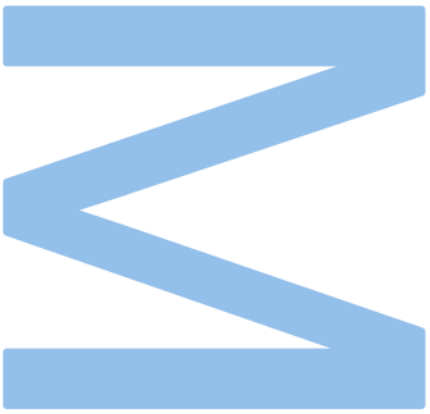


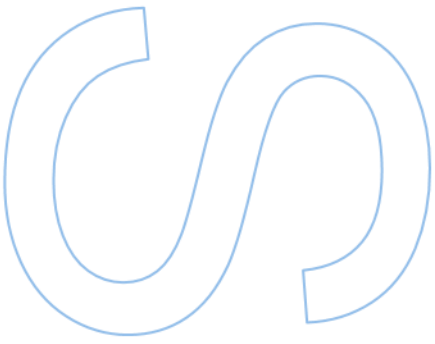
A metabarcoding assessment of the diet of insectivorous bats in a subtropical oceanic island

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Sworn Statement

I, Angelina de Lurdes de Amorim Gonçalves, enrolled in the Master Degree Ecologia e ambiente at the Faculty of Sciences of the University of Porto hereby declare, in accordance with the provisions of paragraph a) of Article 14 of the Code of Ethical Conduct of the University of Porto, that the content of this dissertation reflects perspectives, research work and my own interpretations at the time of its submission.

By submitting this dissertation, I also declare that it contains the results of my own research work and contributions that have not been previously submitted to this or any other institution.

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Angelina Gonçalves

29/09/2022

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Resumo

Compreender a estrutura trófica dos conjuntos de espécies é importante para compreender como as espécies sintrópicas são capazes de co-ocorrer no espaço e no tempo. Os morcegos são o segundo grupo de mamíferos mais diversificados do ponto de vista taxonómico e apresentam uma vasta gama de estratégias alimentares. No entanto, embora os morcegos insulares habitem alguns dos ecossistemas mais vulneráveis do mundo, são relativamente pouco estudados. A sequenciação em massa de códigos de barra do ADN (daqui em diante 'DNA metabarcoding') pode ser utilizada para estudos de ecologia trófica, uma vez que permite uma abordagem fiável e sistemática à análise da dieta, aumenta a probabilidade de identificar a maioria das espécies de presas e não é afectada por enviesamento de dureza dos itens consumidos. Aqui, utilizei o DNA metabarcoding, para investigar a ecologia trófica das três espécies de morcegos encontradas na Ilha da Madeira, nomeadamente *Pipistrellus maderensis*, *Nyctalus leisleri verrucosus*, e *Plecotus austriacus*. No total, identifiquei 110 presas de artrópodes de presa na dieta das três espécies de morcegos encontrados na Madeira, incluindo múltiplas espécies de pragas agrícolas e florestais, uma espécie de relevância para a saúde humana, e numerosos taxa que não tinham sido previamente registados na ilha. Lepidoptera foi a ordem dominante de presas para as três espécies de morcegos. A composição da dieta de *N. l. verrucosus* e *P. maderensis* diferiu significativamente, com *P. maderensis* a consumir mais Diptera, bem como múltiplos taxa de presas não encontrados na dieta de *N. l. verrucosus*. Adicionalmente, encontrei diferenças relacionadas com o sexo na dieta de *N. l. verrucosus*, com os machos a exibirem uma maior amplitude de nicho trófico do que as fêmeas. Este estudo está entre os primeiros a utilizar DNA metabarcoding para avaliar a dieta dos morcegos insulares e oferece a primeira análise aprofundada da dieta dos morcegos Macaronésicos.

Palavras-chave: [Dieta, Ecologia trófica, Ilha da Madeira, DNA metabarcoding, Morcegos da Madeira, Peste]

Abstract

Understanding the trophic structure of species' assemblages is important to understand how syntropic species are capable to co-occur in space and time. Bats are the second most taxonomically diverse group of mammals and display a wide range of dietary strategies. However, although insular bats inhabit some of the world's most vulnerable habitats, they are relatively poorly studied. DNA metabarcoding can be used for trophic ecology studies since it allows for a reliable and systematic approach to diet studies, increases the probability of identifying the majority of the prey species consumed and hardness bias is non-existent. Here, I used DNA metabarcoding to investigate the trophic ecology of the three bats species found in Madeira Island, namely *Pipistrellus maderensis*, *Nyctalus leisleri verrucosus* and *Plecotus austriacus*. In total, I identified 110 arthropod prey in the diet of the three bat species found in Madeira, including multiple agriculture and forestry pest species, a species of human disease relevance, and numerous taxa that had not previously been recorded to the island. Lepidoptera was the dominant prey order for all three bat species. The diet composition of *N. l. verrucosus* and *P. maderensis* differed significantly, with *P. maderensis* consuming more Diptera and multiple prey taxa not found in the diet of *N. l. verucosus*. Additionally, I found sex-related differences in the diet of *N. l. verrucosus*, with males exhibiting a wider niche breadth than females. This study presents the first in-depth analysis of the diet of Macaronesian bats and is amongst the first to use DNA metabarcoding to evaluate the diet of insular bats.

Keywords: [Diet, Madeira Island, Madeiran bats, DNA metabarcoding, Pest, Trophic ecology]

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Analysis of variance: (ANOVA), 15
Barcode of Life Data Systems: (BOLD), 14
Cytochrome oxidase subunit 1: (COI), 13
Deoxyribonucleic acid: (DNA), 8
International Union for Conservation of Nature: (IUCN), 2
Multidimensional scaling: (MDS), 22
Operational Taxonomic Units: (OTU), 14
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Polymerase Chain Reaction: (PCR), 13

1. Introduction

1.1. Predator-prey relationships

Predator-prey interactions are key components of ecosystem dynamics and understanding the trophic structure of biological communities is paramount for species and ecosystem management (Lima, 1998; Jia et al., 2021). From the early days of ecological research ecologists have tried to understand the dynamic nature of trophic interactions. However, despite important theoretical and technological advances (e.g., Pringle et al., 2019), the complex nature of predator-prey interactions continues to hamper a full understanding of the ecological processes underpinning the relationship between predators and their prey.

The age and size of respective predator and prey, hunger levels, population sizes, presence of alternative prey, and behavioural factors are ever changing (Schmidt, 2009). Understanding trophic interactions can be achieved by breaking the predation process down into its component stages. The eight steps suggested by Wootton et al. (2021) (i.e., (1) search, (2) prey detection, (3) attack decision, (4) pursuit, (5) subjugation, (6) ingestion, (7) digestion and (8) nutrient allocation of the pre-consumption and post-consumption phases of a foraging cycle) form a general dynamic model to understand trophic interactions, and how they are moulded by species' traits and environmental factors. Furthermore, predators may have direct effects on prey individuals (e.g., causing loss of fitness, injury, or death), as well as indirect effects on population structure (e.g., selecting particular phenotypes) and on assemblage organization (e.g., reducing the number of potential competitors) (Jaksic & Simonetti, 1987). As a result, predator-prey adaptations often lead to "evolutionary arms races", in which specific traits (e.g., physiology and behaviour) of co-evolving species diversify (Dawkins & Krebs, 1979).

Trophic interactions impact the relationship between biodiversity and the stability of ecosystem processes (Thébault & Loreau, 2005). Research analysing the competition among plants and plant-herbivore interactions indicates that diversity affects multiple ecological facets, including the strength of species interactions and the temporal variability of total biomass of the various trophic levels (Thébault & Loreau, 2005). Trophic interactions are affected by a wide array of factors, including the traits of both predators and prey as well as environmental features. For instance, body sizes condition trophic interactions (Digel et al., 2011; Laigle et al., 2018) and nutritional quality plays an important role in prey selection (Razeng & Watson, 2015). However, notwithstanding an increasing body of literature devoted to predator-prey relationships (e.g., Kasso &

Balakrishnan, 2013; Lima & O'Keefe, 2013; Mikula, 2015; Haarsma & Kaal, 2016; Arndt et al., 2018; Oedin et al., 2021), we still lack a good understanding of trophic ecology, even for more simplified ecosystems, such as oceanic islands.

1.2. Island biodiversity

Although amounting to a small percentage of Earth's terrestrial land area, islands hold a significative number of unique taxa and ecological communities (Jones et al., 2009a). Islands usually have a high degree of endemic taxa due to their geographic isolation and associated restricted interaction with mainland biota (Kier et al., 2009). Therefore, islands are home to a significant proportion of the global biodiversity, hosting high levels of species endemism, unique functional traits and unique evolutionary lineages (Russell & Kueffer, 2019). However, islands have lower levels of species richness in comparison to mainland sites with similar climate (Whittaker & Fernández-Palacios, 2007), and island characteristics (with area and isolation acting as main drivers) significantly contribute to insular species richness (Kreft et al., 2008) and relate to endemism and mammalian biodiversity (Barreto et al., 2021). Being often endemic, islands populations have relatively higher extinction rates than non-island populations and 61% of all species listed by the International Union for Conservation of Nature (IUCN) as extinct and 37% of species listed by the IUCN as critically endangered are confined to islands (Frankham, 1997; Tershy et al., 2015). Hence, islands represent some of the most naturally vulnerable habitats on the planet (Jones et al., 2009a; Nori et al., 2022). Indeed, the number of extinct and critically endangered species is greater on islands than on continents (Tershy et al., 2015) and biological invasions, wildlife exploitation, and habitat loss have been responsible for the majority of insular extinctions (85%) and remain the main threats for insular biodiversity, impacting more than 60% of insular threatened species (Leclerc et al., 2018).

Generally, islands have fewer species than mainland areas of similar size, latitude and topographical complexity and, consequently, individual species tend to have fewer competitors (Lister, 1976). Given that competitors constrain the resource use and abundance of a species, insular species may have higher densities and wider niches than their mainland counterparts. Resource availability and lack of competitors can lead to 'ecological release' - i.e., population-level niche expansions (an increase in niche width, as measured by an increase in the variance of habitats or resources used; Herrmann et al., 2020). Furthermore, when a constraining interspecific interaction is reduced or removed it can induce habitat shifts with organisms expanding their resource use to include resources they do not normally use (Lister, 1976; Herrmann et al., 2020).

Over evolutionary time, this expansion may allow for the exploration of a wider range of resources (Lister, 1976). Insular species often have to adapt their diet to local conditions, and this sometimes leads to unconventional trophic relationships. An interesting example are insular reptiles, which often differ in their diets when compared to continental congeners e.g., insular lizards often display nectarivory (Olesen & Valido, 2003; Hernández-Teixidor et al., 2020), frugivory and ovophagy (Brock et al., 2014).

1.3. Bat trophic relationships

Bats are the second most taxonomically diverse group of mammals, representing one fifth of global mammalian diversity (>1,430 species; Simmons et al., 2021). They display a wide range of dietary strategies including insectivory, carnivory, nectarivory and hematophagy (Altringham, 2011). Additionally, bats are also highly diverse regarding foraging behaviour, roosting ecology and habitat use (Patterson et al., 2003). Globally, they occur in most non-polar habitats and play important roles in arthropod suppression, nutrient recycling, supporting primary production through seed dispersal and pollination, and have a great potential as bioindicators (Jones et al., 2009b; Kunz et al., 2011; Ramírez-fráncel et al., 2022). Around 15% of the global bat diversity is classified as threatened by the IUCN Red List and the conservation status of many bat species is unknown (ca. 18% classified as Data Deficient according to the IUCN Red List) (Frick et al., 2020). This is due to the wide range of threats bats are confronted with, the most conspicuous and widespread being habitat loss and fragmentation (Frick et al., 2020). For instance, deforestation is a key threat as forests provide essential foraging habitats and allow many bat species to roost in plant structures (Rocha et al., 2017a). Agriculture through direct habitat loss and modification also impacts many bat species, as agricultural habitats have limited foraging and roosting resources for many species (Williams-Guillén et al., 2016). In addition, due to insecticides, insectivorous species have fewer resources to feed on and can be directly poisoned (Williams-Guillén et al., 2016). Nonetheless, bat hunting for food, medicine, bushmeat, and cultural beliefs is also a major concern for bat populations, as well as climate change and invasive species (Welch & Leppanen, 2017; Frick et al., 2020; Festa et al., 2022).

Bats are the only mammals capable of powered flight, and although flight is an effective way to cover distances, it is nevertheless associated with an extremely high energy cost per unit of time, making air travel one of the most expensive modes of transport (Alexander, 2002). Therefore, due to flight, bats have a high metabolic rate and consequently have to forage extensively (Thomas, 2004). Insectivorous bats can consume almost their weight in arthropods every night, playing an important role in

controlling arthropod populations (Kurtal et al., 1989; Kunz et al., 1995; Kunz et al., 2011; Kolkert et al., 2019).

Dietary studies are essential to understand the division of resources and niches (Patterson et al., 2003; Emrich et al., 2014). A central principle in ecology, considering that limited resources may prevent the stable coexistence of two species that depend on their similar use, is known as the principle of competitive exclusion (Hardin, 1960). The capacity of bats to move quickly and efficiently over great distances in search of food, roosts and mates has allowed for extensive division of resources between bat species (Patterson et al., 2003). There are several potential non-strictly independent mechanisms for resource partitioning. According to Andriollo et al. (2021), two main measures of the trophic niche are traditionally used to characterize the feeding ecology of interacting species: the trophic niche breadth distinguishing between generalist and specialized feeding strategies and the dietary niche overlap, which measures the extent of food resources shared between species. Understanding the trophic structure of an assemblage (i.e., a taxonomically related group of species populations that occur together in space; Stroud et al., 2015) is important to understand how syntopic species (i.e., a special case of sympatry in which two or more related species occupy the same macrohabitat (Rivas, 1964) are capable to co-occur in space and time). For instance, Mancina and Rivera (2012) demonstrated that although four syntopic bat species were well suited for foraging in cluttered environments, differences in their wing morphologies and echolocation calls suggest that these species should have different foraging strategies and use different microhabitat while occurring sympatrically. Patterson et al. (2003) suggests that the abundance of a species and its morphological separation from competitors may be positively associated. This phenomenon - known as density compensation (i.e., an increase in abundance per unit area in response to the absence of a competitor, such that the increased abundance compensates for the number or biomass of missing competitors; Herrmann et al., 2020) – may cause niche expansion, although it does not have to (Herrmann et al., 2020).

1.4. Female-male differences in bat diet

Evident sexual dimorphism in bats is unusual although it is a common feature in mammals and often seen in sexually segregated species (Altringham et al., 2006). Yet, sex-specific differences in diet composition (Mata et al., 2016; Sánchez-Losada & Mancina, 2020), habitat selection (Hillen et al., 2011; Rocha et al., 2017b), foraging and roosting areas (Encarnacão et al., 2005; Downs et al., 2016), as well as acoustics landmarks (Schmidtke & Esser, 2011) have been described for many species. Levin et

al. (2013) suggested that it is the different physiological and social needs between males and females, and not competition, that drives sexual segregation in greater mouse-tailed bats (*Rhinopoma microphyllum*). According to these authors, sexual segregation may be a behavioural response to reduce male and female competition for food resources, mates, and roosting sites or may be associated with biological differences between the sexes (e.g., thermoregulatory requirements, sociality, and body size). Also, the dietary analysis of Cuban flower bats (*Phyllonycteris poeyi*) in Cuba revealed that although both sexes fed on similar food categories, a significant variation in diet composition between males and females was detected. The study found that throughout the year, females predominantly consumed fruits while males had a preference for floral resources (Sánchez-Losada & Mancina, 2020).

1.5. Insular bats

Islands are often characterized by reduced species richness and taxonomically and ecologically skewed faunas and floras (Fleming & Racey, 2009). Due to their flying ability, bats tend to be good colonizers of oceanic islands, where they usually represent most (or all) of the native, non-marine mammals (Fleming & Racey, 2009). Of the over 1,400 bat species, about 60% occur on islands, and about 25% of all species are island endemics (Jones et al., 2009a; Conenna et al., 2017). Islands have thus played an important role in the overall evolution of bats. However, although insular bats inhabit some of the world's most vulnerable habitats, they are relatively poorly studied (Conenna et al., 2017). As per above, area and isolation influence island species richness, endemism and mammalian diversity. Bats, due to their flight capacity, are more strongly associated with the current isolation than past one, and are also less affected by island area, at least for endemism (Barreto et al., 2021). Island may imply fewer competitors but also a more restricted range of resources (Li & Kokko, 2021). All bat species whose extinction was directly or indirectly associated with anthropogenic factors - 8 species possibly extinct and 4 assessed by the IUCN as Extinct - occurred exclusively on islands (Conenna et al., 2017). Threats caused by human action, by intrinsic (e.g., low reproductive rate) and extrinsic factors (e.g., natural disasters) are significantly more problematic for island species than for species with a distribution not restricted to islands (Jones et al., 2009a). The extinction and population decline of island bats not only contributes to the decline in global biodiversity but also to the loss of important ecological services on the islands where they occur (Fleming & Racey, 2009).

1.6. Madeira Island – general characterization and bat diversity

The archipelago of Madeira is an autonomous region of Portugal located in the North Atlantic Ocean, southwest of the Iberian Peninsula (Figure 1). Of volcanic origin, the archipelago consists of Madeira, Porto Santo and Desertas with only Madeira (741 km²) and Porto Santo (42 km²) being currently inhabited. Madeira Island is ca. 700 km from Africa and 980 km from the Iberian coast. Its topography is characterized by mountains and deep valleys in the interior and rocky cliffs near the coast. Its highest point is Pico Ruivo (1862 m above sea level). Due to the relief and exposure to the prevailing north-easterly trade winds, Madeira Island has several microclimates which allow for a significant variety of ecosystems (Delgado et al., 2006). Indeed, the Laurissilva of Madeira, within Madeira Natural Park was classified by United Nations Educational, Scientific and Cultural Organization as a World Natural Heritage site.

As a consequence of the archipelago's richness of microhabitats, its biodiversity is characterized by a high number of endemisms (Delgado et al., 2006). However, because Madeira was an important centre for commerce, non-native species of fauna and flora including predators, competitors and parasites were brought to the island (Wetterer et al., 2007). Over 7500 terrestrial taxa were estimated to inhabit the archipelagos of Madeira and Selvagens (both part of the Autonomous Region of Madeira) and 19% of the total diversity are endemic species (Borges et al., 2008). Arthropods are the most diverse Phylum both archipelagos, representing ca. 51% of the taxa, most of which belonging to the Orders Coleoptera, Lepidoptera, Diptera and Hymenoptera (Borges et al., 2008).

The diet of insectivorous bats is largely dominated by Lepidoptera (Vaughan, 1997; McWilliams, 2005; Mata et al., 2016; Baroja et al., 2021; Ingala et al., 2021). Because of its isolation and relative small size, the lepidopteran fauna of Madeira is relatively poor - ca. 300 species (Karsholt, 2000; García et al., 2015). These can be divided into i) species endemic to Madeiran - ca. 15% of the species; ii) species endemic to Macaronesia; iii) native species with a wider distribution; iv) non-native, naturalized species; v) immigrants crossing the ocean (some of these are able to establish populations in Madeira, at least for some time); and, vi) accidentally introduced species, which are not able to establish populations in Madeira. Most of the endemic Lepidoptera in Madeira have their strongholds in the mountains covered by Laurissilva in the northern section of the island (Karsholt, 2000). The mammalian fauna of the archipelago is poor regarding specific richness and, with the exception of bats, all terrestrial mammals have been introduced

(Jesus et al., 2009). According to historical and recent records, *Plecotus austriacus* (Fischer, 1829), *Tadarida teniotis* (Rafinesque, 1814), *Hypsugo savii* (Bonaparte, 1837), *Pipistrellus maderensis* (Dobson, 1878) and *Nyctalus leisleri verrucosus* Bowditch, 1825 were recorded in Madeira (Teixeira & Jesus, 2009). However, systematic surveys confirmed the current presence of only three species: *Plecotus austriacus*, *Pipistrellus maderensis* and *Nyctalus leisleri verrucosus* (Jesus et al., 2009; Ferreira et al., 2022). However, there is a lack of information about their trophic relationships.

The Madeira pipistrelle (*Pipistrellus maderensis*) (Dobson, 1878) is endemic to Macaronesia and is classified as Vulnerable by the IUCN Red List (Rocha, 2021). In the Madeira archipelago, the species is restricted to Madeira and Porto Santo. Some authors have, in the past, considered *Pipistrellus maderensis* as an intraspecific form of Kuhl's pipistrelle *Pipistrellus kuhlii* (Corbet, 1978). The species taxonomic status is still unclear and, at the genetic level, Pestano et al. (2003) found that pipistrelle-like bats from the Canary Islands, morphologically identified as Madeiran pipistrelles, seemed to represent a lineage of Kuhl's pipistrelle indicating that perhaps *P. maderensis* has evolved from African *Pipistrellus kuhlii* that colonized the Canary Islands and the archipelago of Madeira. *Pipistrellus maderensis* is very flexible regarding its habitat requirements and they hunt in most of the island's habitats, being relatively abundant in forest, agricultural and urban biotopes (Queiroz et al., 2005; Ferreira et al., 2022). Despite the lack of diet studies, it has been suggested that they feed mainly on mosquitoes (Diptera), caddisflies (Trichoptera), butterflies/moths (Lepidoptera) and small beetles (Coleoptera) (Jesus et al., 2009).

Leisler's bat (*Nyctalus leisleri*) (Kuhl, 1817) is a typical European forest bat. As an aerial hawking species, its foraging habitats are associated with deciduous and coniferous woodlands, as well as open areas, over rivers, lakes or pastures, and around streetlights (Boston et al., 2020). The diet, which can vary between sites and seasons (Waters et al., 1999; Kaňuch et al., 2005) frequently consists of small to medium-sized insects (e.g., Diptera, Trichoptera, Coleoptera, and Lepidoptera). The species is reliant on aquatic insects or insects with aquatic larvae (Vaughan, 1997; Shiel et al., 1998), thus often feeding near freshwater habitats. Shiel et al. (1998) investigated the diet of *N. leisleri* by analysing faecal pellets collected from pastoral sites in Ireland, England and Germany (in forest), and found the species to feed mostly on medium-sized and small insects caught in flight. In Madeira the species is represented by the endemic subspecies *N. leisleri verrucosus* (Palmeirim, 1991).

Plecotus austriacus (Fischer, 1829) is a European long-eared bat. Traditionally, all long-eared bats were assigned as subspecies of *Plecotus auritus*. However, molecular studies based on mitochondrial DNA (mtDNA) identified two major clades in the genus in Europe: the 'auritus' and 'austriacus' clades (Juste et al., 2004; Razgour et al., 2021). Phylogenetic reconstructions suggest that *P. austriacus* arrived to Madeira during its relatively recent westward expansion across Europe (Juste et al., 2004). *Plecotus austriacus* forages mostly on open-edge habitats and is a sedentary species, as most individuals remain close to their natal grounds (Razgour et al., 2021). Although it can vary according to the season, the diet of *P. austriacus* consists primarily on moths (Lepidoptera) (Ashrafi et al., 2011; Razgour et al., 2021) but other insect taxa, such as Diptera (the second taxon more consumed prey group), Neuroptera, Hemiptera, Trichoptera, and Isopoda are also part of its diet in England (Razgour et al., 2011). In addition, 45% of the prey species were habitat generalists, 40% were open grassland specialists, 12% woodland specialists and 3% riparian or water habitat specialists (Razgour et al., 2021). However, unlike in England, in Switzerland there was no significant seasonal variation in the main prey category: Lepidoptera (Ashrafi et al., 2011).

1.7. Metabarcoding

Traditionally, to analyse the diet of a specific animal, direct observation or microscopic examination of prey fragments in the faeces were used. However, since most bats prey aerially at night and masticate the larger prey fragments, these methods are not particularly effective for analysing their diet. Therefore, the emergence of DNA metabarcoding has greatly expanded our understanding of bat trophic ecology. It allows for a more reliable and systematic approach to diet studies (Yoccoz, 2012; Mata et al., 2016) and, relative to diet analysis using more traditional approaches, increases the probability of identifying the majority of the prey species consumed, 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 the presence of distinctive morphological characters (Yoccoz, 2012). DNA metabarcoding is a rapid biodiversity assessment method that combines two technologies: DNA taxonomy and high-throughput DNA sequencing (Ji et al., 2013). This technique uses universal primers to amplify DNA segments and is particularly useful for studying diets of animals with ambiguous lifestyles for which more traditional methods are not as effective (Ingala et al., 2021). It can be used to identify a wide variety of prey. For instance, Ingala et al. (2021) used DNA metabarcoding to investigate plants, arthropods and vertebrates consumed by 25 bat species from tropical dry forests in Belize. The study revealed that

each bat species varied considerably in their associations with invertebrate and plant groups, with no two species completely overlapping in food items detected. DNA metabarcoding also permits the prevision of the consequences of environmental changes on the diet of bats. For instance, Tournayre et al. (2021) found that the greater horseshoe bat (*Rhinolophus ferrumequinum*) feds on a wider range of prey than expected from previous studies and confirmed the high dietary plasticity of the species. Therefore, the abundance and distribution of this species is unlikely to be at high risk when facing environmental changes.

2. Objectives

The main aim of this study is to investigate the trophic ecology of the three bat species found in Madeira Island, using DNA metabarcoding. Specifically, I address the following questions: i) which prey taxa are consumed by *Pipistrellus maderensis* (endemic species to Macaronesia), *Nyctalus leisleri verrucosus* (subspecies endemic to Madeira) and *Plecotus austriacus*; ii) how similar is the diet of *P. maderensis* and *N. l. verrucosus* (the two most common species) in terms of prey composition and diversity; and, iii) are there differences in prey species composition and prey species richness in the diet of females and males of *N. l. verrucosus*? I anticipate that i) all three species prey on a wide diversity of native and non-native prey to Madeira Island, including potential agricultural and agroforestry pests and human disease vectors; ii) due to considerable differences in habitat use and foraging ecology of *P. maderensis* and *N. l. verrucosus*, I hypothesize that the two species are likely to exhibit considerable differences in prey selection; and iii) due to the increased energetic demands of females associated with pregnancy and lactation, I expect sex-specific differences in the diet of *N. l. verrucosus*. This study offers the first insights into the diet of Macaronesian bats, and by providing a holistic overview of the prey consumed by all three bat species found in Madeira and investigating both interspecific (between *P. maderensis* and *N. l. verrucosus*) and intraspecific (between females and males of *N. l. verrucosus*), greatly advances the knowledge regarding the trophic ecology of insular insectivorous bats.

3. Methodology

3.1. Study area

The study was carried out in Madeira, a volcanic island with a maximum elevation of 1,889 m a.s.l., located in the north-eastern Atlantic Ocean. The archipelago of Madeira is part of the Macaronesian biogeographic sub-region, which also includes the archipelagos of Azores, Selvagens, Canary Islands and Cape Verde. Macaronesia comprises 40 islands and islets larger than 1 km² and extends over 3,000 km (Florencio et al., 2021). The climate in Madeira Island is largely conditioned by the anticyclone of the Azores and can range from very humid to arid (Figueira et al., 2006). Additionally, the climate is also conditioned by the orography since the northern slope is more exposed to the dominant winds, more humid, with more rainfall and less solar exposure than the south side. Average annual temperature ranges from 8°C in the highest peaks, and 18-19°C in lower altitudes.

Fieldwork took place in Ecological Park of Funchal (Figure 1), a municipal protected area established in 1994 (Nunes et al., 2021). The park has ca. 1,000 ha and is located north the island's capital, Funchal (Monteiro, 2014). It 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 1818 m a.s.l., at Pico do Areeiro (Camacho & Silva, 2021).



Figura 1: Map of Madeira Island and location of the Ecological Park of Funchal.



Figure 2: Two of the sampling sites at the Ecological Park of Funchal where bats were captured for this study.

3.2. Sample collection

Fieldwork took place from September to October 2021. Mist-nets were used to capture bats in five different sites across the park, each of which was given a name; “Lagoa” (Figure 2a), “Casa do Barreiro” (Figure 2b), “Centro de Receção”, “Centro Temático da Água” and “Sede do Parque Ecologico do Funchal” (Figure 2). Bat capture and handling was conducted following guidelines approved by the American Society of Mammalogists (Sikes & Gannon, 2011). The sex, age (juveniles versus adults), weight, forearm length and reproductive status of each captured individual was recorded. Bats were kept in cloth bags until defecation occurred, after which faecal 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-4 AM) to match with the peak activity pattern of the species. A total of 100 bat pellets were collected during field work, including 75 of *N. leisleri 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).

3.3. DNA Extraction and Amplification

DNA was extracted using the E.Z.N.A tissue DNA kit (Omega Bio-Tek, Norcross, Georgia, USA) following (Mata et al., 2021), except that no Inhibitex tablets (Qiagen, Hilden, Germany) were used. First, one 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 7.5–8) using a sterilized tweezer. After that, faecal 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 x 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 the kit recommendations, except that DNA was eluted two times in 50 µL into different extracts. DNA was extracted in batches of 23 samples plus one negative control in which no faecal 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. 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 ultra-pure 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.

3.4. 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 7bp barcodes. PCR mix consisted in 7 µL KAPA HiFi HotStart ReadyMix (Rocher, KAPA Biosystems, Basel, Switzerland), 2.8 µL of ultra-pure water, 0.7 µL of each P5 and P7 index and 2.8 µL of diluted PCR product. The cycling conditions of the indexing PCR consisted in denaturation at 95º for 3 min, followed by 9 cycles of 95ºC for 30 s, annealing at 55ºC for 30 s and final extension at 72º for 30 s and a final extension at 72ºC for 5 min. Afterwards, indexed PCR products were cleaned with Agencourt AMPure XP beads (Beckman Coulter, Brea, California, USA) 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, the samples were pooled equimolarly and run in TapeStation (Agilent, California, USA) using a High Sensitivity D1000 ScreenTape, to validate the average amplicon length and the absence of small non-target amplicons or primer dimer 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 (2x250bp) along with samples from other projects.

3.5. 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 than predicted, 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 (OTU) 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 mitochondria. 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 - Barcode of Life Data Systems (BOLD) BOLDigger (Buchner & Leese, 2020), manually curated, and classified as diet if identified to the order level or below, while belonging to classes Insecta or Arachnida, with the exception of 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. This was done to reduce false positives associated with events of lab and sequencing cross-contamination.

3.6. Statistical analysis

All statistical analyses were performed in RStudio (v.1.4.1106 2009-2021 RStudio, PBC). Niche breadth, diet diversity, and prey composition were compared among *N.l.verrucosus* and *P.maderensis*, as well as between sexes of *Nyctalus leisleri verrucosus*. Comparisons were always done at three 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 based on 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 linear

model. For this purpose, a linear model was fit using the ‘glm’ function, with a ‘poisson’ distribution, using the number of prey consumed by each individual as response variable, and bat species or sex as 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’ based on Jaccard method to evaluate diet similarity among samples. Differences in the dietary composition between species and, in the case of *Nyctalus leisleri,* sexes, 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 the most to dietary composition disparities across groups.

4. Results

4.1. Overall diet description

DNA material was obtained from 72 of the 100 faecal samples collected, leading to a total of 110 haplotypes and 1,750,053 reads. All three bat species showed a diverse diet, composed by 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 three bat species considered.

In the case of *Nyctalus leisleri verrucosus* – the species with highest sample size – a total of 81 prey items representing 10 orders and 33 families were identified, all belonging

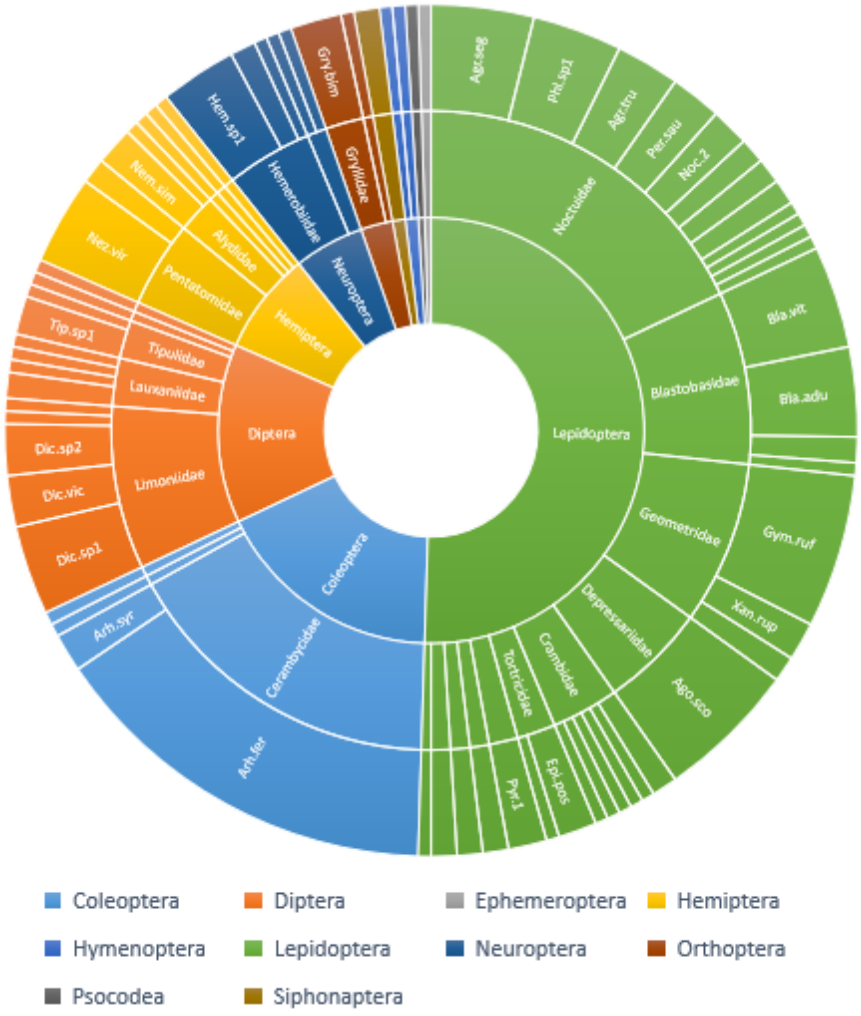


Figure 3: Multi-level circular chart of prey consumed by *Nyctalus leisleri verrucosus*. Species/OTUs names are encoded with the first three letters of the genus and of the specific restrictive, for visualization purposes.

to the class Insecta (Figure 3). 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 and Geometridae (both 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 agriculture known pests (e.g., *Agrotis segetum*) and 15.9% referred to possible pests (Table 1).

In the case of *Pipistrellus maderensis,* a total of 49 prey items of 10 orders and 22 families were identified, associated with two classes: Insecta (95.4%) and Arachnida

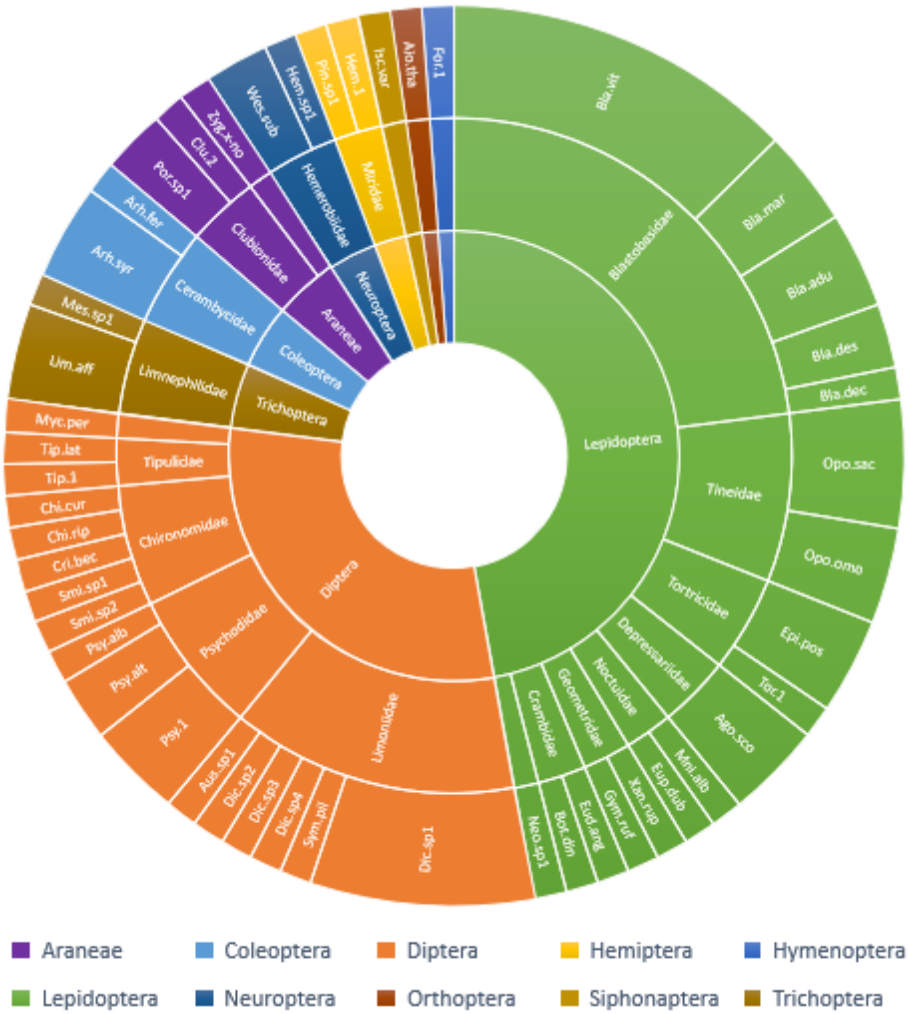


Figure 4: Multi-level circular chart of prey consumed by *Pipistrellus maderensis*. Species/OTUs names are encoded with the first three letters of the genus and of the specific restrictive, for visualization purposes.

(4.6%) (Figure 4). As in *N. leisleri verrucosus,* most prey belonged to the order Lepidoptera (47.1%), followed by Diptera (29.9%), Trichoptera, Coleoptera and Araneae (all three of them 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 commonly observed species/OTUs were *Blastobasis vittate* (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., *Opogona sacchari*), 6.4% were possible agriculture pests, and 3.2% species with human health implications (Table 1).

Only three faecal samples were obtained for *Plecotus austriacus* and thus the results should be interpreted carefully. A total of 8 prey from two orders and three families were identified (Figure 5). Similarly, to the other two species, most prey belonged to the order Lepidoptera (80%), followed by Coleoptera (20%). Noctuidae was the most common family (70%), followed by Cerambycidae (20%) and Geometridae (10%). The species/OTUs most observed were *Arhopalus ferus* and *Mythimna unipuncta* (both 20%). Additionally, of the 6 prey items identified to the species level, at least 50% referred to agriculture known pests (e.g., *Chrysodeixis chalcites*) (Table 1).



Figure 5: Multi-level circular chart of taxa consumed by *Plecotus austriacus*. Species/OTUs names are encoded with the first three letters of the genus and of the specific restrictive, for visualization purposes.

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 attachment 2.

Class	Order	Family	Species	common name	BOLD ID percentage	status	crop/disease	*N. leisleri verrucosus* (51)	*P. maderensis* (18)	*P. austriacus* (3)
Insecta	Coleoptera	Cerambycidae	*Arhopalus ferus*	Burnt pine longhorn beetle	100.00%	known pest	export timber[3], pine[4]	64.6%	5.6%	66.7%
			Arhopalus syriacus		99.51%	possible pest	unknown	6.3%	16.7%	
	Diptera	Psychodidae	*Psychoda albipennis*		100.00%	disease vector	urogenital myiasis[25]		5.6%	
		Tipulidae	*Tipula oleracea*	common crane fly	99.51%	known pest	turfgrass[27]	2.1%		
	Hemiptera	Miridae	*Taylorilygus apicalis*	broken-backed bug	100.00%	possible pest	unknown	2.1%		
		Pentatomidae	*Nezara viridula*	green stink bug	100.00%	known pest	soybean[18], cotton[19]	14.6%		
	Lepidoptera	Blastobasidae	*Blastobasis decolorella*		100.00%	known pest	apple[5]	2.1%	5.6%	
		Crambidae	*Botyodes diniasalis*		100.00%	known pest	poplar plants[6]		5.6%	
			Herpetogramma licarsisalis	grass caterpillar/webworm	100.00%	known pest	turf[13], rice[14]	4.2%		
			Palpita vitrealis	jasmine moth	100.00%	known pest	olive[23]	2.1%		
		Gelechiidae	*Aproaerema anthyllidella*		99.51%	possible pest	unknown	4.2%		
		Geometridae	*Gymnoscelis rufifasciata*	double-striped pug	100.00%	possible pest	unknown	25.0%	5.6%	
		Noctuidae	*Agrotis segetum*	turnip moth	100.00%	known pest	vegetables, cereals[1]	16.7%		
			Caradrina clavipalpis	pale mottled willow	100.00%	possible pest	unknown	4.2%		
			Chrysodeixis chalcites	golden twin-spot moth	100.00%	known pest	Banana, tobacco[7], soybean[8], tomato, greenhouse crops[9], (among others)[10]	4.2%		33.3%
			Cornutiplusia circumflexa		100.00%	possible pest	unknown	2.1%		
			Mythimna unipuncta	true armyworm	100.00%	known pest	small grains[15], corn[16], grass[17]	4.2%		66.7%
			Peridroma saucia	pearly underwing moth	100.00%	known pest	peanuts, sunflower, soybean, grapevine[24] , (among others)	8.3%		
		Tineidae	*Opogona sacchari*	banana moth	100.00%	known pest	ornamental plants[20], banana, sugarcane [21], pineapple[22]		22.2%	
		Tortricidae	*Epiphyas postvittana*	light brown apple moth	100.00%	known pest	horticultural plants[11], apple[12]	6.3%	16.7%	
			Selania leplastriana	Leplastrier's piercer	100.00%	known pest	cabbage, cauliflower[26]	2.1%		
	Orthoptera	Acrididae	*Aiolopus thalassinus*		100.00%	known pest	grasses, cereals, alfalfa, vegetables[2]	2.1%	5.6%	
		Gryllidae	*Gryllus bimaculatus*		100.00%	possible pest	unknown	8.3%		

Table 2: List of the 59 OTUs (species level) found in the diet of *Nyctalus leisleri verrucosus*, *Pipistrellus maderensis* and *Plecotus austriacus*, after data filtering, alongside information regarding the previous knowledge about their presence in Madeira as well as colonization status in the island (according to Borges et al., 2008). MAC represents Macaronesia native endemic species and "—"stands for 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.

OTU	SCIENTIFIC ID	PRESENT IN MADEIRA	STATUS
OTU8	*Agonopterix scopariella* (Heinemann, 1870)	yes	introduced
OTU23	*Agrotis segetum* (Denis & Schiffermuller, 1775)	yes	native non-endemic
OTU103	*Agrotis trux* (Hübner, 1824)	yes	native non-endemic
OTU105	*Aiolopus thalassinus* (Fabricius, 1781)	yes	native non-endemic
OTU37	*Aproaerema anthyllidella* (Hübner, 1813)	yes	MAC
OTU1	*Arhopalus ferus* (Mulsant, 1839)	yes	introduced
OTU15	*Arhopalus syriacus* (Reitter, 1895)	yes	introduced
OTU26	*Ascotis fortunata* (Blachier, 1887)	yes	native endemic
OTU9	*Blastobasis adustella* Walsingham, 1894	yes	native endemic
OTU108	*Blastobasis decolorella* (Wollaston, 1858)	yes	native endemic
OTU98	*Blastobasis desertarum* (Wollaston, 1858)	yes	native endemic
OTU52	*Blastobasis maroccanella* Amsel, 1952	yes	native non-endemic
OTU3	*Blastobasis vittata* (Wollaston, 1858)	yes	native endemic
OTU301	*Botyodes diniasalis* (Walker, 1859)	yes	introduced
OTU29	*Caenopsis fissirostris* (Walton, 1847)	yes	introduced
OTU146	*Caradrina clavipalpis* Scopoli, 1763	yes	native endemic
OTU71	*Chrysodeixis chalcites* (Esper, 1789)	yes	native non-endemic
OTU630	*Cloeon peregrinator* Gattolliat & Sartori, 2008	yes	native endemic
OTU159	*Cornutiplusia circumflexa* (Linnaeus, 1767)	yes	native non-endemic
OTU79	*Cricotopus beckeri* Hirvenoja, 1973	yes	native non-endemic
OTU12	*Dicranomyia vicina* Macquart, 1839	yes	MAC
OTU30	*Epiphyas postvittana* (Walker, 1863)	yes	introduced
OTU128	*Eudonia angustea* (Curtis, 1827)	yes	native non-endemic
OTU114	*Eudonia scoriella* (Wollaston, 1858)	yes	native endemic
OTU102	*Euplexia dubiosa* (Bethune-Baker, 1891)	yes	native endemic
OTU49	*Gryllus bimaculatus* De Geer, 1773	yes	introduced
OTU19	*Gymnoscelis rufifasciata* (Haworth, 1809)	yes	native non-endemic
OTU187	*Hemerobius stigma* Stephens, 1836	yes	native non-endemic
OTU117	*Herpetogramma licarsisalis* (Walker, 1859)	yes	introduced
OTU96	*Limnephilus affinis* Curtis, 1834	yes	—
OTU721	*Mecyna asinalis* (Hübner, 1819)	yes	native non-endemic
OTU59	*Mniotype albostigmata* (Bethune-Baker, 1891)	yes	endemic
OTU358	*Mycetophila perpallida* Chandler, 1993	yes	native non-endemic

OTU	SCIENTIFIC ID	PRESENT IN MADEIRA	STATUS
OTU60	*Mythimna unipuncta* (Haworth, 1809)	yes	migrant
OTU47	*Nemausus simplex* Horváth, 1911	yes	–
OTU17	*Nezara viridula* (Linnaeus, 1758)	yes	introduced
OTU76	*Ocys harpaloides* (Audinet-Serville, 1821)	yes	native non-endemic
OTU7	*Opogona omoscopa* (Meyrick, 1893)	yes	probably introduced
OTU13	*Opogona sacchari* (Bojer, 1856)	yes	introduced
OTU94	*Palpita vitrealis* (Rossi, 1794)	yes	native non-endemic
OTU4	*Peridroma saucia* (Hübner, 1808)	yes	native non-endemic
OTU5077	*Selania leplastriana* (Curtis, 1831)	yes	native non-endemic
OTU124	*Symplecta pilipes* (Fabricius, 1787)	yes	introduced
OTU199	*Taylorilygus apicalis* (Fieber, 1861)	yes	introduced
OTU142	*Uresiphita gilvata* (Fabricius, 1794)	yes	native non-endemic
OTU56	*Voria ruralis* (Fallén, 1810)	yes	native non-endemic
OTU217	*Wesmaelius subnebulosus* (Stephens, 1836)	yes	native non-endemic
OTU89	*Xanthorhoe rupicola* (Wollaston, 1858)	yes	native endemic
OTU253	*Zygiella x-notata* (Clerck, 1757)	yes	introduced
OTU551	*Chironomus curabilis* Beljanina, Loginova, Sigareva, 1990	no	
OTU415	*Chironomus riparius* Meigen, 1804	no	
OTU211	*Chrysoperla nipponensis* (Okamoto, 1914)	no	
OTU196	*Dicranophragma nemorale* (Meigen, 1818)	no	
OTU36	*Ischnopsyllus variabilis* (Wagner, 1898)	no	
OTU121	*Pangaeus piceatus* Stål, 1862	no	
OTU104	*Psychoda albipennis* Zetterstedt, 1850	no	
OTU31	*Psychoda alternata* Say, 1824	no	
OTU113	*Tipula lateralis* Meigen, 1804 / *Tipula intermedia* Eiroa-Álvarez, 1990	no	
OTU279	*Tipula oleracea* Linnaeus, 1758	no	

Arthropod species native but not endemic to Madeira constituted the majority of the prey consumed by the three different species of bats (32.2%), followed by introduced species (23.7%), native endemic species (18.6 %) and a relatively small percentage of native endemic species to Macaronesia (3.4%) and island's suspected migratory species (1.7%) (Table 2).

4.2. Inter-specific 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 (Figure 6). However, at the family level, *N. l. verrucosus* appeared to have a wider niche breadth than *P. maderensis*. Nevertheless, as confidence intervals overlapped in all three levels, the observed differences were not statistically significant.

Average prey diversity per sample was slightly higher for *P. maderensis* than for *N. l. verrucosus* (Figure 7). However, differences were not statistically significant for all taxonomic levels analysed (order: p-value = 0.435; family: p-value = 0.215; OTU: p-value = 0.394). Alternatively put, the mean number of prey consumed by both species was similar.

The diet composition of *P. maderensis* and *N. l.* verrucosus differed at the order (p-value =0.006), family (p-value < 0.001) and OTU level (p-value < 0.001) as the multidimensional scaling (MDS) plot demonstrates (Figure 8). Simper species analysis

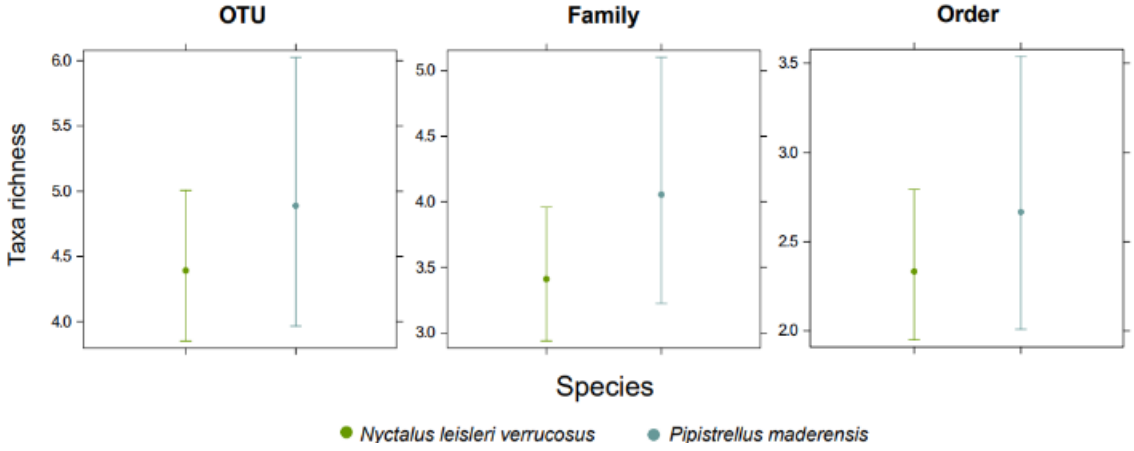


Figure 7: Modelled effect of bat species on the average prey richness per sample at different taxonomic levels.

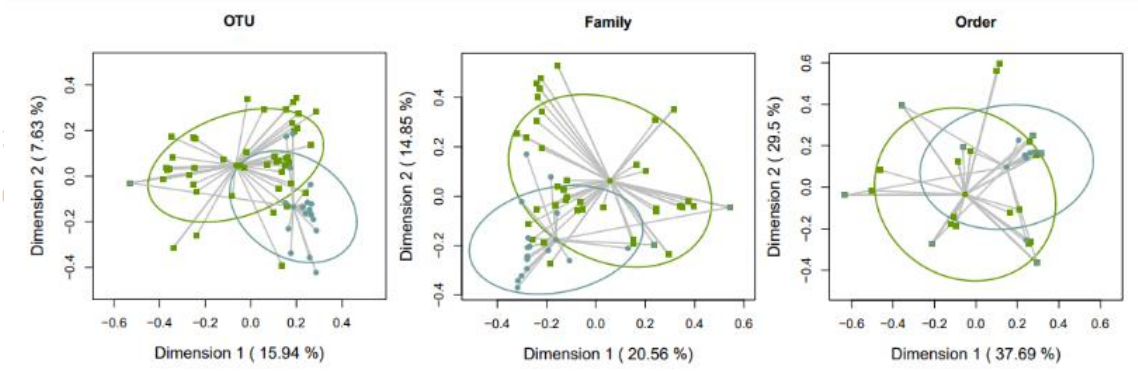


Figure 8: Multidimensional scaling (MDS) plot of the prey composition of *Nyctalus leisleri verrucosus* (green) and *Pipistrellus maderensis* (blue) at different taxonomic levels. The variance explained by each axis is indicated within parenthesis.

showed that at the order level, *P. maderensis* consumed more Diptera (p-value = 0.018; 77% of samples vs 39% in *N. l. verrucosus*). Moreover, Trichoptera (p-value = 0.004; 16%) and Araneae (p-value = 0.008; 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* (p-value < 0.001; 33% vs 4% in *N. l. verrucosus)*, along with Blastobasidae (p-value < 0.001; 72% vs 25% in *N. l. verrucosus*), Chironomidae (p-value < 0.001; 27% vs 1.9% in *N. l. verrucosus*), Psychodidae (p-value < 0.001; 27% vs 0% in *N. l. verrucosus*), and Limnephilidae (p-value = 0.002; 16% vs 0% in *N. l. verrucosus*). Finally, simper analysis evidenced that at the OTU level, *N. l. verrucosus* consumed more *Arhopalus ferus* (p-value = 0.020; 60% vs 5% in *P. maderensis*). On the other hand, *Blastobasis maroccanella* was more common on the diet of *P. maderensis* (p-value = 0.010; 16% vs 4% in *N. l. verrucosus*), along with *Opogona omoscopa* (p-value = 0.027; 16% vs 4% in *N. l. verrucosus*), *Blastobasis vittata* (p-value < 0.001; 61% vs 15% in *N. l. verrucosus*), *Dicranomyia.*sp 1 (p-value = 0.047; 38% vs 13% in *N. l. verrucosus*), *Opogona sacchari* (p-value = 0.002; 22% vs 0% in *N.l.verrucosus*), *Limnephilus affinis* (p-value = 0.004 ; 16% vs 0% in *N.l.verrucosus*), and *Psychodidae* 1 (p-value = 0.005; 16% vs 0% in *N. l. verrucosus*).

4.3. Intra-specific variation in 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 (non-overlapping confidence intervals; Figure 9). Yet, although the mean number of prey consumed by males was higher than that consumed by females (Figure 10) these differences were not significant for any of the analysed taxonomic levels considered (order p-value = 0.902; family p-value = 0.225; and, OTUs p-value = 0.207). Lastly, the diet composition of male and female *N. l. verrucosus* (Figure 11) was not significantly different at neither the order, family or OTUs levels (p-value = 0.622, 0.314 and 0.586, respectively).

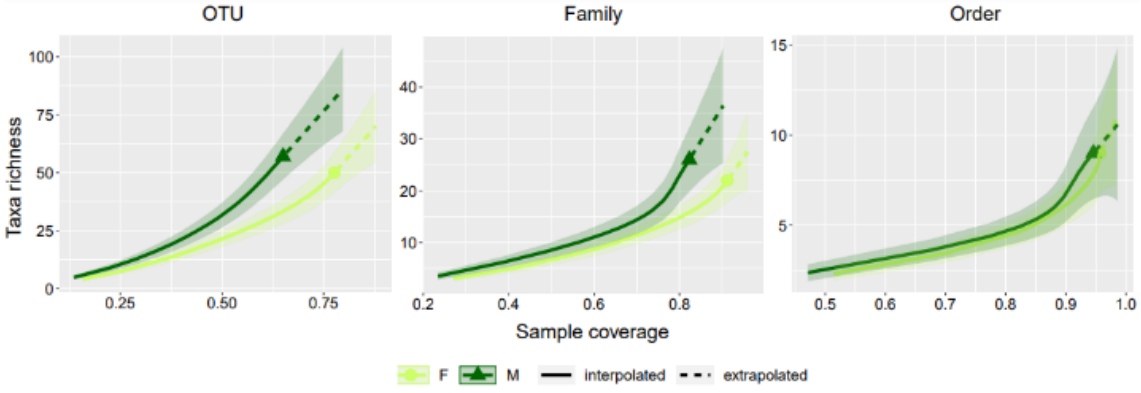


Figure 9: Rarefaction curves of prey taxa richness at different taxonomic levels for both females (F) and males (M) of *Nyctalus leisleri verrucosus*. Shaded area represents 95% confidence intervals.

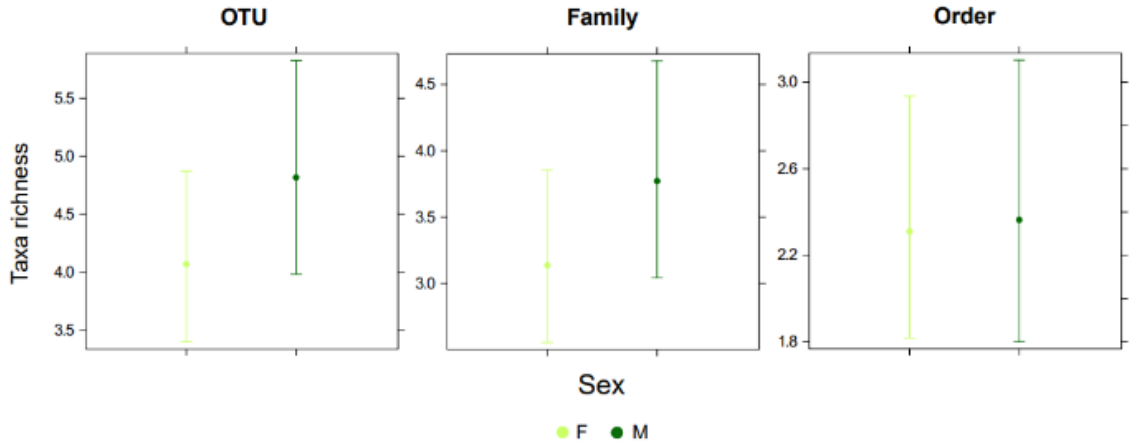


Figure 10: Modelled effect of bat sex on the average prey richness per sample of *Nyctalus leisleri verrucosus* at different taxonomic levels.

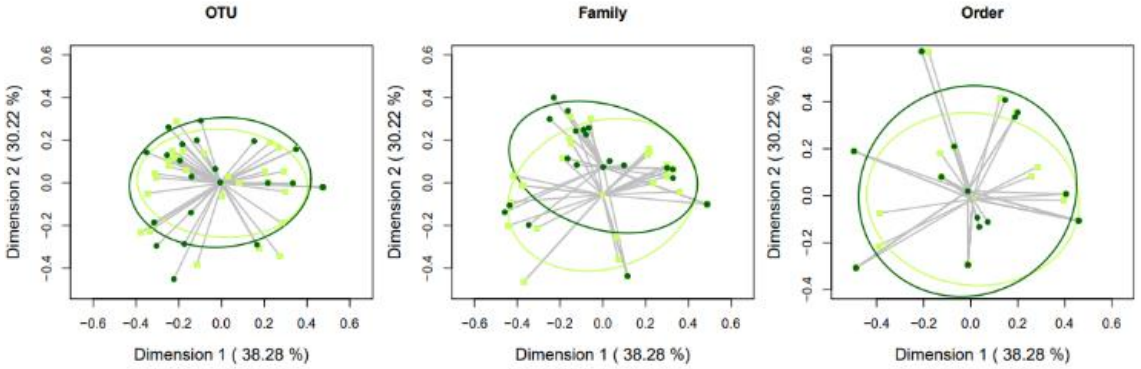


Figure 11: Multidimensional scaling (MDS) plot of the prey composition of females (light green) and males (dark green) of *Nyctalus leisleri verrucosus* at different taxonomic levels. The variance explained by each axis is indicated within parenthesis.

5. 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 the Madeira bats, including multiple agricultural and forestry pest species, a species of human disease relevance and numerous taxa non-previously recorded in Madeira.

5.1. Prey consumed by the insectivorous bats found in Madeira

All three Madeiran bat species prey on a wide diversity of native and non-native prey to Madeira Island, including potential agricultural and agroforestry pests and pests with human health implications. Our findings indicate that the diet composition of the *N. l. verrucosus* in Madeira is – to a certain extent – in line with other non-molecular dietary studies of *N. leisleri* populations from mainland Europe (Vergari & Dondini, 1999; Kaňuch et al., 2005). Except for the order Neuroptera, all orders identified in Vergari and Dondini (1999) as prey of *N. leisleri* were also detected in this study. However, whereas some previous studies identified Diptera (Vaughan, 1997; Waters et al., 1999) as primary prey of *N. leisleri*, our results indicate that Lepidoptera is the dominant order consumed by *N. l. verrucosus*. Four of the six 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*. Also, we identified that 0.5% of diet of *N. l. verrucosus* was composed by Ephemeroptera, aligning with Shiel et al. (1998) assertion that insects with aquatic larvae may be part of the diet of *N. leisleri*. A bat flea (*Ischnopsyllus variabilis*) was also identified in the diet of *N. l. verrucosus*, which may have been ingested during grooming. Jesus et al. (2009) had suggested that in addition to Lepidoptera and Coleoptera *N. l. verrucosus* was also likely to prey Trichoptera. However, although we found evidence for Lepidoptera and Coleoptera, we could not detect Trichoptera on the species' diet. Moreover, *N. l. verrucosus* was found to share several prey species – e.g., *Aiolopus thalassinus, Caradrina clavipalpis, Gymnoscelis rufifasciata, Herpetogramma licarsisalis, Nezara viridula, Opogona sacchari, Opogona omoscopa, Blastobasis desertarum* and *Blastobasis* maroccanella - with a recently introduced gecko (*Tarentola mauritanica*) 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 the expectations for an aerial hawking foraging bat and matching previous research findings (Boston & Dechmann, 2020).

The diet of *Pipistrellus maderensis* is poorly documented. However, all of the prey families previously suggested to be consumed by the species (Diptera, Trichoptera, Lepidoptera and Coleoptera; Jesus et al., 2009) were identified in the diet of *P. maderensis* in this study. Additionally, the findings of Goiti et al. (2003) regarding the diet of *Pipistrellus kuhlli* (a close relative to *P. maderensis*) match our findings, as the diet of *P. kuhlii* was found to be dominated by the prey orders Lepidoptera, Hymenoptera, Coleoptera and Diptera and the families Tipulidae, Chironomidae and Aranea, that were also found to constitute a significant portion of the diet of *P. maderensis*. However, unlike Goiti et al. (2003), the suborder Brachycera was identified in this study. Also, Safi and Kerth (2004) identified Diptera as the most common order consumed by *P. khulli*, followed by Lepidoptera. Similarly, previous study also identified Diptera as the main order consumed by *Pipistrellus pipistrellus* (Barlow, 1997) and by *Pipistrellus pygmaeus* (Bartonička et al., 2008). In this study, Lepidoptera was found to be the most consumed order by *P. maderensis*, with Diptera ranking second.

Regarding the diet of *Plecotus austriacus*, the identification of Lepidoptera and in particular Noctuidae as the main prey is 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., *Mythimna unipuncta* and *Chrysodeixis chalcites* (percent frequency of interaction: 20% and 10% of the diet, respectively) - had already been identified in previous dietary analysis of this species (Borg & Sammut, 2002). However, our limited number of samples collected for *P. austriacus* precluded a robust assessment of the diet of this species in Madeira.

Our findings show that all three insectivorous bat species found in Madeira feed on a wide variety of prey, including several economically important insect pest species that affect a variety of crops, and insects that can spread diseases affecting humans (Table 1). As examples, *N. l. verrucosus* has found to consume turnip moths (*Agrotis segetum*), one of the most damaging insects to vegetables and cereals in the world (Esbjerg & Sigsgaard, 2014). Turnip moths affect a broad range of plant hosts (at least 25 plant families are known to be impacted by this species; June, 2011), and are difficult to control due to their soil-dwelling habits (El-Salamouny et al., 2003). *Agrotis segetum* is widespread in Europe, Asia and Africa where it can cause significant (up to 30% of production) crop losses (June, 2011). *N. l. verrucosus* was also found to prey on the grass webworm (*Herpetogramma licarsisalis),* an economically important rice pest (Barrion et al., 1987) and a major pest of turfgrass (Tashiro, 1976) and on the southern

green stink bug (*Nezara viridula*), one of the most important pest species impacting soybeans. The grass webworm is suspected to impact on dairy production in New Zealand, where it damages kikuyu (*Pennisetum clandestinum*), an important turf species to pastoral farming (Willoughby & Barns, 2002), whereas *Nezara viridula* is known to impact over 145 plant species within 32 families. This pest is constantly spreading as soybean producing acreage expands, notably in South America (Werdin González et al., 2011). *Nezara viridula* transmits the pathogenic bacteria (*Pantoea agglomerans*) into cotton bolls (*Gossypium hirsutum*) (Medrano et al., 2007), the main species of cotton currently cultivated (Kouakou et al., 2007). In addition to agricultural pests, we have also detected agroforestry pests in the diet of *N. l. verrucosus.* For instance, *Arhopalus ferus*, the most frequent prey of *N. l. verrucosus* (frequency of interaction = 15%) affects New Zealand's forest industry (particularly after windthrow or fire) and can cause significant economic loss during the salvage of windthrown or fire-killed trees (Hosking & Bain, 1977). A particularly relevant agricultural pest found in the diet of *P. maderensis* is the banana moth (*Opogona sacchari*), which attacks a wide variety of plants worldwide. In Madeira, this pest 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). Another particularly interesting prey of *P. maderensis* is *Psychoda albipennis*, which can cause urogenital myiasis in humans, resulting in abdominal pain, diarrhea and dysuria (Hazratian et al., 2020). Lastly, *Chrysodeixis chalcites*, an important pests of banana crops in the Canary Islands (Fuentes et al., 2018) and one of the four most important pests of European greenhouse crops (Simón et al., 2015) was found in both the diet of *P. austriacus* and *N. l. verrucosus*. In total, 23 of the 59 species found in the three bats diet (38.9%), are known or potential agricultural/forestry pest species or species known to transmit diseases to humans. These high diversity of agricultural/forestry pest species and of species of human health relevance consumed by bats in Madeira highlights the potential role of insectivorous bats as suppressors of arthropods with negative economic and human health impacts (Kunz et al., 2011; Kemp et al., 2019; Pedro et al., 2020; Ramírez-fráncel et al., 2022).

Ten species found to be consumed by bats in Madeira had not previously been described to the island (Table 2). However, most of the prey consumed by the three bat species consisted of arthropod species native but non endemic to Madeira, followed by introduced species, native endemic species and a rather small percentage of species native endemic to Macaronesia and species suspected to be migratory in the island. In Madeira Island Arthropoda accounts for over 85% of endemic animals (species and subspecies) (Borges et al., 2008). Given the high number of endemic arthropods in

Madeira, it is not surprising that the three Madeiran bats consume a significant number of endemic arthropods species.

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

Sympatric species (i.e., species with overlapping ranges and that live in the same local community, close enough to interact; Marko (2008) are likely to have a different use of the limited resources available – resource partitioning – either or both dietary or temporal (Matthews et al., 2010), hence reducing overlapping niches and stabilizing competition. There are several potential non-strictly independent mechanisms for resource partitioning. For insectivorous bats, differences in wing morphology, echolocation call structure, temporal activity patterns and habitat partitioning, may lead to resource partitioning (Mancina & Rivera, 2012; Emrich et al., 2014; Rocha et al., 2020). Focusing on the latter, *N. l. verrucosus* and *P. maderensis* are known to respond differently to landscape composition thus it is likely that they feed on different prey (Ferreira et al., 2022). Indeed, notwithstanding some conspicuous differences in the diet of *N. l. verrucosus* and *P. maderensis*, our results indicate that the niche breadth and the diet diversity of both species is rather similar. Similarly to what suggested by Divoll et al. (2022), *N. l. verrucosus* and *P. maderensis* may use different hunting techniques or forage at different habitat strata, but both may be capturing the same arthropod species, while looking for their specific preferred prey. Yet, the diet composition of *N. l. verrucosus* and *P. maderensis* was considerably different at the order, family and OTU levels. For instance, *N. l. verrucosus* consumed fewer Diptera prey than *P. maderensis* and, contrary to *P. maderensis*, it did not consume spiders (Aranae) or Trichoptera. Emrich et al. (2014) showed that bats with different morphologies and echolocation tend to differ in diet and habitat use, therefore reducing overlap in resource use. Habitat segregation may thus be a factor contributing to different dietary composition between *N. l. verrucosus* (more associated with production forests; Ferreira et al., 2022) and *P. maderensis* (more associated to agricultural areas, laurel forest and shrubland habitats ;Ferreira et al., 2022).

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

We detected some sex-related differences in the diet of *N. l. verrucosus*. Although the diet composition and diversity of both sexes did not differ much, male bats had a wider

niche breadth than females at both the family and OTUs level. These findings differ from those published by Mata et al. (2016) in their investigation of *Tadarida teniotis'* dietary sexual segregation using metabarcoding. Females of *T. teniotis* were found to consume larger moths than males, probably because 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 favourable foraging areas (Rocha et al., 2017b). Furthermore, male and female bats may exhibit differing temporal distributions of activity. According to Thies et al. (2006), females of long-legged bats (*Macrophyllum macrophyllumi*) were more active at dawn, whereas male activity was more evenly distributed throughout the night. However, as the activity pattern of the different sexes of Madeiran bats has not yet been study, nothing can be said about whether the observed differences in the diet of female and male bats might relate to different activity patterns.

Males of some bat species are thought to forage closer to day roosts than females in order to dedicate more time to roost vigilance and female defence, getting access to females in the day roost and attempting sneak matings (Meyer et al., 2005). By being more spatially constricted, it is possible that male bats might in fact consume a wider diversity of prey, as they cannot select more nutritious/energy-rich prey. Likewise, Nunes et al. (2022) observed a more specialized diet in female insular wall lizards (*Podarcis bocagei berlengensis*) in Portugal, which was justified by the possible selection of more nutritious prey, likely due to high energy demands associated with reproduction. On the other hand, males of *P. b. berlengensis* had a wider diversity of prey (i.e., less specialized diet), likely reflecting that they were less selective while foraging, as they were more spatially constrained due to the need of defending their territory (Nunes et al., 2022).

Female Natal long-fingered bats (*Miniopterus natalensis*) in different reproductive phases were found to have different isotopic diet ratios, which was likely reflected different levels of nutritional investment in reproduction (Pretorius et al., 2022). Little is known about the reproductive phenology of *N. l. verrucosus*. However, in Azores, the first pregnant females of the island-restricted sister species *Nyctalus azoreum* were detected by the end of May (Leonardo & Medeiros, 2011). However, *Nyctalus* bats are known to exhibits delayed fertilization (i.e., the sequence of events from insemination to birth is interrupted, after copulation but before fertilization) (Orr & Zuk, 2014). Therefore, it is possible that the observed differences in the diet of males and females, which are

based on sampled collected between September and October might not reflect the full spectrum of sex-related differences throughout the year.

6. Conclusion

This study shows that all the bat species found in Madeira - *P. maderensis*, *N. l. verrucosus* and *P. austriacus* – prey on a wide diversity of arthropod species and may benefit humans through the potential suppression of agricultural and forestry pests. Moreover, the detection of numerous previously unrecorded species to Madeira in the diet of the three Madeiran bats suggests that island aerial insectivorous bats may serve as potential samplers and early warners of non-native arthropods, including potential invasive pests. We show that although *N. l. verrucosus* and *P. maderensis* are largely sympatric throughout the island, their diets differ considerably, likely reflecting differences in habitat use. Furthermore, we detected that in *N. l. verrucosus* males exhibited a larger niche breadth than females, potentially reflecting female-male differences in habitat use or activity patterns. This research considerably expands our understanding of the trophic ecology of insular insectivorous bats by giving the first insights into the diet of Macaronesian bats, offering a comprehensive review of the prey ingested by all three bat species present in Madeira, and analysing both intraspecific and interspecific differences in the diet of island-restricted bats.

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Attachment 1

Supplementary Table S1: List of all 110 OTUs found on the diet of the three studied bat species. 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.

Class	Order	Family	OTU	*N. l. verrucosus* (51)	*P. maderensis* (18)	*P. austriacus* (3)
Arachnida	**Araneae**	Araneidae	*Zygiella x-notata*		5.6%	
		Clubionidae	Clubionidae 2		5.6%	
			Porrhoclubiona sp.1		11.1%	
Insecta	**Coleoptera**	Carabidae	*Ocys harpaloides*	2.0%		
		Cerambycidae	*Arhopalus ferus*	60.8%	5.6%	66.7%
			Arhopalus syriacus	5.9%	16.7%	
		Curculionidae	*Caenopsis fissirostris*	2.0%		
	Diptera	Chironomidae	*Chironomus curabilis*	2.0%	5.6%	
			Chironomus riparius		5.6%	
			Cricotopus beckeri		5.6%	
			Smittia sp.1		5.6%	
			Smittia sp.2		5.6%	
		Lauxaniidae	Lauxaniidae 1	3.9%		
			Lauxaniidae 2	2.0%		
			Minettia sp.1	2.0%		
			Sapromyza sp.2	2.0%		
		Limoniidae	*Austrolimnophila sp.1*		5.6%	
			Dicranomyia sp.1	13.7%	38.9%	
			Dicranomyia sp.2	7.8%	5.6%	
			Dicranomyia sp.3	2.0%	5.6%	
			Dicranomyia sp.4	2.0%		
			Dicranomyia vicina	7.8%		
			Dicranophragma nemorale		5.6%	
			Symplecta pilipes		5.6%	
		Mycetophilidae	*Mycetophila perpallida*		5.6%	
		Psychodidae	*Psychoda albipennis*		5.6%	
			Psychoda alternata		11.1%	
			Psychodidae 1		16.7%	
		Tachinidae	*Voria ruralis*	2.0%		
		Tipulidae	*Tipula lateralis/intermedia*		5.6%	
			Tipula oleracea	2.0%		
			Tipula sp.1	5.9%		
			Tipulidae 1		5.6%	
		Unidentified	Diptera 3	5.9%		
			Diptera 4	5.9%		
			Diptera 5	3.9%		
			Diptera 6	2.0%		

Class	Order	Family	OTU	*N. l. verrucosus* (51)	*P. maderensis* (18)	*P. austriacus* (3)
			Diptera 7	2.0%		
			Diptera 8		5.6%	
			Diptera 9	3.9%		
			Diptera 10	2.0%		
			Diptera 11	2.0%		
			Diptera 12	2.0%		
			Diptera 14	2.0%		
	Ephemeroptera	Baetidae	*Cloeon peregrinator*	2.0%		
	Hemiptera	Alydidae	Alydidae 1	2.0%		
			Nemausus simplex	5.9%		
		Cicadellidae	Cicadellidae 1	2.0%		
		Cydnidae	*Pangaeus piceatus*	2.0%		
		Miridae	*Pinalitus sp.1*		5.6%	
			Taylorilygus apicalis	2.0%		
		Pentatomidae	*Nezara viridula*	13.7%		
			Pentatomidae 1	3.9%		
		Unidentified	Hemiptera 1		5.6%	
	Hymenoptera	Braconidae	*Homolobus sp.1*	2.0%		
		Formicidae	Formicidae 1		5.6%	
			Nylanderia sp.1	2.0%		
	Lepidoptera	Blastobasidae	*Blastobasis adustella*	13.7%	16.7%	
			Blastobasis decolorella	2.0%	5.6%	
			Blastobasis desertarum		11.1%	
			Blastobasis maroccanella	3.9%	16.7%	
			Blastobasis vittata	15.7%	61.1%	
		Crambidae	*Anania sp.1*	2.0%		
			Botyodes diniasalis		5.6%	
			Eudonia angustea		5.6%	
			Eudonia scoriella	2.0%		
			Herpetogramma licarsisalis	3.9%		
			Mecyna asinalis	2.0%		
			Palpita vitrealis	2.0%		
			Uresiphita gilvata	2.0%		
		Depressariidae	*Agonopterix scopariella*	21.6%	16.7%	
		Gelechiidae	*Aproaerema anthyllidella*	3.9%		
		Geometridae	*Ascotis fortunata*	3.9%		33.3%
			Gymnoscelis rufifasciata	23.5%	5.6%	
			Xanthorhoe rupicola	5.9%	5.6%	
		Noctuidae	*Agrotis segetum*	15.7%		
			Agrotis trux	9.8%		
			Caradrina clavipalpis	3.9%		
			Chrysodeixis chalcites	3.9%		33.3%
			Cornutiplusia circumflexa	2.0%		

Class	Order	Family	OTU	*N. l. verrucosus (51)*	*P. maderensis (18)*	*P. austriacus (3)*
			Euplexia dubiosa	2.0%	5.6%	33.3%
			Mniotype albostigmata	2.0%	5.6%	33.3%
			Mythimna unipuncta	3.9%		66.7%
			Noctuidae 1	2.0%		
			Noctuidae 2	5.9%		33.3%
			Peridroma saucia	7.8%		
			Phlogophora sp.1	13.7%		33.3%
		Praydidae	*Prays sp.1*	3.9%		
		Pterophoridae	*Amblyptilia sp.1*	2.0%		
		Pyralidae	Pyralidae 1	5.9%		
		Stathmopodidae	*Neomariania sp.1*		5.6%	
		Tineidae	*Opogona omoscopa*	3.9%	16.7%	
			Opogona sacchari		22.2%	
		Tortricidae	*Epiphyas postvittana*	5.9%	16.7%	
			Selania leplastriana	2.0%		
			Tortricidae 1		5.6%	
		Unidentified	Lepidoptera 1	2.0%		
	Neuroptera	Chrysopidae	*Chrysoperla nipponensis*	2.0%		
			Chrysopidae 1	2.0%		
		Hemerobiidae	Hemerobiidae 1	2.0%		
			Hemerobius sp.1	11.8%	5.6%	
			Hemerobius stigma	3.9%		
			Wesmaelius subnebulosus		11.1%	
	Orthoptera	Acrididae	*Aiolopus thalassinus*	2.0%	5.6%	
		Gryllidae	*Gryllus bimaculatus*	7.8%		
		Unidentified	Orthoptera 1	2.0%		
	Psocodea	Liposcelididae	*Liposcelis sp.1*	2.0%		
	Siphonaptera	Ischnopsyllidae	*Ischnopsyllus variabilis*	*3.9%*	*5.6%*	
	Trichoptera	Limnephilidae	*Limnephilus affinis*		*16.7%*	
			Mesophylax sp.1		5.6%	

Attachment 2

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