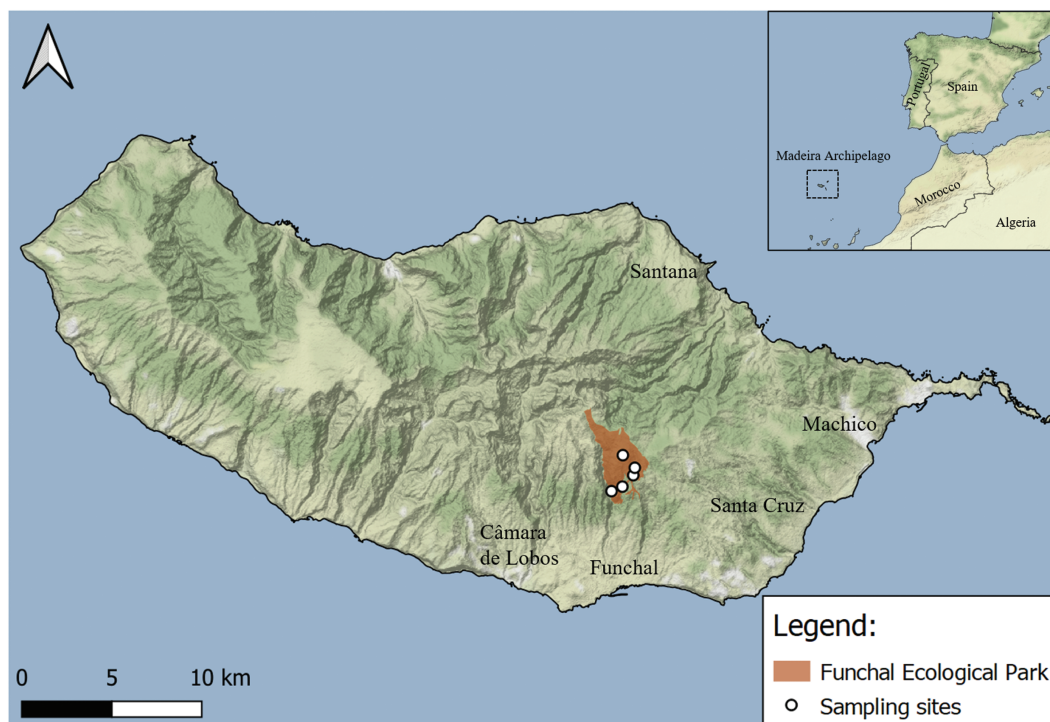
Fig. 1. Map of Madeira Island and location of the Ecological Park of Funchal. Sampling sites are indicated by a white dot.

2010). The park—encompassing ca. 1,000 ha and located north of the island capital of Funchal—is bounded to the North by the municipality of Santana, to the East by Machico and Santa Cruz, and to the Northwest by Câmara de Lobos. The park extends from 470 m a.s.l. at the confluence of the Pisão stream with the Santa Luzia stream to 1,818 m a.s.l. at Pico do Areeiro (Soto et al. 2023).

Sample collection

Fieldwork took place from September to October 2021. Mist nets were used to capture bats at 5 different sites across the park (Fig. 1). Bat capture and handling was conducted following guidelines approved by the American Society of Mammalogists (Sikes et al. 2016). The sex, age (juveniles vs. adults), weight, forearm length, and reproductive status of each captured individual was recorded. Bats were kept in cloth bags until defecation occurred, after which fecal samples were collected using sterilized forceps directly from the bottom of the bags. Pellets were stored in tubes containing silica gel and refrigerated until DNA extraction was performed. Sampling was performed at night (6 PM to 4 AM) to match with the peak activity pattern of the species. A total of 100 bat pellets were collected during fieldwork, including 75 of *N. l. verrucosus* (49 females and 26 males), 22 of *P. maderensis* (7 females and 15 males), and 3 of *P. austriacus* (2 females and 1 male).

DNA extraction and amplification

DNA was extracted using the E.Z.N.A. tissue DNA kit (Omega Bio-Tek, Norcross, Georgia) following (Mata et al. 2021), except that no Inhibitex tablets (Qiagen, Hilden, Germany) were used. First, 1 pellet per sample was removed from the original tube with silica gel to an extraction tube with 650 µL of lysis buffer (0.1 M Tris–HCl, 0.1 M EDTA, 0.01 M NaCl, 1% N-lauroylsarcosine, pH 7.5 to 8) using a sterilized tweezer. After that, fecal samples were homogenized with a sterilized spatula, vortexed, and left in a dry bath at 56 °C for 30 min. Samples were then vortexed for 1 min and centrifuged at $12,000 \times g$ for 30 s. Up to 500 µL of supernatant was transferred to a new tube and 25 µL of OB Protease was added. The remaining steps followed kit recommendations, except that DNA was eluted 2 times in 50 µL into different extracts. DNA was extracted in batches of 23 samples plus 1 negative control in which no fecal pellet was added. Extracted DNA was distributed in 96-well plates where the last well was left empty for polymerase chain reaction (PCR) negative control. DNA extracts were stored at -20 °C until amplification. Mitochondrial DNA was amplified by PCR using arthropod general cytochrome oxidase subunit 1 (*COI*) primers fwhF2-R2n (Vamos et al. 2017) modified with Illumina overhangs. PCR reactions were carried out in volumes of 10 µL, including 5 µL of QUIAGEN Multiplex PCR Master Mix, 0.25 µL of forward primers, 0.25 µL of reverse primers, 2.5 µL of ultrapure water, and 2 µL of DNA extract. PCR cycling conditions consisted in an initial denaturing at 95 °C for 15 min, 42 cycles of 95 °C denaturing for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 60 °C for 10 min. Amplification success and quality was checked by visualizing PCR products in 2% agarose gels stained with GelRed.

Library preparation

PCR products were diluted 1:4 with water and tagged for individual identification in a second amplification step, this time with P5 and P7 Illumina adaptors along with unique combinations of 7-bp barcodes. PCR mix consisted in 7 µL KAPA HiFi HotStart ReadyMix (Rocher, KAPA Biosystems, Basel, Switzerland), 2.8 µL of ultrapure water, 0.7 µL of each P5 and P7 index, and 2.8 µL of diluted PCR product. Cycling conditions of the indexing PCR consisted of denaturation at 95° for 3 min, followed by 9 cycles of 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72° for 30 s, and a final extension at 72 °C for 5 min. Afterwards, indexed PCR products were cleaned with Agencourt AMPure XP beads (Beckman Coulter, Brea, California) with a 1:0.8 ratio, following the manufacturer's instructions, to remove remaining nucleotides, primers, and primer dimers. Next, libraries were quantified using Epoch (dsDNA) followed by normalization to 25 nM for a volume of 10 µL. Finally, samples were pooled equimolarly and run in TapeStation (Agilent, Santa Clara, California) using a High Sensitivity D1000 ScreenTape, to validate average amplicon length and absence of small nontarget amplicons or primer dimers that might interfere with sequencing. DNA concentration was further validated by qPCR (KAPA Library Quant Kit qPCR Mix, Rocher) and diluted to 4 nM. Libraries were finally sequenced in a MiSeq desktop sequencer (Illumina) using a MiSeq Reagent Kit v3 (2 × 250 bp) along with samples from other projects with a target depth of 25k reads/sample.

Bioinformatics

Bioinformatic processing of raw sequencing data was done using Obitools (Boyer et al. 2016) and VSEARCH (Rognes et al. 2016). Paired reads were first combined using the command "illuminapairedend" and then, removal of primer sequences and sample-tagging of reads was done with "ngsfilter." Next, the command "obiuniq" was used to individually dereplicate reads for each sample. Potential PCR and sequencing artifacts, as well as sequences shorter or longer than predicted (202 to 208 bp), were removed with the command "–cluster_ unoise," followed by chimeral removal with "–uchime3_denovo." Finally, sequences were clustered at 99% similarity using "–cluster_size" and initial reads mapped back to the retained Operational Taxonomic Units (OTUs) using "–usearch_global" with an identity level of 99%. The R package "LULU" (Frøslev et al. 2017) was used to further remove PCR and sequencing artifacts, along with nuclear copies of the mitochondrial gene. OTUs with a sequence similarity above 84% co-occurring in over 95% of the samples were merged into a single unit and their reads summed. OTUs were taxonomically assigned using "BOLDigger" (Buchner and Leese 2020) and manually curated. When different OTUs were assigned to the same taxon but at the genus level or above, they were numbered differently to keep them differentiated during statistical analysis (e.g., if OTU1 and OTU2 were both identified at family level to the taxon Noctuidae, they were classified as Noctuidae 1 and Noctuidae 2). Each OTU was further classified as diet if identified to the order level or below, while belonging to classes Insecta or Arachnida, except for known parasites like Trombidiformes, Mesostigmata, Ixodida, and Siphonaptera. OTU reads observed in extraction and PCR negative controls were subtracted from the corresponding samples, while dietary OTUs representing less than 1% of the total dietary reads of each sample were removed in order to reduce false positives associated with events of lab and sequencing cross-contamination.

Statistical analysis

Due to limitations in sample size, comparisons of niche breadth, diet diversity, and prey composition were limited to comparisons among *N. l. verrucosus* and *P. maderensis*, as well as between sexes of *N. l. verrucosus*. Comparisons were always done at 3 taxonomic levels: order; family; and species/OTU. Statistical significance was considered at an alpha value of 0.05.

Niche breadth (i.e., prey gamma diversity) was estimated using rarefaction curves based on Hill numbers using the package and function "iNEXT" (Hsieh et al. 2016). The diversity of ingested prey items was considered significantly different between categories if the 95% confidence intervals did not overlap at similar levels of sample coverage. Next, diet diversity (i.e., prey alpha diversity) was compared across groups using a generalized linear model. For this, a linear model was fit using the "glm" function, with a "poisson" distribution, using the number of prey consumed by each individual as the response variable, and bat species or sex as the explanatory variable. An analysis of variance (ANOVA) was then used to test the significance of the model using the function "anova" from the package "car".

To assess differences in diet composition, the number of reads per sample and prey item was converted into a presence (>0) and absence (0) matrix. Then, using the "vegan" package (Dixon 2003), a distance matrix between samples was created using the command "vegdist." Differences in dietary composition between species and, between sexes in *N. l. verrucosus* were tested using a permutational multivariate analysis of variance (PERMANOVA) with the function "adonis." The command "betadisper" was then used to test for differences in variance homogeneity across groups. This test was conducted to determine the degree to which the findings of the PERMANOVA were reliable. Finally, the command "simper" was used to identify the prey items that contributed most to dietary composition disparities across groups. A multidimensional scaling (MDS) plot was further created by using the R base function "plot" on the "betadisper" object created earlier to visualize diet similarity among samples.

Unless otherwise specified, all analyses were conducted in R 4.1.3 (R Development Core Team 2022).

Results

Overall diet description

DNA material was obtained from 72 of the 100 fecal samples collected, leading to a total of 110 haplotypes and 1,750,053 reads. All 3 bat species showed a diverse diet composed of multiple arthropod orders and families. In total, 110 OTUs of at least 12 orders, 39 families, 63 genera, and 59 species were identified to be consumed by the 3 bat species considered.

In the case of *N. l. verrucosus*—the species with highest sample size—a total of 81 prey items representing 10 orders and 33 families were identified, all belonging to the class Insecta (Fig. 2). Most prey belonged to the order Lepidoptera (50.5%), followed by Coleoptera (17.5%), Diptera (13.6%), Hemiptera (7.8%), and Neuroptera (5.3%). Noctuidae was the most consumed family (18%), followed by Cerambycidae (16.5%), Blastobasidae (8.7%), Limoniidae (8.3%), and Geometridae (8.3%). The most common species/OTUs in the diet of *N. l. verrucosus* was *Arhopalus ferus* (15.0%), followed by *Gymnoscelis rufifasciata* (5.8%), and *Agonopterix scopariella* (5.3%). Additionally, of the 44 consumed species, 29.5% referred to known agriculture pests (e.g., *Agrotis segetum*) and 15.9% referred to possible agricultural pests (Table 1). In the case of *P. maderensis*, a total of 49 prey items of 10 orders and 22 families were identified, associated with classes Insecta (95.4%) and Arachnida (4.6%; Fig. 2). As in *N. l. verrucosus*, most prey belonged to the order Lepidoptera (47.1%), followed by Diptera (29.9%), Trichoptera, Coleoptera, and Araneae (all 3 at 4.6%). Blastobasidae was the most consumed family (23.0%), followed by Limoniidae (13.8%), Tineidae (8.0%), and Psychodidae (6.9%).

Lastly, the most observed species/OTUs were *Blastobasis vittata* (12.6%), followed by *Dicranomyia* sp. 1 (8.0%), and *Opogona sacchari* (4.6%). Additionally, of the 31 prey species identified, at least 19.3% were known agriculture pests (e.g., *O. sacchari*), 6.4% were possible agriculture pests, and 3.2% species with human health implications (Table 1). Only 3 fecal samples were obtained for *P. austriacus* and thus results should be interpreted carefully. A total of 8 prey from 2 orders and 3 families were identified (Fig. 2). Similar to the other 2 species, most prey belonged to the order Lepidoptera (80%), followed by Coleoptera (20%); with Noctuidae being the most common family (70%), followed by Cerambycidae (20%), and Geometridae (10%). The species/OTUs most observed were *A. ferus* and *Mythimna unipuncta* (both 20%). Additionally, of the 6 prey items identified to the species level, at least 50% referred to known agriculture pests (e.g., *Chrysodeixis chalcites*; Table 1).

Arthropod species native but not endemic to Madeira constituted most of the prey consumed by the 3 different species of bats (32.2%), followed by introduced species (23.7%), native endemic species (18.6 %), and a relatively small percentage of native species endemic to Macaronesia (3.4%) and suspected migratory species (1.7%; Supplementary Data SD1).

Interspecific variation in diet of *N. l. verrucosus* and *P. maderensis*

For similar sampling coverage levels, *P. maderensis* exhibited a wider dietary niche breadth than *N. l. verrucosus* at the order and OTU level (Supplementary Data SD2). However, at the family level, *N. l. verrucosus* appeared to have a wider niche breadth than *P. maderensis*. Nevertheless, as confidence intervals overlapped at all 3 levels, the observed differences were not statistically significant. Average prey diversity per sample was slightly higher for *P. maderensis* than for *N. l. verrucosus* (Supplementary Data SD3). However, these differences were not statistically significant for all taxonomic levels analyzed (order, $P = 0.435$; family, $P = 0.215$; OTU, $P = 0.394$).

The diet composition of *P. maderensis* and *N. l. verrucosus* differed at the order ($P = 0.006$), family ($P < 0.001$), and OTU level ($P < 0.001$) as the MDS plot demonstrates (Fig. 3). Simper species analysis showed that at the order level, *P. maderensis* consumed more Diptera than did *N. l. verrucosus* ($P < 0.05$; 77% of samples vs. 39%). Moreover, Trichoptera ($P < 0.01$; 16%) and Araneae ($P < 0.01$; 16%) were consumed by *P. maderensis* but absent from the diet of *N. l. verrucosus.* At the family level, Tineidae was more frequent on the diet of *P. maderensis* than *N. l. verrucosus* ($P < 0.001$; 33% vs. 4%), along with Blastobasidae ($P < 0.001$; 72% vs. 25%), Chironomidae ($P < 0.001$; 27% vs. 1.9%), Psychodidae ($P < 0.001$; 27% vs. 0%), and Limnephilidae ($P < 0.01$; 16% vs. 0%). Finally, simper analysis evidenced that at the OTU level, *N. l. verrucosus* consumed more *A. ferus* than did *P. maderensis* ($P < 0.05$; 60% vs. 5%). On the other hand, *B. maroccanella* was more common in the diet of *P. maderensis* than in that of *N. l. verrucosus* ($P < 0.05$; 16% vs. 4%), along with *O. omoscopa* ($P < 0.05$; 16% vs. 4%), *B. vittata* ($P < 0.001$; 61% vs. 15%), *Dicranomyia* sp. 1 ($P < 0.05$; 38% vs. 13%), *O. sacchari* ($P < 0.01$; 22% vs. 0%), *Limnephilus affinis* ($P < 0.01$; 16% vs. 0%), and *Psychodidae* 1 ($P < 0.01$; 16% vs. 0%).

Intraspecific variation in the diet of *N. l. verrucosus*

For similar sampling coverage levels, male *N. l. verrucosus* presented a significantly wider dietary niche breadth than females at both family and OTU levels (nonoverlapping confidence intervals; Fig. 4). Yet, although the mean number of prey consumed by
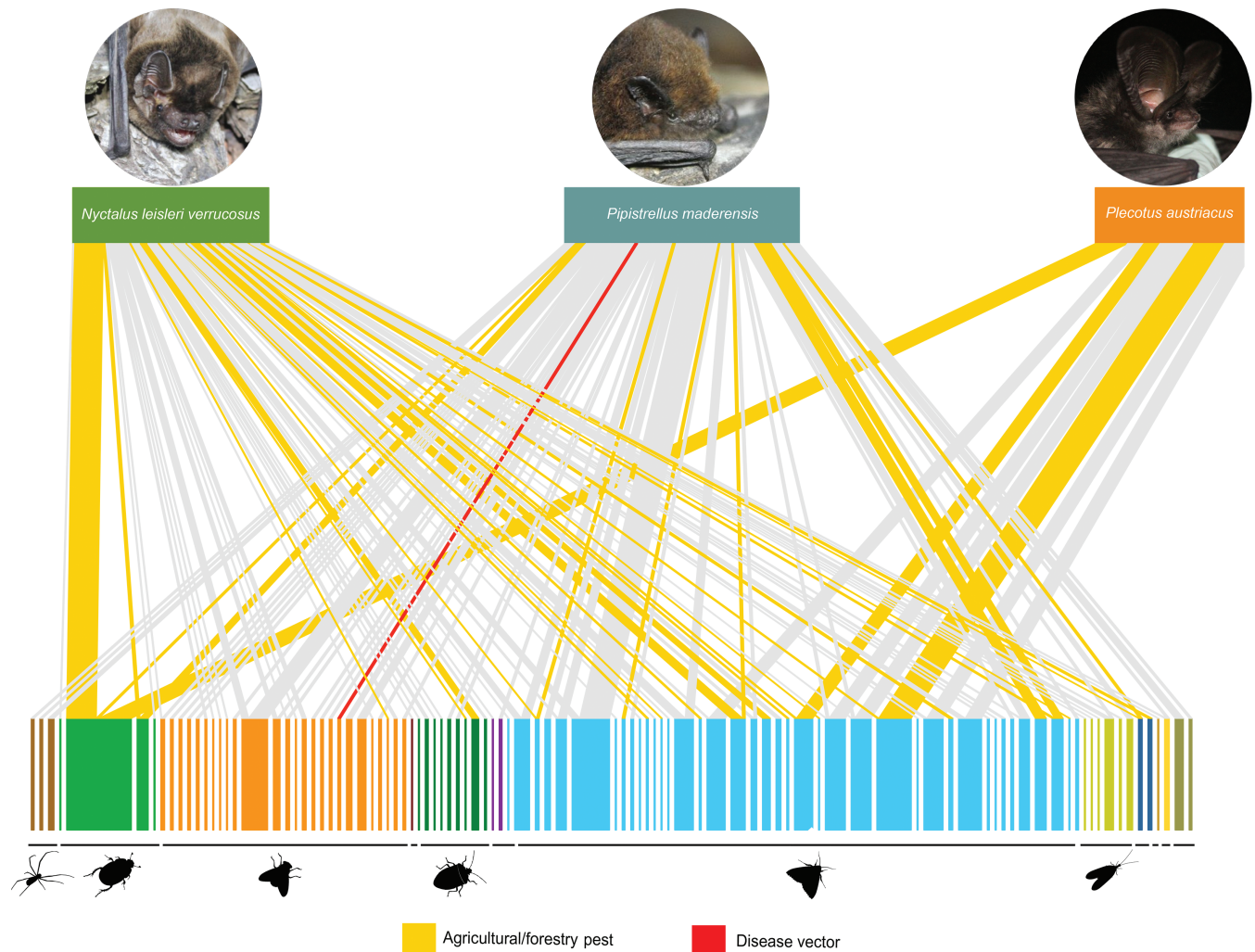
Fig. 2. Foodweb displaying the Operational Taxonomic Units (OTUs) consumed by *Nyctalus leisleri verrucosus*, *Pipistrellus maderensis*, and *Plecotus austriacus* on Madeira Island. Link width between bats and their prey is proportional to its frequency of occurrence in fecal samples. Different OTUs are separated by white lines and different colors denote different taxonomic orders.

males was higher than that consumed by females (Fig. 4) these differences were not significant for any of the analyzed taxonomic levels considered (order, $P = 0.902$; family, $P = 0.225$; OTU, $P = 0.207$). Lastly, the diet composition of male and female *N. l. verrucosus* (Supplementary Data SD4) was not significantly different at either the order, family, or OTU levels ($P = 0.622$, 0.314, and 0.586, respectively).

Discussion

This study provides the first detailed analysis of the diet of Macaronesian bats and represents one of the first studies to use DNA metabarcoding to assess the diet of bat species inhabiting an oceanic island. Overall, we detected 110 prey items in the diet of Madeira Island bats, including multiple agricultural and forestry pest species, a species of human disease relevance, and numerous taxa nonpreviously recorded on Madeira.

### Prey consumed by the insectivorous bats found on Madeira Island

Our findings indicate that diet composition of the *N. l. verrucosus* is—to a certain extent—in line with other non-molecular dietary

studies of *N. leisleri* populations from mainland Europe (Vergari and Dondini 1999; Kaňuch et al. 2005). Other than Neuroptera, all other orders identified as prey of *N. leisleri* by Vergari and Dondini (1999) were also detected in this study. However, whereas some previous studies identified Diptera as its primary prey (Vaughan 1997; Waters et al. 1999), our results indicate that Lepidoptera is the dominant order consumed by *N. l. verrucosus*. Four of the 6 orders identified as prey of *N. leisleri* by Kaňuch et al. (2005; Neuroptera, Coleoptera, Lepidoptera, and Diptera) were also detected in the diet of *N. l. verrucosus*. Moreover, *N. l. verrucosus* was found to share several prey species (e.g., *Aiolopus thalassinus*) with a nocturnal gecko (*Tarentola mauritanica*) recently introduced to Madeira Island (Martins et al. 2022). Moreover, our results show that *N. l. verrucosus* prey mostly on flying arthropods (Noctuidae, Blastobasidae, and Cerambycidae), aligning with expectations for an aerial-hawking foraging bat, and matching previous research findings (Boston et al. 2020).

All arthropod families previously suggested to be consumed by *P. maderensis* (Diptera, Trichoptera, Lepidoptera, and Coleoptera; Jesus et al. 2009) were confirmed as prey in this study. Additionally, our findings align with diet studies for *P. kuhlii* (a close relative to *P. maderensis*) as its diet was found to be dominated by the orders

Table 1. List of known and suspected agriculture/forestry pest species and species of human health concern found on the diet of the *Nyctalus leisleri verrucosus*, *Pipistrellus maderensis*, and *Plecotus austriacus*. The percentage of samples for which each prey was detected in each bat species is also indicated. Sample size of each bat species is indicated within parenthesis. References in Supplementary Data SD5.

Order	Family	Species	Common name	BOLD ID percentage	Status	Crop/disease	N. leisleri verrucosus (51)	P. maderensis (18)	P. austriacus (3)
Coleoptera	Cerambycidae	*Arhopalus ferus*	Burnt pine longhorn beetle	100%	Known pest	Export timber[3], pine[4]	64.6%	5.6%	66.7%
		Arhopalus syriacus		99.5%	Possible pest	Unknown	6.3%	16.7%	
Diptera	Psychodidae	*Psychoda albipennis*		100%	Human parasite	Urogenital myiasis[25]		5.6%	
	Tipulidae	*Tipula oleracea*	Common crane fly	99.5%	Known pest	Turfgrass[27]	2.1%		
Hemiptera	Miridae	*Taylorilygus apicalis*	Broken-backed bug	100%	Possible pest	Unknown	2.1%		
	Pentatomidae	*Nezara viridula*	Green stink bug	100%	Known pest	Soybean[18], cotton[19]	14.6%		
Lepidoptera	Blastobasidae	*Blastobasis decolorella*		100%	Known pest	Apple[5]	2.1%	5.6%	
	Crambidae	*Botyodes diniasalis*		100%	Known pest	Poplar plants[6]		5.6%	
		Herpetogramma licarsisalis	Grass caterpillar/webworm	100%	Known pest	Turf[13], rice[14]	4.2%		
		Palpita vitrealis	Jasmine moth	100%	Known pest	Olive[23]	2.1%		
	Gelechiidae	*Aproaerema anthyllidella*		99.5%	Possible pest	Unknown	4.2%		
	Geometridae	*Gymnoscelis rufifasciata*	Double-striped pug	100%	Possible pest	Unknown	25.0%	5.6%	
	Noctuidae	*Agrotis segetum*	Turnip moth	100%	Known pest	Vegetables, cereals[1]	16.7%		
		Caradrina clavipalpis	Pale mottled willow	100%	Possible pest	Unknown	4.2%		
		Chrysodeixis chalcites	Golden twin-spot moth	100%	Known pest	Banana, tobacco[7], soybean[8], tomato, greenhouse crops[9] (among others)[10]	4.2%		33.3%
		Cornutiplusia circumflexa		100%	Possible pest	Unknown	2.1%		
		Mythimna unipuncta	True armyworm	100%	Known pest	Small grains[15], corn[16], grass[17]	4.2%		66.7%
		Peridroma saucia	Pearly underwing moth	100%	Known pest	Peanuts, sunflower, soybean, grapevine[24] (among others)	8.3%		
	Tineidae	*Opogona sacchari*	Banana moth	100%	Known pest	Ornamental plants[20], banana, sugarcane[21], pineapple[22]		22.2%	
	Tortricidae	*Epiphyas postvittana*	Light brown apple moth	100%	Known pest	Horticultural plants[11], apple[12]	6.3%	16.7%	
		Selania leplastriana	Leplastrier's piercer	100%	Known pest	Cabbage, cauliflower[26]	2.1%		
Orthoptera	Acrididae	*Aiolopus thalassinus*		100%	Known pest	Grasses, cereals, alfalfa, vegetables[2]	2.1%	5.6%	
	Gryllidae	*Gryllus bimaculatus*		100%	Possible pest	Unknown	8.3%		
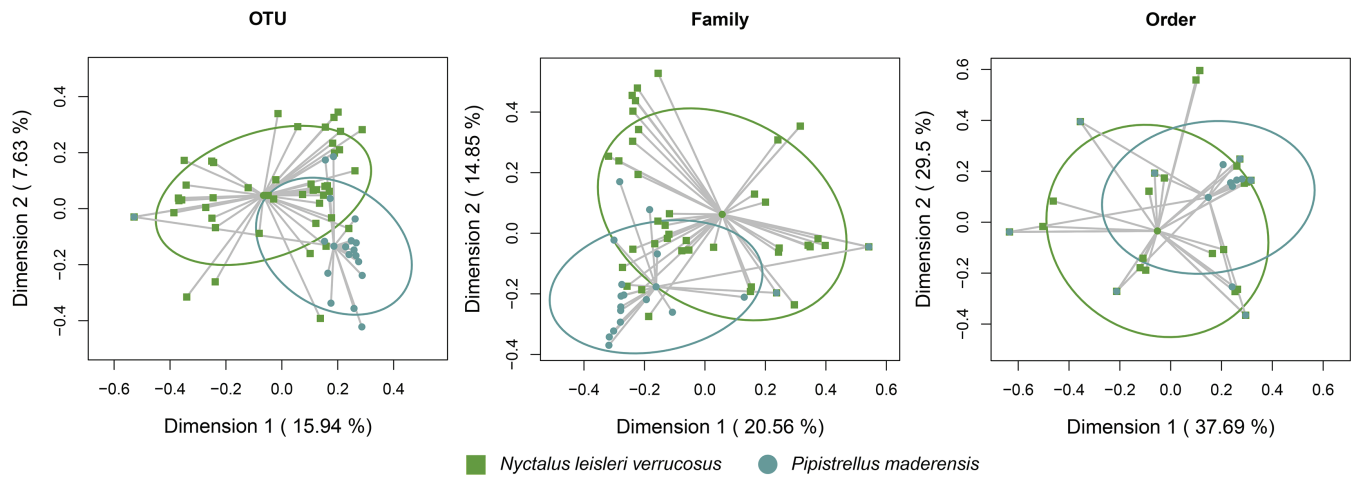
Fig. 3. MDS plot of the prey composition of *Nyctalus leisleri verrucosus* (green) and *Pipistrellus maderensis* (blue) at different taxonomic levels. The variance explained by each axis is indicated within parenthesis.
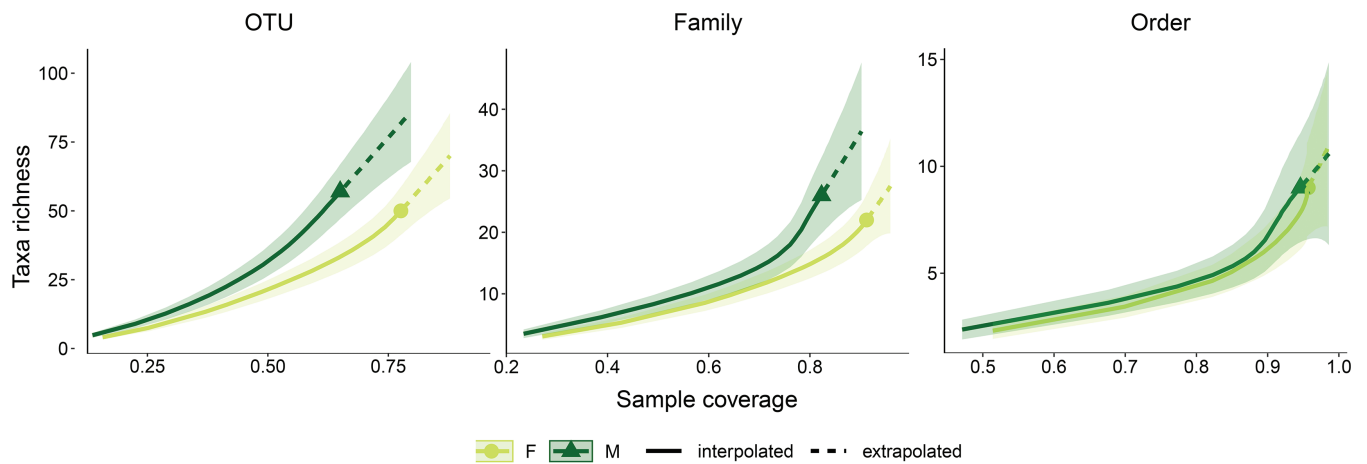
Fig. 4. Rarefaction curves of prey taxa richness at different taxonomic levels for both female (F) and male (M) *Nyctalus leisleri verrucosus*. Shaded area represents 95% confidence intervals.

Lepidoptera, Hymenoptera, Coleoptera, and Diptera; and the families Tipulidae, Chironomidae, and Aranea that constitute a significant portion of the diet of *P. maderensis* (Goiti et al. 2003; Safi and Kerth 2004).

Lepidoptera (and in particular Noctuidae) as the main prey of *P. austriacus* is well in line with previous diet studies for this wide-ranging species (Bartonička et al. 2008; Ashrafi et al. 2011; Razgour et al. 2011). Additionally, some of the species identified to be consumed by *P. austriacus* in this study (e.g., *M. unipuncta* and *C. chalcites*—percent frequency of interaction 20% and 10% of the diet, respectively) had already been identified in previous dietary analysis of this species (Borg and Sammut 2002). However, a limited number of samples collected for *P. austriacus* precluded a robust assessment of the diet of this species on Madeira Island.

Our findings also showed that bats on Madeira Island feed on a wide variety of economically important agricultural and forestry pests (Table 1). For example, *N. l. verrucosus* was found to consume turnip moths (*A. segetum*), one of the most damaging insects to vegetables and cereals in the world (Esbjerg and Sigsgaard 2014); and *P. maderensis* preys on the banana moth (*O. sacchari*), which in Madeira affects banana plantations and can impact on multiple

ornamental species of high economic relevance such as Bird of Paradise flowers (*Strelitzia reginae*; Gaag et al. 2013). Furthermore, *P. maderensis* was found to consume *Psychoda albipennis*, which can cause urogenital myiasis in humans, resulting in abdominal pain, diarrhea, and dysuria (Hazratian et al. 2020). Likewise, *P. austriacus* (and *N. l. verrucosus*) was found to feed on *C. chalcites*, an important pest of banana crops in the Canary Islands (Fuentes et al. 2018) and 1 of the 4 most important pests of European greenhouse crops (Simón et al. 2015). In total, 23 of the 59 species found in the diet of the 3 bat species (38.9%) are known or potential agricultural/forestry pest species or species known to transmit diseases to humans and 10 species found in bat diets had not previously been described to Madeira Island (Supplementary Data SD1). These findings highlight the potential role of insectivorous bats as suppressors of arthropods with negative economic and human health impacts and their potential as an early warning signal to presence of non-native taxa (Kemp et al. 2019; Ancillotto et al. 2022, 2023; Ramírez-Fráncel et al. 2022). Although our findings of arthropod taxa not yet recorded on Madeira Island are not surprising, we emphasize that targeted efforts to search for these species may be advisable before considering them as present on the island.

Interspecific differences in the diet of *N. l. verrucosus* and *P. maderensis*

Sympatric species are likely to partition dietary resources available to reduce niche overlap and avoid competition. In insectivorous bats, differences in wing morphology, echolocation call structure, temporal activity patterns, and habitat use may lead to resource partitioning (Mancina and Rivera 2012; de Oliveira et al. 2020; Rocha et al. 2020). Although our results indicate that the niche breadth and the diet diversity of *N. l. verrucosus* and *P. maderensis* is rather similar, their diet was considerably different at the order, family, and OTU levels (e.g., *N. l. verrucosus* consumed fewer Diptera prey than *P. maderensis* and, contrary to *P. maderensis*, Aranae or Trichoptera were not consumed). Both species differ in their habitat use—*N. l. verrucosus* is more associated with production forests, while *P. maderensis* is more active in agricultural areas, laurel forest, and shrubland habitats (Ferreira et al. 2022)—suggesting that habitat segregation may be a factor contributing to their different dietary composition.

Differences in the diet of female and male *N. l. verrucosus*

Although the diet composition and diversity of female and male *N. l. verrucosus* did not differ greatly, male bats had a wider niche breadth than females at both the family and OTU level. Female-male dietary differences have been studied in multiple bat species with contrasting results (e.g., Arrizabalaga-Escudero et al. 2019; Ancillotto et al. 2023). Our findings somewhat align with small diet composition differences found between male and female Savi's Pipistrelle (*Hypsugo savii*; Ancillotto et al. 2023) but differ from the results obtained by Mata et al. (2016) in their investigation of the dietary sexual segregation of *Tadarida teniotis*. In the latter, females of *T. teniotis* were found to consume larger moths than males, probably due of their higher energetic needs during pregnancy and lactation. Additionally, differences in the diet of female and male bats might reflect differences in habitat use (e.g., the energetic demands of pregnancy and lactation can limit females to foraging within highest quality habitats; Lintott et al. 2014); or alternatively it could drive female bats to select particularly favorable foraging areas (Rocha et al. 2017). None of the captured female *N. l. verrucosus* were either pregnant or lactating, suggesting that potential differences in the diet of males and females might be more conspicuous during more reproductively active phases of the phenological cycle.

Supplementary data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—List of all 110 OTUs found in the diet of the 3 bat species, alongside information regarding previous knowledge about their presence on Madeira Island as well as information on the history of colonization of the island (according to Borges et al. 2008). The percentage of samples for which each prey was detected in each bat species is also indicated. Sample size of each bat species is indicated within parentheses. MAC represents Macaronesia endemic species and "NA" refers to species whose colonization status is not reported in Borges et al. (2008). The status of species *Zygiella x-notata* was disclosed by Pedro Cardoso and the status of *Agonopterix scopariella*, *Epiphyas postvittana*, *Botyodes diniasalis*, and *Opogona omoscopa* by Ysabel Gonçalves, both through personal communication. Potential first records for the island are indicated as "NEW."

Supplementary Data SD2.—Rarefaction curves of prey taxa richness at different taxonomic levels for both *Nyctalus leisleri verrucosus* and *Pipistrellus maderensis*. Shaded area represents 95% confidence intervals.

Supplementary Data SD3.—Modeled effect of bat species on average prey richness per sample at different taxonomic levels.

Supplementary Data SD4.—Multidimensional scaling (MDS) plot of prey composition of females (light green) and males (dark green) of *Nyctalus leisleri verrucosus* at different taxonomic levels. The variance explained by each axis is indicated within parentheses.

Supplementary Data SD5.—References supporting the classification of several prey items as potential agricultural pest species or disease vectors.

Author contributions

RR, AG, and VAM conceived and designed the methodology. RR, AG, and EKN conducted fieldwork. AG and VAM performed the laboratory analyses. AG and VAM analyzed the data and AG and RR led the writing. All authors contributed critically to the drafts and gave final approval for publication.

Conflict of interest

None declared.

Data availability

Raw sequencing data and sample information data can be found on https://www.ebi.ac.uk/biostudies/studies/S-BSST1337

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