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A Metabarcoding Approach to the Diet of Invasive Rodents in Ecological Park of Funchal (Madeira Island)

MASTER DISSERTATION

João Miguel Gouveia Nunes
MASTER IN APPLIED BIOLOGY

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(blue bars), and negative values indicate higher occurrence in *Rattus rattus* (red bars). Significant contrasts (permutation $p < 0.05$) are marked for each species.

List of Abbreviations

BOLD	Barcode of Life Data Systems
COI	Cytochrome oxidase subunit 1
DNA	Deoxyribonucleic acid
EU	European Union
ITS2	Internal Transcribed Spacer 2
IUCN	International Union for Conservation of Nature
Jaccard	Jaccard index
NCBI	National Center for Biotechnology Information
NMDS	Non-metric Multidimensional Scaling
OUT	Operational Taxonomic Unit
PECOF	Ecological Park of Funchal
PERMANOVA	Permutational multivariate analysis of variance
PCR	Polymerase Chain Reaction
SIMPER	Similarity Percentage analysis
SAC	Special Area of Conservation
SPA	Special Protection Area

Abstract

Invasive rodents are among the main drivers of biodiversity loss on islands. Their omnivorous diet includes plants, invertebrates, and occasionally vertebrates, producing strong ecological impacts. On Madeira Island, the diet of *Rattus rattus* and *Mus musculus* remains poorly studied, and the only previous work, based on morphological methods, reported a generalist diet with no evidence of vertebrate predation.

Using a DNA metabarcoding approach, 49 samples were analysed (31 from *Mus musculus* and 18 from *Rattus rattus*). A total of 78 dietary species were identified, belonging to 60 families and 78 genera. Both rodents showed generalist feeding, but *M. musculus* consumed more plants, while *R. rattus* fed mostly on invertebrates. Endemic plants of conservation interest, such as *Rubus bollei*, *Erica platycodon* and *Vaccinium padifolium*, were detected, together with invasive species like *Acacia melanoxylon* and *Ageratina adenophora*. Several Madeiran endemic invertebrates were also recorded, including *Acalles oblitus*, *Calliptamus madeirae* and *Hipparchia maderensis*. Vertebrate DNA was not detected in the samples, but field observations suggest that predation events may occasionally occur.

This study provides the first molecular baseline for rodent diet in Madeira and shows potential impacts on both native and exotic *taxa*. By consuming endemic plants, dispersing invasive species, and preying on invertebrate communities, rodents may compromise habitat recovery, especially in the Ecological Park of Funchal, already severely affected by wildfires. These results confirm that invasive rodents, through their consumption of both animal and plant resources, are responsible for ecological changes and highlight the need for directed conservation actions.

Keywords: Diet, Madeira Island, Invasive rodents, *Rattus rattus*, *Mus musculus*, DNA metabarcoding

Resumo

Os roedores invasores estão entre os principais responsáveis pela perda de biodiversidade em ilhas. A sua dieta omnívora inclui plantas, invertebrados e, ocasionalmente, vertebrados, produzindo fortes impactos ecológicos. Na ilha da Madeira, a dieta de *Rattus rattus* e *Mus musculus* continua pouco estudada, e o único trabalho anterior, baseado em métodos morfológicos, descreveu uma dieta generalista sem evidência de predação de vertebrados.

A partir de 49 amostras, foram identificadas 78 espécies alimentares pertencentes a 60 famílias e 78 géneros. Ambas as espécies revelaram dietas generalistas, mas *M. musculus* consome mais plantas, enquanto *R. rattus* alimenta-se sobretudo de invertebrados. Foram detetadas espécies endémicas de interesse para a conservação, como *Rubus bollei*, *Erica platycodon* e *Vaccinium padifolium*, bem como espécies invasoras como *Acacia melanoxylon* e *Ageratina adenophora*. Também foram registados invertebrados endémicos da Madeira, incluindo *Acalles oblitus*, *Calliptamus madeirae* e *Hipparchia maderensis*. Não foi detetado DNA de vertebrados nas amostras, mas observações de campo sugerem que eventos de predação podem ocorrer ocasionalmente.

Este estudo fornece a primeira referência molecular para a dieta de roedores na Madeira e evidencia impactos potenciais sobre espécies nativas e exóticas. Ao consumir plantas endémicas, dispersar espécies invasoras e predar comunidades de invertebrados, os roedores podem comprometer a recuperação dos habitats, especialmente no Parque Ecológico do Funchal, já fortemente afetado por incêndios. Estes resultados confirmam que os roedores invasores, através do consumo de recursos animais e vegetais, são responsáveis por alterações ecológicas e reforçam a necessidade de ações de conservação direcionadas.

Palavras-chave: Dieta, Madeira, Roedores invasores, *Rattus rattus*, *Mus musculus*, DNA metabarcoding

Chapter I - Introduction

Invasive species are among the major threats to global biodiversity and have caused important ecological impacts, including many cases of extinction (Bellard et al., 2016; Clavero et al., 2009; Doherty et al., 2016). Among them, non-native mammalian predators are particularly harmful and have been involved in more than half of the recorded vertebrate extinctions in the past five centuries (Doherty et al., 2016). On islands their effect is even stronger, being associated with around seventy five percent of all terrestrial vertebrate extinctions (McCreless et al., 2016).

Islands contain high numbers of endemic species that are the result of long evolutionary isolation (Kier et al., 2009). This isolation produced unique communities with limited genetic diversity and with adaptations shaped by the absence of mainland predators (Paulay, 1994; Whittaker & Fernández-Palacios, 2007). At the same time, it makes these ecosystems fragile and more vulnerable to invasive species, particularly mammals, which interfere with ecological processes through predation, competition, disease transmission and cascading effects across different levels of the ecosystem(Ceia et al., 2017; Drake & Hunt, 2009; Russell & Kueffer, 2019; Shiels & Drake, 2011).

Among invasive mammals, rodents such as *Rattus rattus*, *Rattus norvegicus* and *Mus musculus* are considered the most destructive. They are generalist feeders with broad dietary flexibility (Ceia et al., 2017; Gabrielson et al., 2024) and produce strong impacts on ecological processes, including seed predation and dispersal (Shiels & Drake, 2011). Their activity can also disrupt nutrient cycles, pollination networks and vegetation dynamics, and in some cases they may promote the spread of invasive plants by dispersing their seeds (Pejchar & Mooney, 2009).

Studies from the Hawaiian and Canary Islands using both traditional approaches and DNA metabarcoding have shown that invasive rodents feed on a wide range of resources, including seeds of native plants, arthropods and even toxic or chemically defended species, which demonstrates their ecological versatility and the challenges they pose to conservation (Gabrielson et al., 2024; Pomeda-Gutiérrez et al., 2021). In several island ecosystems *Rattus rattus* and *Mus musculus* also prey on seabird eggs, chicks and sometimes adults, leading to strong population declines and even local extinctions (Jones et al., 2008; Towns et al., 2006). For example, predation on ground nesting seabirds such as *Pterodroma madeira* has contributed to its critical conservation status (Spatz et al., 2017; Zino et al., 2001).

Understanding the trophic role of invasive rodents is essential for the development of management and conservation strategies(Ceia et al., 2017; Pomeda-Gutiérrez et al., 2021), and recent advances in molecular methods, particularly DNA metabarcoding, now allow diets to be studied with much higher resolution (Gabrielson et al., 2024; Shiels & Drake, 2011).

1.1 - Impact on forest ecosystems

Invasive rodents also have strong impacts on island flora through the consumption of seeds, seedlings and other vegetative parts, which prevents natural regeneration and changes the structure of vegetation communities, creating additional risks for endemic species and reducing biodiversity (Gabrielson et al., 2024; Pomeda-Gutiérrez et al., 2021; Shiels et al., 2013; Shiels & Drake, 2011). Many native island plants evolved without mammalian herbivores and therefore lack defensive traits to resist rodent predation (Traveset et al., 2009). By feeding on seeds and dispersing them, rodents can further promote the spread of exotic plants that compete with native flora for light, water and nutrients, thereby intensifying ecosystem degradation (Pomeda-Gutiérrez et al., 2021; Shiels et al., 2013). In this way, rodents contribute not only to the direct reduction of native plant populations, but also to wider changes in plant community composition and ecological functioning. While these effects are often negative, particularly in insular ecosystems, rodents may also act as seed dispersers for some plant species, including native taxa, depending on seed traits and local ecological context(Ceia et al., 2017; Gabrielson et al., 2024).

1.2 - Cascading Ecological Effects

The ecological consequences of invasive rodents extend well beyond direct herbivory and seed predation (Gabrielson et al., 2024; Shiels et al., 2013; Towns et al., 2006). As generalist consumers they exert top-down pressure across several trophic levels, disturbing food web dynamics and altering the balance of ecological interactions (Jones et al., 2008; Schuette et al., 2022). By preying on seeds, invertebrates and small vertebrates they reduce biodiversity and shift community structures, and these effects cascade through ecosystems by altering nutrient cycling (St. Clair, 2011), vegetation structure and species composition (Pomeda-Gutiérrez et al., 2021; Shiels & Drake, 2011).

Rodents may also disturb predator prey relationships and create feedback loops that weaken ecological networks (Schuette et al., 2022; Towns et al., 2006). A particularly important effect is the predation of pollinators such as insects and small birds, which disrupts pollination networks, reduces plant reproductive success and contributes to further biodiversity loss (Kaiser-Bunbury et al., 2010; Traveset et al., 2009). These examples illustrate the high level of interconnection of island ecosystems, where disturbances created by invasive rodents can spread rapidly across the system and amplify ecological decline (Gabrielson et al., 2024; Towns et al., 2006).

1.3 - Rodents in Madeira Island

Madeira Island is inhabited by three introduced rodent species: *Rattus rattus*, *Rattus norvegicus* and *Mus musculus*. Molecular analysis and radiocarbon dating of ancient bones suggest that the house mouse (*M. musculus*), the only representative of its genus on the island, was introduced before European colonisation, probably through early maritime activities about one thousand years ago (Rando et al., 2014). With the arrival of European settlers in the fifteenth century, black rats *(Rattus rattus*) were also brought to Madeira (Mathias, 1988;Rando et al., 2014), and in the following century Norway rats (*Rattus norvegicus*) became established, increasing the pressure on native ecosystems (Mathias, 1988; Rando et al., 2014). Despite their long history on the island, the diet of these invasive rodents remains poorly studied, and information on their impacts on Madeira's biodiversity is very limited (Nunes et al., 2010). In the Ecological Park of Funchal, Nunes et al. (2010) analysed the stomach contents of *Rattus rattus* and *Mus musculus* using morphological methods and found a generalist diet based on plants, fruits and invertebrates, with no signs of vertebrate predation. The present study builds on that work by applying DNA metabarcoding, which allows higher resolution and the detection of prey items that cannot be identified by morphology. Some of the best documented cases of rodent impact in Madeira include predation by *R. rattus* on chicks and eggs of the critically endangered Zino's petrel *Pterodroma madeira* (Spatz et al., 2017; Zino et al., 2001). Other observations also suggest threats to different endemic vertebrates. Oliveira & Menezes (1999) reported predation on the endemic Madeira pigeon *Columba trocaz*, and local evidence indicates that native gastropods may also be vulnerable to rodent predation (D. Teixeira, personal communication, 2023). These examples highlight the broad and often underestimated ecological footprint of invasive rodents in Madeira.

A similar situation is described in the Canary Islands, where invasive rodents are widespread and strongly affect native fauna. Black rats are the main predators of eggs of the endemic pigeons *Columba bollii* and *Columba junoniae* and have also restricted several seabird species to offshore islets. These impacts are comparable to those affecting Zino's petrel in Madeira and confirm that rodent predation is a recurrent conservation threat in Macaronesia (Nogales et al., 2006).

1.4 - Importance of Metabarcoding in Diet Studies

Knowing the diet of invasive species, especially generalist feeders like rodents, is important to understand their ecological role and impacts on native ecosystems (Gabrielson et al., 2024; Pomeda-Gutiérrez et al., 2021; Shiels & Drake, 2011). Traditional approaches, such as visual inspection of stomach or faecal samples, have been widely used but often give low taxonomic resolution. Soft bodied or highly digested prey are usually missed, which leads to underestimation of dietary diversity (Pompanon et al., 2012; Yoccoz, 2012).

DNA metabarcoding is a molecular method that uses high-throughput sequencing of short DNA fragments to identify prey in diet samples. The workflow includes extraction of DNA, PCR amplification with universal primers, sequencing, and comparison with reference databases to assign *taxa* (Pompanon et al., 2012; Mata et al., 2019). This technique increases the accuracy of diet studies because it can detect cryptic or morphologically indistinguishable prey. It also reduces observer bias, since identification is based on DNA sequences instead of morphological expertise (Pompanon et al., 2012; Mata et al., 2019).

Still, metabarcoding has some limitations. Reference databases are incomplete, and primer bias can affect the detection of some *taxa*. Contamination is a risk, and it is often not possible to separate primary from secondary predation. The method also does not provide information on prey biomass or quantities consumed (Pompanon et al., 2012; Yoccoz, 2012).

Even with these problems, metabarcoding has already revealed complex and previously hidden trophic interactions. It has become a powerful tool to inform conservation,

especially in islands where food webs are fragile and many species are endemic (Gabrielson et al., 2024; Ingala et al., 2021).

In this study, DNA metabarcoding was applied to faecal samples of Rattus *rattus* and *Mus musculus* to characterise their diet in the Ecological Park of Funchal, focusing on plant and invertebrate prey, which are expected to represent the main components of rodent diets in insular ecosystems.

1.5 - Broader Conservation Implications

Revealing trophic interactions using DNA metabarcoding can directly support conservation by improving our understanding of which species and ecological processes are most exposed to invasive predators. By identifying native and endemic taxa consumed by invasive rodents, this approach helps to highlight species that may be particularly vulnerable, even when direct predation events are rarely observed (Pompanon et al., 2012; Schuette et al., 2022). Such information can be used to prioritise conservation actions towards habitats or species of higher concern (Russell & Kueffer, 2019).

Metabarcoding can also inform management by identifying the key resources that sustain invasive rodent populations. Knowledge of dietary composition, including the relative importance of plants and invertebrates, may help managers understand why rodents persist in certain areas and guide the timing and spatial targeting of control actions (Ceia et al., 2017; Gabrielson et al., 2024). For example, control measures may be more effective when implemented during periods of reduced food availability, potentially limiting population recovery.

In addition, the detection of invasive plant species in rodent diets can indicate a potential role of rodents as seed dispersers, which has implications for habitat restoration and invasive plant management (Shiels & Drake, 2011; Traveset et al., 2009). This is particularly relevant in post-fire landscapes, such as the Ecological Park of Funchal, where regeneration dynamics are fragile and easily disrupted by biotic interactions (Fontinha et al., 2014).

Overall, DNA metabarcoding provides conservation practitioners with a high-resolution tool to detect trophic interactions that are difficult to observe using traditional methods

(Deagle et al., 2013; Pompanon et al., 2012). When combined with field observations and ecological monitoring, this information can support more targeted and evidence-based management decisions, helping to reduce cascading effects and to promote the long-term resilience of island ecosystems (Russell & Kueffer, 2019; Shiels et al., 2014).

1.6 - Aim of this study

In Madeira Island, *Rattus rattus* and *Mus musculus* occur sympatrically and are commonly found in the same habitats, including forested and disturbed areas, where they often exploit similar food resources. This spatial overlap makes them suitable models to compare dietary composition and potential ecological impacts within the same insular context.

This study investigates the diet and ecological effects of invasive rodents in the Ecological Park of Funchal, Madeira Island, using DNA metabarcoding of faecal contents. The main aim was to characterise the dietary composition of both species and to explore how their feeding habits may affect native biodiversity.

Specifically, we addressed the following research questions and hypotheses:

1. **What is the composition and diversity of the diet of *Rattus rattus* and *Mus musculus* in the study area?**
   We hypothesised that both species would show a generalist diet, consuming a wide range of plant and invertebrate taxa, including native and introduced species.
2. **Do diets differ between the two rodent species, and are there intraspecific differences (e.g., among individuals)?**
   We expected some degree of dietary overlap between species due to habitat sharing, but also differences in diet composition reflecting species-specific foraging strategies and potential niche partitioning.
3. **How do the detected diet items indicate potential ecological impacts on native biodiversity and ecosystem processes?**
   We hypothesised that the consumption of native and endemic taxa, particularly plants and invertebrates, could indicate potential impacts on ecosystem functioning and conservation-relevant species.

By addressing these questions, the study links rodent foraging behaviour with conservation challenges in insular ecosystems such as Madeira, providing baseline information to support future management and conservation actions.

Chapter II - Materials and Methods

2.1 - Study Area

This study was conducted on Madeira Island (Figure 1), a volcanic island in the northeastern Atlantic Ocean that forms part of the Macaronesian biogeographic region, which also includes the Azores, Canary Islands, Cape Verde and the Selvagens. The region is recognised for its high biodiversity and remarkable levels of endemism (Florencio et al., 2021). Madeira's climate is strongly influenced by the Azores anticyclone and by the rugged topography of the island, producing marked environmental gradients. The northern slopes are generally cooler and wetter, whereas the southern slopes are warmer and drier, with annual average temperatures ranging from about 8 °C in mountainous areas to 18–19 °C at lower elevations (Prada et al., 2009).

The Ecological Park of Funchal (Figure 2) is a municipal protected area of approximately 729 hectares located north of the city of Funchal. The park borders the municipalities of Santana to the north, Machico and Santa Cruz to the east and Câmara de Lobos to the northwest. Elevation ranges from 470 m at the confluence of the Pisão and Santa Luzia streams to 1,818 m at Pico do Areeiro (Barros, 1946), encompassing the Central Mountain Massif, which corresponds to the highest elevations of the park. This area is included in the Natura 2000 network and is designated as both a Special Protection Area (SPA) under the Birds Directive and a Special Area of Conservation (SAC PTMAD0002) under the Habitats Directive (European Commission, 2013; IFCN, 2012).

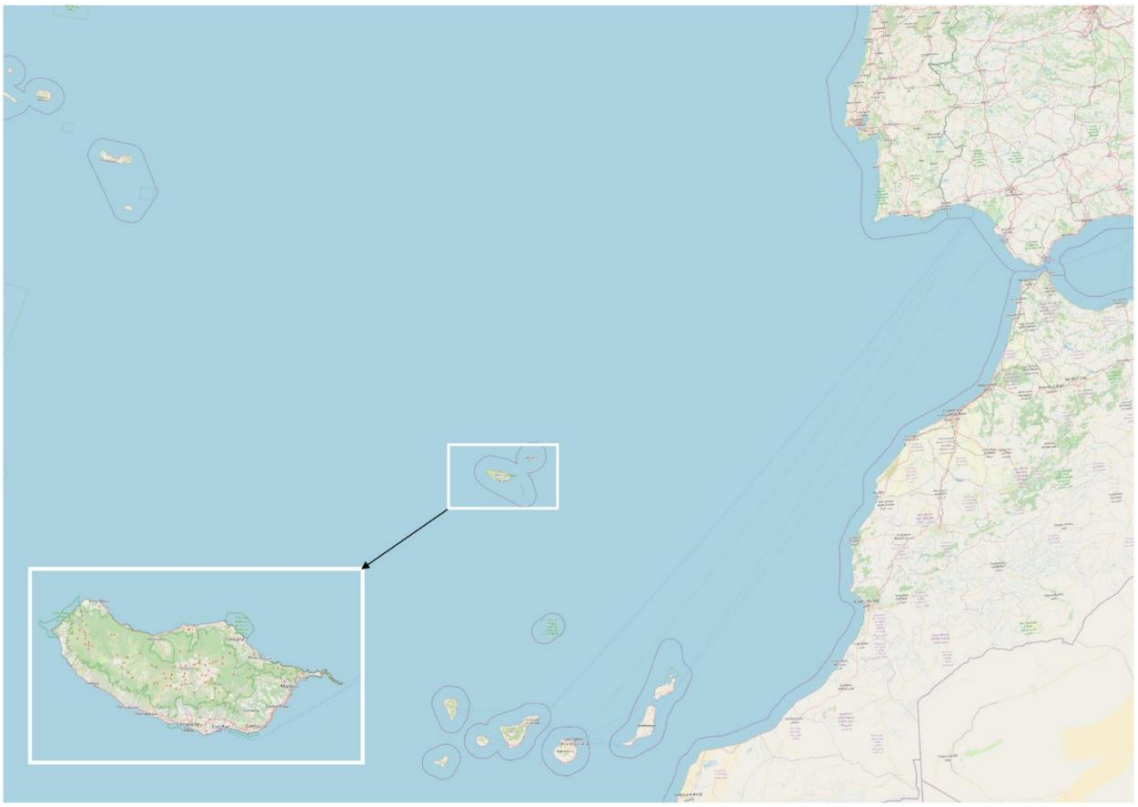

Figure 1. Location of Madeira Island in the northeastern Atlantic Ocean, within the Macaronesian biogeographic region.

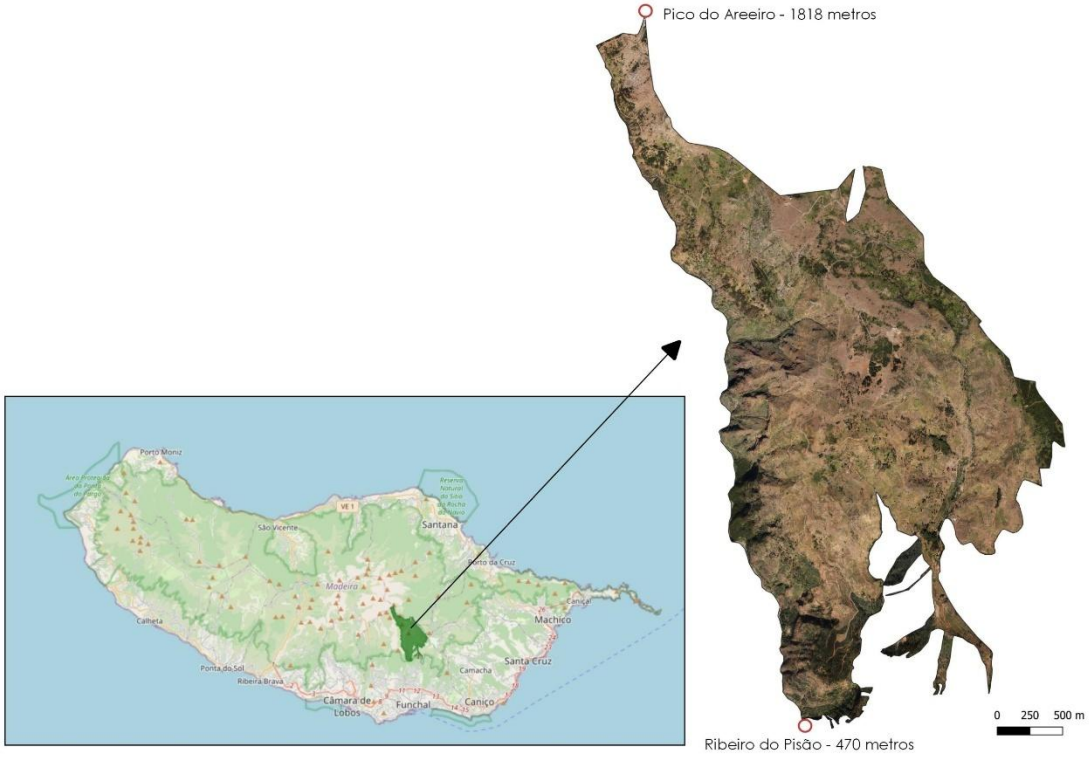

Figure 2. Location and boundaries of the Ecological Park of Funchal (PECOF), Madeira Island. The map shows the position of the park on Madeira and its extent (729 ha), located north of Funchal city, ranging from 470 m to 1,818 m in elevation.

2.2 - Flora and Vegetation

The flora of the Ecological Park of Funchal has historically included several endemic plant species, many of which have been negatively affected by habitat destruction, competition with invasive species and catastrophic wildfires, particularly those of 2010 and 2016 (Fontinha et al., 2014). One illustrative example is **Sorbus maderensis**, which is currently classified as Critically Endangered and has suffered severe population declines, with only a few individuals remaining (Fontinha et al., 2014). Madeira's flora includes about 1,200 vascular plant species, of which 154 are endemic (Borges et al., 2008). Post-fire surveys in the park identified 202 vascular plant taxa, including 52 endemic and 41 alien species, of which 23 were classified as invasive (Fontinha et al., 2014).

A number of habitat types occurring in the park are included in Annex I of the EU Habitats Directive. These comprise *endemic Macaronesian heaths* (4050*), *endemic forests with Juniperus spp.* (9560*), *Macaronesian mesophile grasslands* (6180), and *siliceous rocky slopes with chasmophytic vegetation* (8220), mainly associated with the high altitude areas of the Central Mountain Massif. In the Macaronesian biogeographic region, some of these habitats have been assessed as having an unfavourable conservation status (European Commission, 2013; IFCN, 2012).

Vegetation is distributed along an altitudinal gradient shaped by climatic, topographic and ecological factors (Capelo et al., 2005; Fontinha et al., 2014). These gradient preserves remnant of Madeira's original vegetation, which are essential habitats for biodiversity and for the stability of ecological processes (Borges et al., 2008; Capelo et al., 2005). Between 470 and 1,000 m the dry laurel forest, part of the *Semele androgynae–Apollonietum barbujanae* series, is characterised by species such as barbusano (*Apollonias barbujana*), Canary laurel (*Laurus novocanariensis*) and Madeira wax myrtle (*Myrica faya*). These forests contribute to soil stability and biodiversity maintenance under drier environmental conditions (Capelo et al., 2004).

The endemic flora of the park includes several species with high conservation concern, such as *Berberis maderensis*, *Erica maderensis*, *Monizia edulis*, *Sorbus maderensis* and *Taxus baccata* (Critically Endangered), *Juniperus maderensis* and *Musschia wollastonii* (Endangered), and *Heberdenia excelsa* (Vulnerable). In total, eight taxa present

threatened status, emphasising the ecological and conservation importance of these habitats.

Historical reforestation programmes introduced species such as maritime pine (*Pinus pinaster*), eucalyptus (*Eucalyptus globulus*), acacias (*Acacia dealbata* and *Acacia melanoxylon*) and holm oak (*Quercus ilex*). Although originally planted to prevent erosion and to supply timber, these species became problematic and today represent challenges related to invasive plant dynamics (Fontinha et al., 2014).

2.3 - Fauna

The Ecological Park of Funchal supports diverse ecosystems and is home to several native vertebrates, including the Madeira wall lizard *Teira dugesii* and three bat species: *Nyctalus leisleri verrucosus* (**Near Threatened**), *Plecotus austriacus* (**Near Threatened**) and *Pipistrellus maderensis* (**Vulnerable**) (Gonçalves et al., 2024). Birds are the most representative group of vertebrates on Madeira and within the park species such as the Madeiran firecrest *Regulus madeirensis* and the recently recognised Madeira chaffinch *Fringilla maderensis* are common residents. The park also provides habitat for the Madeira pigeon *Columba trocaz* and for the sparrowhawk *Accipiter nisus granti*, a subspecies restricted to Madeira and the Canary Islands (Fagundes et al., 2008).

The invertebrate fauna is also diverse, including the Madeiran speckled wood *Pararge xiphia* (**Endangered**), commonly observed in the park (Dawson et al., 2001). Of the 57 arthropod species recorded, 13 are diurnal butterflies, including 4 *taxa* with threatened status, and in total 14 arthropod species are considered endemic (Silva et al., unpublished data).

Invasive mammals are also present and include *Rattus rattus*, *Rattus norvegicus*, *Mus musculus*, the European rabbit *Oryctolagus cuniculus*, the ferret *Mustela furo* and the feral cat *Felis catus*. These species contribute to the decline of native vertebrates through predation and through indirect effects such as competition and disease transmission, thereby intensifying pressures on the ecosystems of the park (Galão et al., 2025; Soto et al., 2024).

In total, four species recorded in the Ecological Park of Funchal are currently listed under threatened categories of the IUCN Red List (IUCN, 2025), including one classified as

Endangered (*Pararge xiphia*), one Vulnerable (*Pipistrellus maderensis*) and two Near Threatened (*Nyctalus leisleri verrucosus* and *Plecotus austriacus*).

2.4 - Sampling Collection

Rodents were captured in five trapping lines distributed along the altitudinal gradient of the Ecological Park of Funchal (Figure 3). The location of the traps was initially based on predator control plan implemented within the Puffinus conservation project, aimed at protecting the Manx shearwater colony in the park. However, the distribution of each trap line was adjusted to cover a broader area and to include higher altitudes, where control actions are usually conducted only sporadically. Fieldwork was carried out between January and November 2023 as part of the park's rodent control actions, and extended to new areas to ensure full altitudinal coverage. Each line contained fifteen automatic A24 traps baited with peanut butter and spaced at intervals of 80 m. The traps were generally active for three consecutive nights, although in some cases they remained for up to five nights to increase sample numbers. They were checked daily to record captures, counter activity, bait condition and CO_2 consumption. Captured individuals were frozen at −20 °C until processing in the laboratory. Each specimen received an identification number and was identified to species, with sex determined from reproductive organs following Cunningham and Moors (1993). Biometric data included body weight, total length, tail length, hind leg length and ear length. For diet analysis, a portion of the faecal contents was preserved in 96% ethanol.

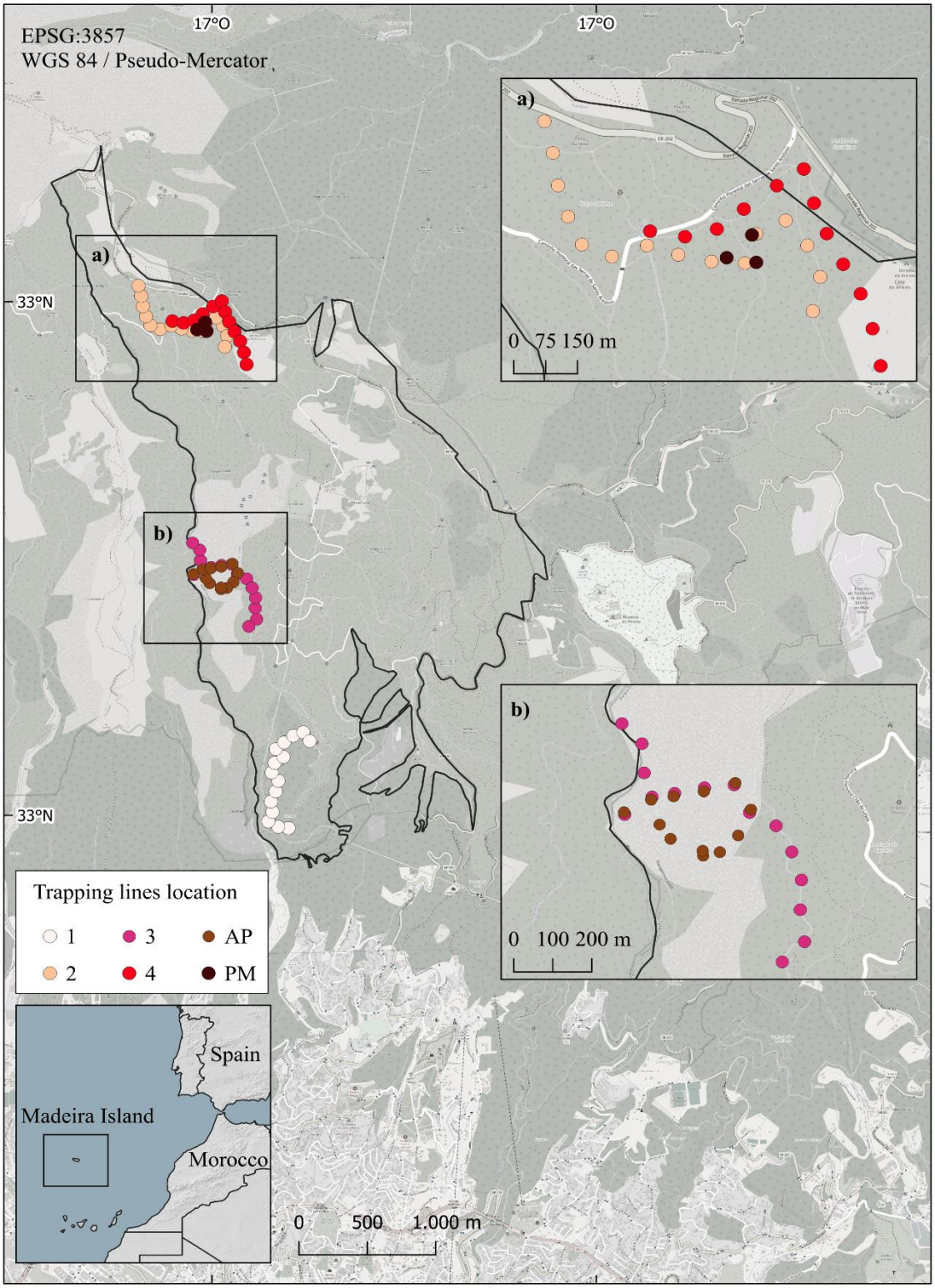

Figure 3. Location of the four trapping lines in the Ecological Park of Funchal (PECOF), Madeira Island. Each dot represents a Goodnature A24 trap. Panels (a and b) show the detailed arrangement of traps in the northern and southern sections of the park.

2.5 - Laboratory Analysis

DNA extraction was performed using the Norgen Stool Kit (Norgen Biotek), following the manufacturer's instructions. In total, 57 faecal samples were collected in the field. Of these, 56 samples generated sequencing data and entered the metabarcoding pipeline, while one sample did not yield sufficient molecular data for downstream processing. Subsequent quality filtering and bioinformatic curation were applied prior to statistical analyses.

A single pellet was collected from the large intestine of each individual. This gut section was selected to standardise sampling across individuals and because intestinal and faecal material is commonly used in diet metabarcoding studies, as it integrates digested food items over time and reduces bias associated with the most recent feeding event (Pompanon et al., 2012; Pinho et al., 2022). In contrast, stomach contents may reflect only the last meal and can be influenced by bait consumption during trapping. Nevertheless, diet composition may vary among gut sections, and this methodological choice can influence the detectability of some taxa (Pinho et al., 2022).

The diet of rats and mice was analysed using two primer sets: FwhF1/FwhR1 (Vamos et al., 2017), targeting a short fragment (~178 bp) of the mitochondrial cytochrome oxidase subunit I (COI) region of animals, and Uniplant-F/Uniplant-R (Moorhouse-Gann et al., 2018), targeting the internal transcribed spacer 2 (ITS2) region of plants. This primer combination was selected to provide a general overview of rodent diet in the study area, focusing on the dietary components expected to be most frequently consumed and most reliably detected in faecal samples, namely plants and invertebrates (Pompanon et al., 2012; Gabrielson et al., 2024). Vertebrates were not specifically targeted using a dedicated vertebrate marker; therefore, the absence of vertebrate detections should be interpreted cautiously and does not exclude occasional predation events.

Both primer pairs were modified to include Illumina overhang adapters. PCR amplifications were carried out in 10 μL reactions containing 5 μL of Qiagen Multiplex Master Mix, 0.25 μL of each 10 nM primer for Fwh1 or 0.3 μL for Uniplant, 2 μL of DNA extract, and nuclease-free water to the final volume. To minimise amplification of predator (rodent) mitochondrial DNA and to increase sequencing depth for prey taxa, species-specific blocking primers were included in all Fwh1 reactions (Vamos et al., 2017). The use of blocking primers is recommended in diet metabarcoding studies

because host DNA is typically abundant in gut-derived samples and can otherwise dominate PCR products, even when primers are optimised for invertebrate detection (Pompanon et al., 2012; Pinho et al., 2022).

DNA extracts were processed in batches of 23 samples, each including a negative extraction control in which no biological material was added. In addition, a negative PCR control (no DNA template) was included in all amplification runs. Thermal cycling conditions consisted of an initial denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, annealing at 50 °C for Fwh1 or 56 °C for Uniplant for 30 s, and extension at 72 °C for 30 s, with a final extension step of 10 min at 72 °C.

PCR products obtained with the Fwh1 primer set were purified using Agencourt AMPure XP beads (Beckman Coulter) at a 1:0.97 bead-to-sample ratio, whereas Uniplant amplicons were diluted 1:4 prior to indexing. A second PCR was then performed to attach 7 bp dual indices and Illumina P5/P7 adapters using KAPA HiFi HotStart ReadyMix (Roche), under similar cycling conditions but with only eight amplification cycles and an annealing temperature of 55 °C. Indexed products were purified using AMPure XP beads at a 1:0.8 ratio for both primer sets.

Purified libraries were quantified spectrophotometrically, normalised to 15 nM, pooled, and quantified by quantitative PCR using the KAPA Library Quantification Kit (Roche). Sequencing was carried out on an Illumina MiSeq platform using a 500-cycle kit, together with other libraries, targeting approximately 30,000 paired-end reads for Fwh1 libraries and 20,000 paired-end reads for Uniplant libraries.

2.6 - Bioinformatic Analysis

Bioinformatic processing of raw sequencing data was done using a series of commonly used tools. First, primer sequences were removed with cutadapt (Martin, 2011), followed by alignment of paired-end reads with flash2 (Magoč and Salzberg, 2011). Sequences were then filtered based on reading quality using VSEARCH (Rognes et al, 2016) with the function '--fastx_filter' and a '--fastq_maxee' of 1. The function '--derep_fulllength' was then used to dereplicate sequences into exact sequence variants (ESV), while removing singletons per sample. ESVs of each sample were then merged and further dereplicated across the entire dataset for each primer set. Potential PCR and sequencing artifacts, as well as sequences outside the expected length range (fwh1: 170-206bp;

uniplant: 187-387bp), were filtered out using '--cluster_unoise', with chimeric sequences removed via '--uchime3_denovo'. Clustering was performed at 99% similarity using '--cluster_size', and dereplicated reads without singletons were mapped back to retained Operational Taxonomic Units (OTUs) using '--usearch_global' at 99% identity to build an OTU table.

To further remove PCR and sequencing artifacts and possible nuclear copies of the targeted regions, the R package LULU (Frøslev et al., 2017) was applied with default options on the produced OTU table. For taxonomic assignment, animal COI OTUs were identified using BOLDigger3 (Buchner and Leese, 2020) with custom thresholds (99% for species, 97% for genus, 95% for family, and 90% for order), and plant ITS2 OTUs were blasted against NCBI and classified using the 'metabin' function from metabinkit (Egeter et al., 2022), with similarity thresholds equal to the ones used for COI. All OTU identifications were manually reviewed to improve taxonomic accuracy, considering known geographic distributions and potential database errors. To address potential contamination issues, the read counts of each OTU detected in extraction and PCR negative controls were subtracted from the corresponding samples. Based on identity, OTUs were categorized as 'target' (e.g., most arthropods, snails, vertebrates, and land plants) or 'non-target' (e.g., fungi, parasites). Finally, we removed target OTUs comprising less than 1% of the total target reads per sample (Drake et al., 2022). Samples with fewer than 100 target reads were considered to have failed and were excluded.

2.7 - Statistical Analysis

All statistical analyses were performed in RStudio (v.1.4.1106, RStudio PBC). Differences in dietary richness, diversity, and composition were examined between predator species (*Mus musculus* and *Rattus rattus*), as well as between sexes.

Dietary richness was defined as the number of unique prey *taxa* detected per faecal sample. To evaluate dietary niche width, incidence-based rarefaction and extrapolation curves based on Hill numbers (q = 0) were generated with the package iNEXT (Hsieh et al., 2016). Differences were considered relevant when 95% confidence intervals of the curves did not overlap.

To provide a standardized measure of prey importance, we calculated the frequency of occurrence (FO) for each taxon, expressed as the proportion of faecal samples in which

the taxon was detected. This metric was used to compare the relative importance of dietary categories and to facilitate comparisons with other studies on invasive rodents and carnivores using DNA metabarcoding of faeces (e.g., Pomeda-Gutiérrez et al., 2021; Gabrielson et al., 2024; Galão et al., 2025).

Comparisons of plant versus animal prey were conducted using Wilcoxon tests: the rank-sum test for interspecific comparisons and the signed-rank test for intraspecific contrasts.

For dietary composition, read counts were transformed into a presence/absence matrix. Distances between samples were calculated using the Jaccard index with the function vegdist from the package vegan (Dixon, 2003). Differences in composition were tested using a PERMANOVA (adonis function, *vegan*), with permutations constrained within trapping lines to account for spatial non-independence. The assumption of homogeneity of multivariate dispersion was checked with betadisper.

To visualise patterns of dietary composition and support the interpretation of multivariate analyses, a non-metric multidimensional scaling (NMDS) ordination was performed using the Jaccard dissimilarity matrix and the function *metaMDS* from the package vegan. Ordinations were run with random starts to ensure convergence on a stable solution, and stress values were used to assess the goodness of fit of the ordination.

To further explore which prey *taxa* contributed most to interspecific dissimilarities, a SIMPER analysis (*simper* function, vegan) was performed, based on Jaccard

Chapter III - Results

3.1 - Distribution of rodent captures across trapping lines

The number of rodents captured in each trapping line and species is shown in Figure 4. Captures were not evenly distributed across the study area, with Line 1 (L1) and Line 4 (L4) producing the highest numbers, while Lines 2 (L2), 3 (L3), AP, and PM registered fewer individuals.

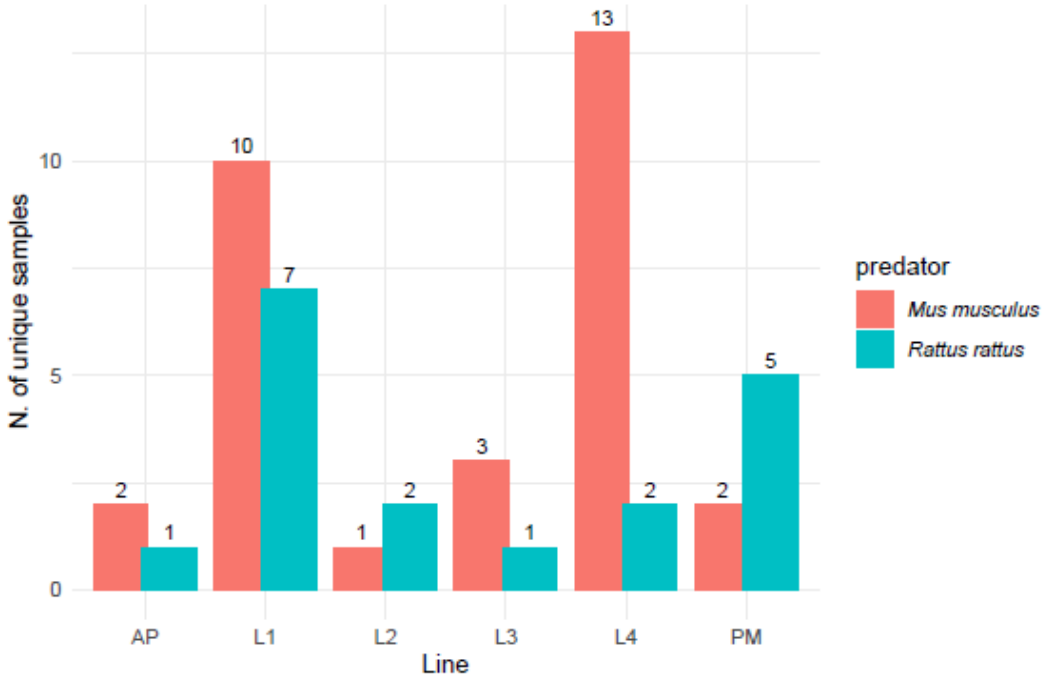

Figure 4. Number of rodents captured per trapping line, separated by species (*Mus musculus* and *Rattus rattus*).

3.2 - Number of samples by predator species and sex

The distribution of samples by species and sex is summarised in Table 1. Sample sizes were unbalanced between species and sexes, reflecting differences in capture success across trapping lines. For *Mus musculus*, 31 samples were obtained, including 19 from females, 10 from males, and 2 from individuals of undetermined sex. For *Rattus rattus*, 18 samples were analysed, of which 14 corresponded to males and 4 to females. This imbalance was addressed by using incidence-based metrics (e.g., frequency of occurrence and rarefaction), non-parametric tests, and permutation-based multivariate analyses, which are robust to unequal sample sizes.

Table 1. Number of unique samples per predator species and sex.

Predator	**Sex**	**n**
Mus musculus	F	19
Mus musculus	M	10
Mus musculus	NA	2
Rattus rattus	M	14
Rattus rattus	F	4
Total		49

3.3 - Overall diet description

After bioinformatic filtering and quality control, a total of 49 unique faecal samples were retained for analysis, corresponding to 31 *Mus musculus* and 18 *Rattus rattus*. Across all samples, 78 prey species were identified, belonging to 78 genera and 60 families. The diet included both plant and animal taxa, and there was considerable dietary overlap between the two rodent species.

Detected arthropod taxa included *Acalles oblitus*, *Calliptamus madeirae*, *Hipparchia maderensis*, *Ctenoplusia limbirena*, *Crocidosema plebejana*, *Piezodorus lituratus*, *Antaxius spinibrachius*, *Calliphora vicina* and *C. vomitoria*. The complete list of detected taxa and their occurrence within the Ecological Park of Funchal (PECOF) is provided in Table 2.

Table 2. List of animal OTUs identified in the diet of *Mus musculus* and *Rattus rattus*, including information on their presence in Madeira and colonisation status according to Borges et al. (2008) and unpublished records from the Ecological Park of Funchal (PECOF). Species are presented by biogeographic status: endemics (END), Macaronesian endemics (MAC), native (NAT), introduced (INT), and uncertain status ("–"). Taxa marked with (*) do not have confirmed records for Madeira and are reported based on molecular assignment only. These detections should be interpreted cautiously and do not imply confirmed new occurrences, reflecting incomplete taxonomic and molecular coverage in regional reference databases.

OTU	Scientific ID	Present in Madeira	Present in PECOF	Status
OTU410	*Acalles oblitus* Wollaston, 1854	Yes	No	END
OTU58	*Agonopterix scopariella* (Heinemann, 1870)	Yes	No	—
OTU28	*Antaxius spinibrachius* (Fischer, 1853)	Yes	Yes	INT
OTU21	*Arion intermedius* Normand, 1852	Yes	No	—
OTU10	*Calliphora vicina* Robineau-Desvoidy, 1830	Yes	Yes	INT
OTU51	*Calliphora vomitoria* (Linnaeus, 1758)	Yes	Yes	INT

OTU	Scientific ID	Present in Madeira	Present in PECOF	Status
OTU253	*Calliptamus madeirae* Uvarov, 1937	Yes	Yes	END
OTU83	*Crocidosema plebejana* Zeller, 1847	Yes	No	NAT
OTU78	*Cryptops hortensis* (Donovan, 1810)	Yes	No	INT
OTU116	*Ctenoplusia limbirena* (Gueneé, 1852)	Yes	Yes	NAT
OTU104	*Dendrobaena octaedra* (Savigny, 1826)	No	No	*
OTU39	*Deroceras invadens* Reise et al., 2011	Yes	No	INT
OTU161	*Deroceras reticulatum* (Müller, 1774)	Yes	No	—
OTU32	*Drosophila suzukii* (Matsumura, 1931)	Yes	Yes	INT
OTU149,172	*Dysdera crocata* C.L. Koch, 1838	Yes	No	—
OTU9	*Eluma caelatum* (Miers, 1877)	Yes	No	—
OTU393	*Forficula auricularia* Linnaeus, 1758	Yes	No	INT
OTU272	*Hipparchia maderensis* (Bethune-Baker, 1891)	Yes	Yes	END
OTU389	*Holotrichia parallela* (Motschulsky, 1854)	Yes	No	—
OTU164	*Lasius grandis* Forel, 1909	Yes	No	NAT
OTU74	*Lehmannia nyctelia* (Bourguignat, 1861)	No	No	*
OTU91	*Lithobius pilicornis* Newport, 1844	Yes	No	INT
OTU50	*Lumbricus castaneus* (Savigny, 1826)	No	No	—
OTU20	*Lumbricus terrestris* Linnaeus, 1758	No	No	*

OTU	Scientific ID	Present in Madeira	Present in PECOF	Status
OTU228	*Milax gagates* (Draparnaud, 1801)	Yes	No	—
OTU513	*Octolasion tyrtaeum* (Savigny, 1826)	No	No	*
OTU3	*Ommatoiulus moreletii* (Lucas, 1860)	Yes	No	INT
OTU89	*Oxychilus alliarius* (Miller, 1822)	Yes	No	—
OTU15	*Oxychilus cellarius* (Müller, 1774)	Yes	No	INT
OTU34	*Piezodorus lituratus* (Fabricius, 1794)	Yes	No	NAT
OTU644	*Scolopostethus affinis* (Schilling, 1829)	Yes	No	—
OTU135	*Tegenaria pagana* (C.L. Koch, 1840)	Yes	No	—
OTU147	*Tenuiphantes tenuis* (Blackwall, 1852)	Yes	No	—
OTU592	*Thrips tabaci* Lindeman, 1889	Yes	No	NAT
OTU8	*Xanthorhoe rupicola* (Wollaston, 1858)	Yes	No	END

Detected plant taxa included *Erica platycodon*, *Melanoselinum decipiens*, *Vaccinium padifolium*, *Phyllis nobla*, *Rubus bollei*, *Tolpis succulenta*, *Erica arborea*, *Geranium robertianum*, *Galactites tomentosa*, *Morella faya*, *Sonchus oleraceus*, *Umbilicus rupestris*, *Acacia longifolia*, *Acacia melanoxylon*, *Ageratina adenophora*, *Eucalyptus globulus*, *Quercus ilex*, and *Zea mays*. Presence in the PECOF area is documented for several taxa, including *Erica arborea*, *Erica platycodon*, *Acacia melanoxylon*, and *Ageratina adenophora*. In terms of biogeographic status, 22.6% of taxa were endemic, 5.5% Macaronesian endemics, 34.8% introduced, 3.7% native non-endemic, and 33.2% of unknown origin (Table 3).

Table 3. List of plant OTUs identified in the diet of *Mus musculus* and *Rattus rattus*, including information on their presence in Madeira and colonisation status according to (Borges et al., 2008; Press & Short, 1994; Fontinha et al., 2014) and unpublished records from the Ecological Park of Funchal (PECOF). Species are presented by biogeographic status: endemics (END), Macaronesian endemics (MAC), native (NAT), introduced (INT), and species with uncertain status ("-").

OTU	Scientific ID	Present in Madeira	Present in PECOF	Status
OTU25	*Acacia longifolia* (Andrews) Willd.	Yes	Yes	INT
OTU27	*Acacia melanoxylon* R. Br.	Yes	Yes	INT
OTU18	*Ageratina adenophora* (Spreng.) R.M. King & H. Rob.	Yes	Yes	INT
OTU13	*Agrostis castellana* Boiss. & Reut.	Yes	Yes	NAT
OTU4	*Andryala integrifolia* L.	Yes	No	INT
OTU36	*Bidens pilosa* L.	Yes	Yes	INT
OTU169	*Brassica juncea* (L.) Czern.	No	No	INT
OTU5	*Briza maxima* L.	Yes	No	NAT
OTU150	*Bromus diandrus* Roth	Yes	No	NAT
OTU52	*Cerastium fontanum* subsp. *vulgare* (Hartm.) Greuter & Burde	Yes	No	NAT
OTU12	*Cerastium glomeratum* Thuill.	Yes	No	NAT
OTU138	*Citrus sinensis* (L.) Osbeck	Yes	No	INT
OTU19	*Corylus avellana* L.	Yes	Yes	INT
OTU50	*Dactylis glomerata* L.	Yes	No	—
OTU31,68	*Echium plantagineum* L.	Yes	No	NAT
OTU17	*Erica arborea* L.	Yes	Yes	NAT

OTU	Scientific ID	Present in Madeira	Present in PECOF	Status
OTU44	*Erica platycodon* subsp. *maderincola*	Yes	Yes	END
OTU70	*Eucalyptus globulus* Labill.	Yes	Yes	INT
OTU33,38	*Galactites tomentosa* Moench	Yes	Yes	NAT
OTU9	*Geranium robertianum* L.	Yes	Yes	NAT
OTU10	*Holcus lanatus* subsp. *lanatus* L.	Yes	No	NAT
OTU1	*Hypochaeris radicata* L.	Yes	No	NAT
OTU51	*Leontodon taraxacoides*	Yes	Yes	NAT
OTU46	*Melanoselinum decipiens* (Schrad. & J.C. Wendl.) Hoffm.	Yes	Yes	END
OTU37	*Mercurialis annua* L.	Yes	No	NAT
OTU22	*Morella faya* Aiton	Yes	Yes	NAT
OTU40	*Orobanche minor* Sm.	Yes	No	NAT
OTU14	*Phyllis nobla* L.	Yes	Yes	MAC
OTU82	*Pittosporum undulatum* Vent.	Yes	Yes	INT
OTU8	*Plantago lancelota* L.	Yes	Yes	NAT
OTU81	*Polygonum capitatum* Buch.-Ham. Ex D.Don	Yes	No	INT
OTU92	*Quercus ilex* L. subsp. *ballota* (Desf.) Samp.	Yes	Yes	INT
OTU15	*Rubus bollei* Focke	Yes	Yes	MAC
OTU2	*Rubus ulmifolius* Schott	Yes	Yes	NAT
OTU53	*Rumex acetosella* subsp. *angiocarpus* (Murb.) Murb.	Yes	No	NAT

OTU	Scientific ID	Present in Madeira	Present in PECOF	Status
OTU20	*Silene gallica* L.	Yes	No	NAT
OTU11	*Sonchus oleraceus* L.	Yes	Yes	NAT
OTU21	*Taraxacum officinale* Weber agg.	Yes	No	NAT
OTU16,3	*Tolpis succulenta* (Dryand. in Aiton) Lowe	Yes	Yes	MAC
OTU75	*Torilis arvensis* (Huds.) Link	Yes	No	NAT
OTU28	*Umbilicus rupestris* (Salisb.) Dandy	Yes	Yes	NAT
OTU62	*Vaccinium padifolium* Sm.	Yes	Yes	END
OTU216	*Zea mays* L.	Yes	No	INT

3.4 - Proportion of animal-based dietary items

The proportion of animal-based dietary items was compared between *Mus musculus* and *Rattus rattus* using the relative contribution of animal items per sample (animals / [animals + plants]). The boxplot in figure 5 illustrates the distribution of this proportion for both predator species.

No statistically significant differences were detected between *Mus musculus* and *Rattus rattus* in the proportion of animal-based dietary items consumed (Wilcoxon rank-sum test, $W = 182.5$, $p = 0.1429$), despite a slightly higher median value observed for *R. rattus*. Considerable overlap between species indicates high inter-individual variability.

At the intraspecific level, *Mus musculus* consumed significantly more plant than animal items (Wilcoxon signed-rank test, $V = 63.5$, $p = 0.00145$). In contrast, no significant difference between plant and animal consumption was detected for *Rattus rattus* (Wilcoxon signed-rank test, $V = 45.5$, $p = 0.6816$).

On average, *M. musculus* consumed 2.23 ± 1.61 animal items and 4.00 ± 2.03 plant items per sample ($n = 31$), whereas *R. rattus* consumed 3.38 ± 1.71 animal items and 3.69 ± 2.09 plant items per sample ($n = 16$).

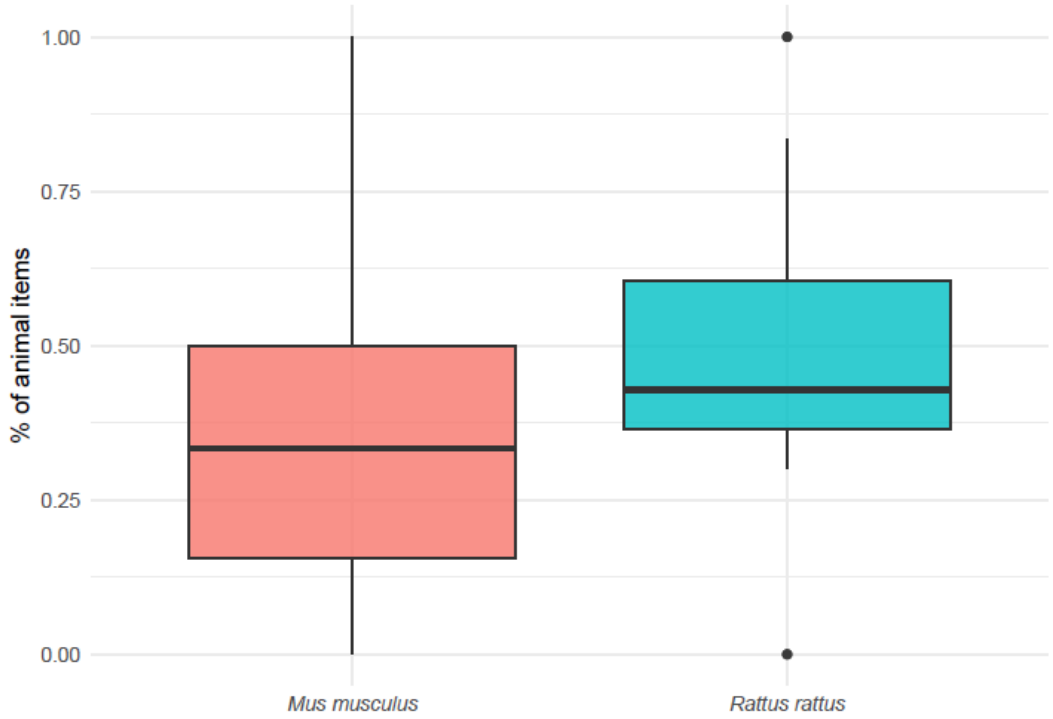

Figure 5. Proportion of animal-based dietary items per sample for Mus musculus and Rattus rattus. Boxes represent the interquartile range, the horizontal line indicates the median, and whiskers show the range of values.

3.5 - Diet of *Mus musculus*

In *Mus musculus*, a total of 61 prey taxa were identified, belonging to 64 genera and 51 families. Prey taxa were reported at the lowest reliable taxonomic level, including species, genus, and family level assignments. The number of prey taxa per faecal sample ranged from 1 to 12.

The diet was dominated by Diplopoda, with *Ommatoiulus moreleti* detected in 55% of the samples. Isopoda was the second most frequent group, represented by *Eluma caelatum* in 23% of the samples. Orthoptera were also detected, mainly *Antaxius spinibrachius* (9.7%). Among Lepidoptera, *Agonopterix scopariella* and *Xanthorhoe rupicola* were each present in 6.5% of the samples. Other taxa occurred at lower frequencies (3.2%), including representatives of Coleoptera, Diptera, Hemiptera, and Arachnida (Table 4).

Table 4. Frequency of occurrence (FO, %) of prey *taxa* identified in the diet of *Mus musculus* (n = 31) using the FWH1 marker.

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Arachnida	Araneae	Dysderidae	otu149,172	*Dysdera crocata* C.L. Koch, 1838	3,2
		Linyphiidae	otu147	*Tenuiphantes tenuis* (Blackwall, 1852)	3,2
Chilopoda	Lithobiomorpha	Lithobiidae	otu91	*Lithobius pilicornis* Newport, 1844	3,2
	Scolopendromorpha	Cryptopidae	otu78	*Cryptops hortensis* (Donovan, 1810)	6,5
Clitellata	Crassiclitellata	Lumbricidae	otu104	*Dendrobaena octaedra* (Savigny, 1826)	6,5
			otu50	*Lumbricus castaneus* (Savigny, 1826)	3,2
			otu20	*Lumbricus terrestris* Linnaeus, 1758	6,5
Diplopoda	Julida	Julidae	otu3	*Ommatoiulus moreletii* (Lucas, 1860)	54,8
Gastropoda	Stylommatophora	Arionidae	otu21	*Arion intermedius* Normand, 1852	3,2
		Milacidae	otu228	*Milax gagates* (Draparnaud, 1801)	3,2
		Oxychilidae	otu89	*Oxychilus alliarius* (Miller, 1822)	3,2
		Oxychilidae	otu15	*Oxychilus cellarius* (Muller, 1774)	3,2

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Insecta	Coleoptera	Curculionidae	otu410	*Acalles oblitus* Wollaston, 1854	3,2
		Scarabaeidae	otu389	*Holotrichia parallela* (Motschulsky, 1854)	3,2
	Diptera	Calliphoridae	otu10	*Calliphora vicina* Robineau-Desvoidy, 1830	6,5
		Drosophilidae	otu32	*Drosophila suzukii* (Matsumura, 1931)	3,2
	Hemiptera	Pentatomidae	otu34	*Piezodorus lituratus* (Fabricius, 1794)	3,2
		Rhyparochromidae	otu644	*Scolopostethus affinis* (Schilling, 1829)	3,2
	Hymenoptera	Formicidae	otu164	*Lasius grandis* Forel, 1909	3,2
	Lepidoptera	Depressariidae	otu58	*Agonopterix scopariella* (Heinemann, 1870)	6,5
		Geometridae	otu8	*Xanthorhoe rupicola* (Wollaston, 1858)	6,5
		Noctuidae	otu116	*Ctenoplusia limbirena* (Gueneé, 1852)	3,2
		Nymphalidae	otu272	*Hipparchia maderensis* (Bethune-Baker, 1891)	3,2
	Lepidoptera	Tortricidae	otu83	*Crocidosema plebejana* Zeller, 1847	3,2
	Orthoptera	Tettigoniidae	otu28	*Antaxius spinibrachius* (Fischer, 1853)	9,7

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Insecta	Thysanoptera	Thripidae	otu592	*Thrips tabaci* Lindeman, 1889	3,2
Malacostraca	Isopoda	Armadillidiidae	otu9	*Eluma caelatum* (Miers, 1877)	22,6

The plant component of the diet (Table 5) was dominated by Poales and Asterales. Within Poaceae, *Briza maxima* (38.7%) and *Agrostis castellana* (29.0%) showed the highest frequencies. Among Asteraceae, *Hypochaeris radicata* (35.5%) and *Tolpis succulenta* (25.8%) were the most frequent taxa. Other plant groups also contributed, such as Plantaginaceae (*Plantago lanceolata*, 19.4%), Rosaceae (*Rubus bollei* and *Rubus ulmifolius*, 19.4% and 16.1%, respectively), and Rubiaceae (*Phyllis nobla*, 16.1%). Additional records included Ericaceae (*Erica arborea*, 12.9%), Myricaceae (*Morella faya*, 12.9%), and Fabaceae (*Acacia longifolia*, 3.2%). The dataset also included Macaronesian endemics (*Tolpis succulenta*, *Phyllis nobla*), Madeiran endemics (*Rubus bollei*), native species (*Erica arborea*, *Geranium robertianum*), and introduced taxa (*Acacia longifolia*, *Eucalyptus globulus*).

Table 5. Frequency of occurrence (FO, %) of plant *taxa* identified in the diet of *Mus musculus* (n = 31) using the ITS2 marker.

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Magnoliopsida	Apiales	Apiaceae	otu75	*Torilis arvensis* (Huds.) Link	3,2
	Asterales	Asteraceae	otu18	*Ageratina adenophora* (Spreng.) R.M. King & H. Rob.	12,9
			otu4	*Andryala integrifolia* L.	3,2
			otu36	*Bidens pilosa* L.	3,2
			otu33, 38	*Galactites tomentosa* Moench	3,2
			otu1	*Hypochaeris radicata* L.	35,5
			otu51	*Leontodon taraxacoides* (Vill.) Mérat subsp. *longirostris* Finch & P.D. Sell	3,2
			otu11	*Sonchus oleraceus* L.	6,5
			otu16, 3	*Tolpis succulenta* (Dryand. in Aiton) Lowe	25,8
	Boraginales	Boraginaceae	otu31, 68	*Echium plantagineum* L.	6,5
	Brassicales	Brassicaceae	otu169	*Brassica juncea* (L.) Czern.	3,2
	Caryophyllales	Caryophyllaceae	otu12	*Cerastium glomeratum* Thuill.	6,5

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Magnoliopsida	Caryophyllales	Polygonaceae	otu81	*Polygonum capitatum* Buch.-Ham. ex D.Don	3,2
			otu53	*Rumex acetosella* L. *subsp. angiocarpus* (Murb.) Murb.	3,2
	Ericales	Ericaceae	otu17	*Erica arborea* L.	12,9
			otu44	*Erica platycodon* (Webb & Berthel.) Rivas Mart., Wildpret, del Arco, O. Rodr., P. Pérez, García Gallo, Acebes, T.E. Díaz & Fern. Gonz. subsp. Maderincola (D.C. McClint.) Rivas Mart., Capelo, J.C. Costa, Lousã, Fontinha, R. Jardim & M. Seq.	3,2
			otu62	*Vaccinium padifolium* Sm.	3,2
	Fabales	Fabaceae	otu25	*Acacia longifolia* (Andrews) Willd.	3,2
	Fagales	Betulaceae	otu19	*Corylus avellana* L.	6,5
	Fagales	Myricaceae	otu22	*Morella faya* Aiton	12,9
	Gentianales	Rubiaceae	otu14	*Phyllis nobla* L.	16,1
	Geraniales	Geraniaceae	otu9	*Geranium robertianum* L.	16,1
	Lamiales	Plantaginaceae	otu8	*Plantago lanceolata* L.	19,4

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Magnoliopsida	Malpighiales	Euphorbiaceae	otu37	*Mercurialis annua* L.	3,2
	Myrtales	Myrtaceae	otu70	*Eucalyptus globulus* Labill.	3,2
	Poales	Poaceae	otu13	*Agrostis castellana* Boiss. & Reut.	29,0
			otu5	*Briza maxima* L.	38,7
			otu150	*Bromus diandrus* Roth	3,2
			otu50	*Dactylis glomerata* L.	3,2
			otu10	*Holcus lanatus* L. *subsp.* lanatus L.	22,6
	Rosales	Rosaceae	otu15	*Rubus bollei* Focke	19,4
			otu2	*Rubus ulmifolius* Schott	16,1
	Saxifragales	Crassulaceae	otu28	*Umbilicus rupestris* (Salisb.) Dandy	3.2

The overall taxonomic composition of the diet of *Mus musculus* is represented in a hierarchical form in Figure 6.

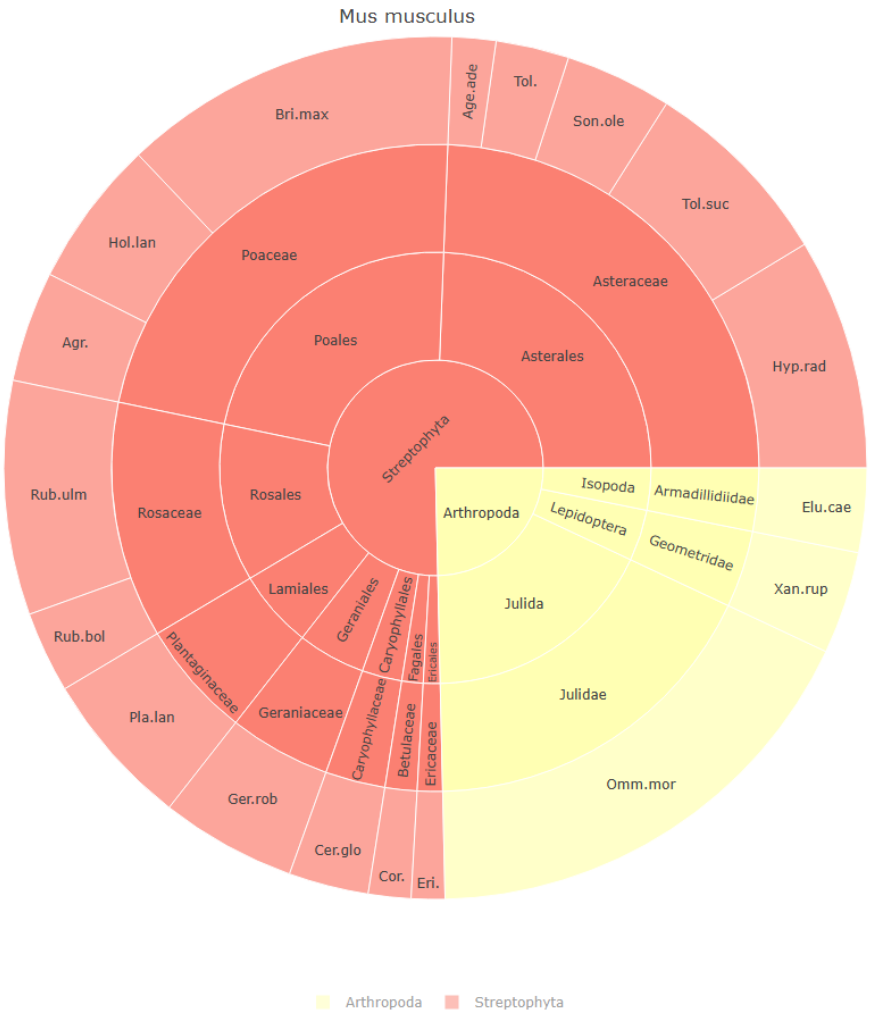

Figure 6. Multi-level circular chart (sunburst) of dietary items identified in *Mus musculus* faecal samples. Colours correspond to the main taxonomic groups detected.

3.6 - Diet of *Rattus rattus*

In Rattus rattus, 47 prey species were identified, corresponding to a total of 49 dietary items when taxa above species level were also considered, distributed across 24 orders and 30 families. The order Julida was again the most frequent (61.1%), mainly represented by *Ommatoiulus moreleti*. Other relevant groups were Stylommatophora (55.6%), Crassiclitellata (38.9%), and Isopoda (11.1%). At the family level, the most frequent were Julidae (61.1%), Agriolimacidae and Arionidae among gastropods, and Lumbricidae among annelids (Table 6).

Table 6. Frequency of occurrence (FO, %) of *taxa* identified in the diet of *Rattus rattus* (n = 18) using the FWH1 marker.

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Arachnida	Araneae	Agelenidae	otu135	*Tegenaria pagana* (C.L. Koch, 1840)	5,6
		Dysderidae	otu149,172	*Dysdera crocata* C.L. Koch, 1838	5,6
Clitellata	Crassiclitellata	Lumbricidae	otu50	*Lumbricus castaneus* (Savigny, 1826)	11,1
			otu20	*Lumbricus terrestris* Linnaeus, 1758	27,8
			otu513	*Octolasion tyrtaeum* (Savigny, 1826)	5,6
Diplopoda	Julida	Julidae	otu3	*Ommatoiulus moreletii* (Lucas, 1860)	61,1
Gastropoda	Stylommatophora	Agriolimacidae	otu39	*Deroceras invadens* Reise et al., 2011	11,1
			otu161	*Deroceras reticulatum* (Müller, 1774)	5,6
		Arionidae	otu21	*Arion intermedius* Normand, 1852	16,7
		Limacidae	otu74	*Lehmannia nyctelia* (Bourguignat, 1861)	5.6
		Oxychilidae	otu89	*Oxychilus alliarius* (Miller, 1822)	11,1
			otu15	*Oxychilus cellarius* (Müller, 1774)	5,6
Insecta	Dermaptera	Forficulidae	otu393	*Forficula auricularia* Linnaeus, 1758	5,6

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Insecta	Diptera	Calliphoridae	otu10	*Calliphora vicina* Robineau-Desvoidy, 1830	44,4
			otu51	*Calliphora vomitoria* (Linnaeus, 1758)	5,6
		Drosophilidae	otu32	*Drosophila suzukii* (Matsumura, 1931)	16,7
	Orthoptera	Acrididae	otu253	*Calliptamus madeirae* Uvarov, 1937	5,6
		Tettigoniidae	otu28	*Antaxius spinibrachius* (Fischer, 1853)	5,6
Malacostraca	Isopoda	Armadillidiidae	otu9	*Eluma caelatum* (Miers, 1877)	11,1

For *Rattus rattus*, plant records were dominated by Asterales, particularly *Hypochaeris radicata* (50.0%), *Tolpis succulenta* (33.3%), and *Andryala integrifolia* (33.3%). Rosales also contributed substantially, with *Rubus bollei* and *Rubus ulmifolius* both present in 16.7% of the samples. Other families were detected at lower frequencies, including Caryophyllaceae (*Cerastium glomeratum*, *Silene gallica*, 11.1%), Rubiaceae (*Phyllis nobla*, 11.1%), and Myricaceae (*Morella faya*, 11.1%). Several additional taxa occurred sporadically (5.6%), such as *Plantago lanceolata*, *Umbilicus rupestris*, *Taraxacum officinale*, and *Orobanche minor* (Table 7).

The overall taxonomic composition of the diet of *Rattus rattus* is represented in a hierarchical form in Figure 7.

Table 7. Frequency of occurrence (FO, %) of plant *taxa* identified in the diet of *Rattus rattus* (n = 18) using the ITS2 marker.

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Magnoliopsida	Apiales	Apiaceae	otu46	*Melanoselinum decipiens* (Schrad. & J.C. Wendl.) Hoffm.	5,6
		Pittosporaceae	otu82	*Pittosporum undulatum* Vent.	5,6
	Asterales	Asteraceae	otu18	*Ageratina adenophora* (Spreng.) R.M. King & H. Rob.	5,6
			otu4	*Andryala integrifolia* L.	33,3
			otu33,38	*Galactites tomentosa* Moench	11,1
			otu1	*Hypochaeris radicata* L.	50,0
			otu11	*Sonchus oleraceus* L.	5,6
			otu21	*Taraxacum officinale* Weber agg.	5,6
			otu16,3	*Tolpis succulenta* (Dryand. in Aiton) Lowe	33,3
	Caryophyllales	Caryophyllaceae	otu52	*Cerastium fontanum* subsp. *Vulgare* (Hartm.) Greuter & Burde	5,6
			otu12	*Cerastium glomeratum* Thuill.	11,1

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Magnoliopsida			otu20	*Silene gallica* L.	11,1
	Ericales	Ericaceae	otu17	*Erica arborea* L.	5,6
	Fabales	Fabaceae	otu25	*Acacia longifolia* (Andrews) Willd.	5,6
			otu27	*Acacia melanoxylon* R. Br.	5,6
	Fagales	Fagaceae	otu92	*Quercus ilex* L. subsp. *ballota* (Desf.) Samp.	5,6
		Myricaceae	otu22	*Morella faya* Aiton	11,1
	Gentianales	Rubiaceae	otu14	*Phyllis nobla* L.	11,1
	Lamiales	Orobanchaceae	otu40	*Orobanche minor* Sm.	5,6
		Plantaginaceae	otu8	*Plantago lanceolata* L.	5,6
	Poales	Poaceae	otu5	*Briza maxima* L.	5,6
			otu216	*Zea mays* L.	5,6
	Rosales	Rosaceae	otu15	*Rubus bollei* Focke	16,7
			otu2	*Rubus ulmifolius* Schott	16,7
	Sapindales	Rutaceae	otu138	*Citrus sinensis* (L.) Osbeck	5,6

Class	Order	Family	OTU(s)	Scientific ID	FO (%)
Magnoliopsida	Saxifragales	Crassulaceae	otu28	*Umbilicus rupestris* (Salisb.) Dandy	5.6

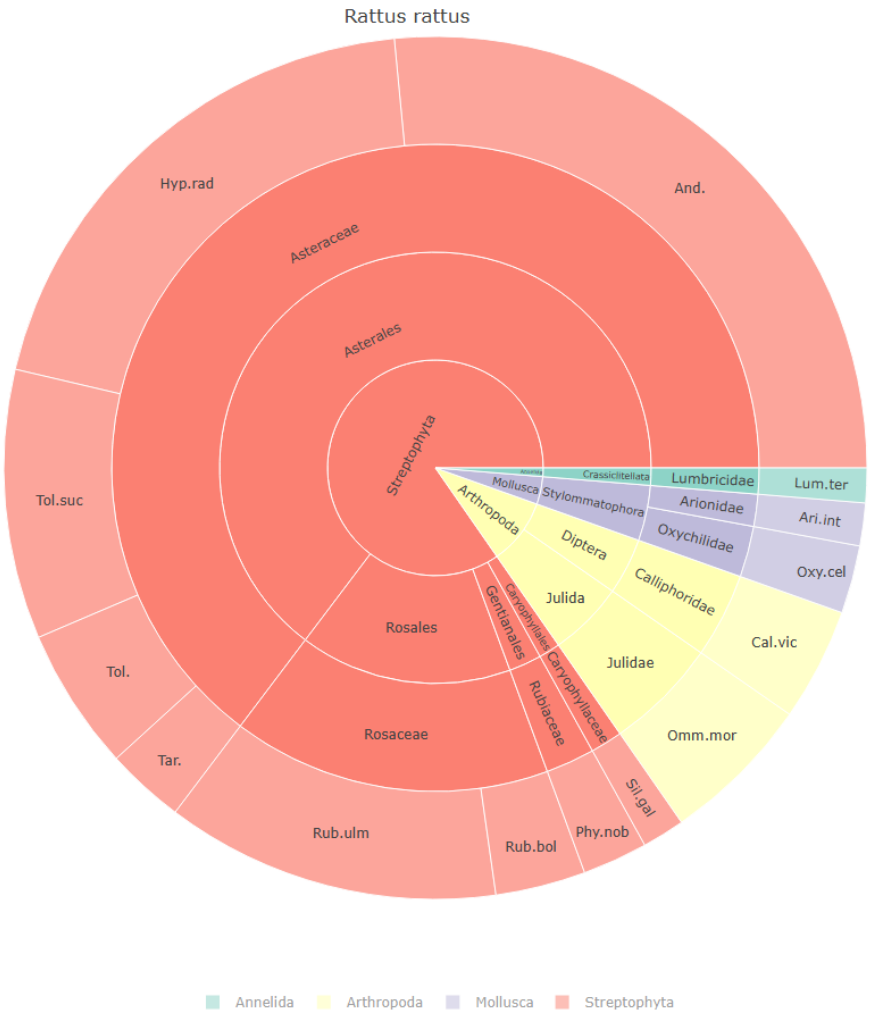

Figure 7. Multi-level circular chart (sunburst) of dietary items identified in *Rattus rattus* faecal samples. Colours correspond to the main taxonomic groups detected.

3.7 - Niche width differences across predator species

Based on incidence data (q = 0), the rarefaction and extrapolation curves show that dietary richness increased steadily with sample size for both predator species and that neither curve reached an asymptote, indicating that sampling effort was insufficient to capture the full dietary diversity (Figure 8). The curves of Mus musculus and Rattus rattus largely overlapped, and no significant differences in dietary richness were detected between the two species.

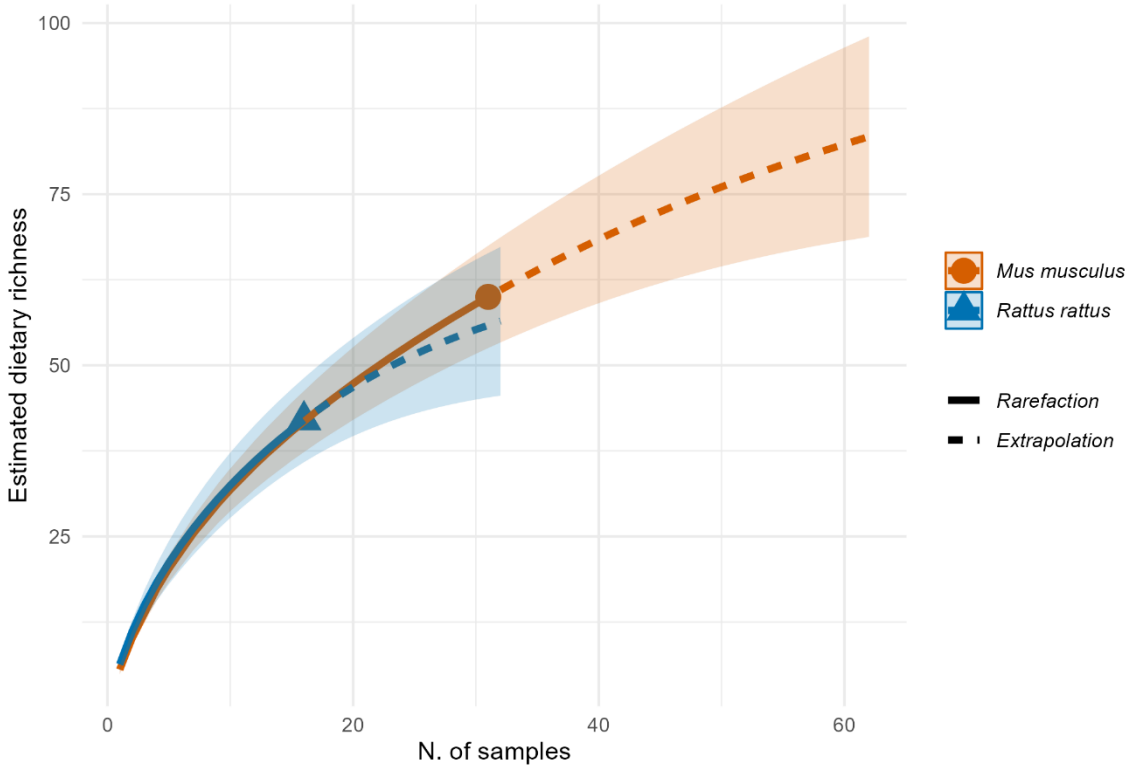

Figure 8. Rarefaction and extrapolation curves of dietary richness (q = 0) for *Mus musculus* and *Rattus rattus.*

3.8 - Diet composition based on the top 15 dietary items

The top 15 dietary items revealed distinct but overlapping patterns between the two rodent species (Figure 9). In *Mus musculus*, the most frequent prey was the millipede *Ommatoiulus moreleti*, occurring in 54.8% of the samples. Among plants, the grasses *Briza maxima* (38.7%) and *Agrostis castellana* (29.0%) were important, together with the composite *Hypochaeris radicata* (35.5%). Endemic taxa were also recorded, such as *Tolpis succulenta* (25.8%) and *Rubus bollei* (19.4%).

In *Rattus rattus*, *Ommatoiulus moreleti* was likewise the most common prey, present in 61.1% of the samples. Other frequent items included the plant *Hypochaeris radicata* (50.0%), the fly *Calliphora vicina* (44.4%), and the endemic *Tolpis succulenta* (33.3%). Additional taxa such as *Andryala integrifolia* (33.3%) illustrate that this species also consumed a mixture of endemic, native, and introduced resources.

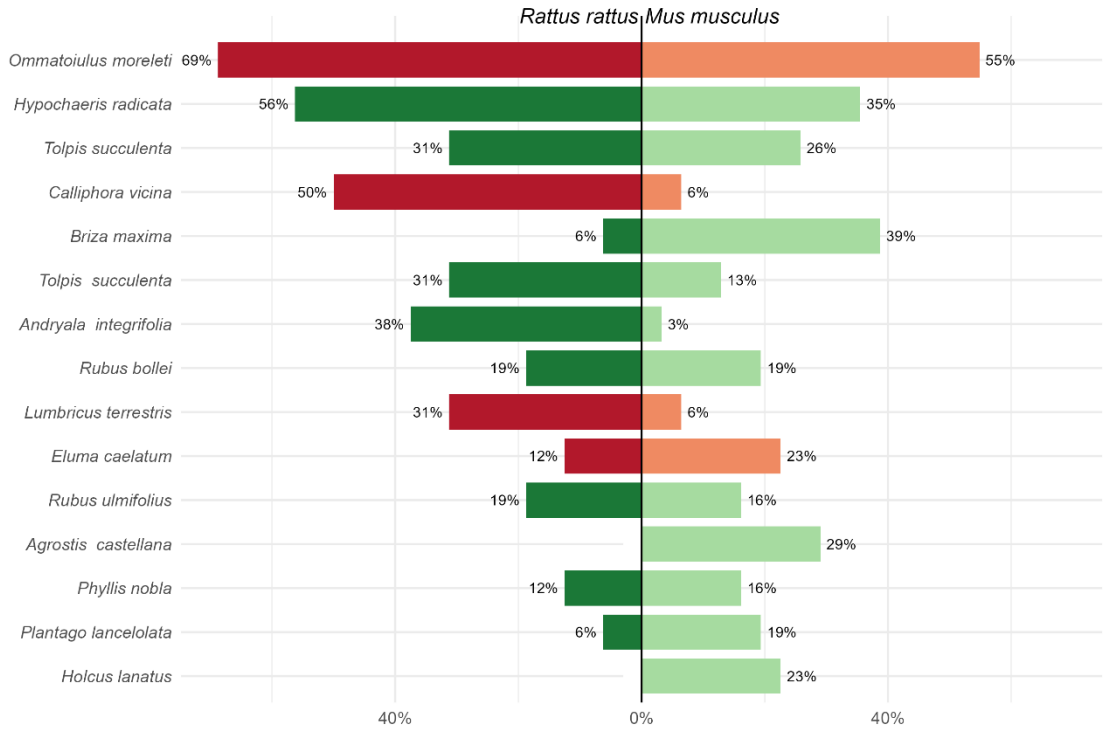

Figure 9. Top 15 dietary items identified in the diet of *Mus musculus* and *Rattus rattus*. Bars represent the percentage of samples in which each taxon was detected, relative to the total number of samples per predator. Red bars indicate animal prey, and green bars indicate plant prey.

3.9 - Dietary richness by sex and species

The boxplot (Figure 10) shows dietary richness of Mus musculus and Rattus rattus separated by sex. Dietary richness was defined as the number of unique prey taxa detected per faecal sample, including OTUs identified at species, genus, or family level. Richness ranged from 1 to 12 items per sample, with both extremes found in M. musculus. No statistically significant differences were detected either between the two species or between sexes. In both species, males tended to have slightly higher median values than females, and males of R. rattus also showed greater variability. Samples of M. musculus with undetermined sex showed intermediate values.

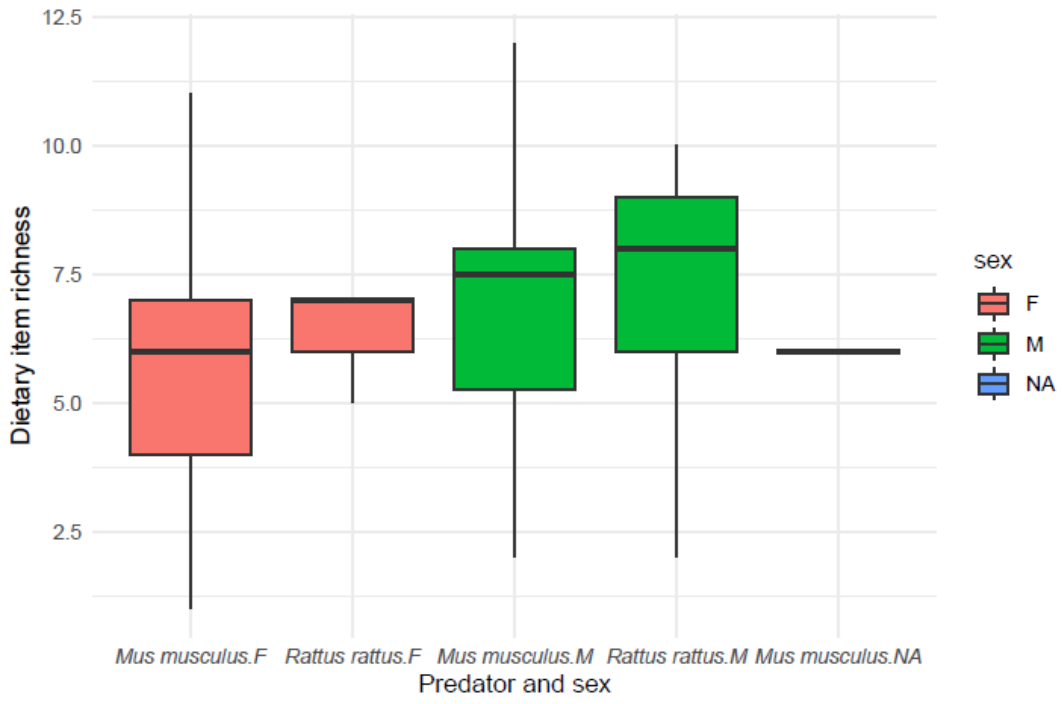

Figure 10. Boxplot of dietary item richness in *Mus musculus* and *Rattus rattus*, separated by sex (F = female, M = male, NA = undetermined).

When looking at the diet composition by phylum (Figure 11), both rodent species consumed mainly Streptophyta (plants) and Arthropoda. Some differences between sexes were observed. In *M. musculus*, males consumed a higher percentage of arthropods and plants compared to females, while females consumed slightly more annelids. In *R. rattus*, the pattern was more balanced, but females consumed more arthropods and annelids, while males had similar values for plants and molluscs. These results indicate that, even if the total dietary richness did not differ significantly between sexes, there were small differences in the types of prey consumed.

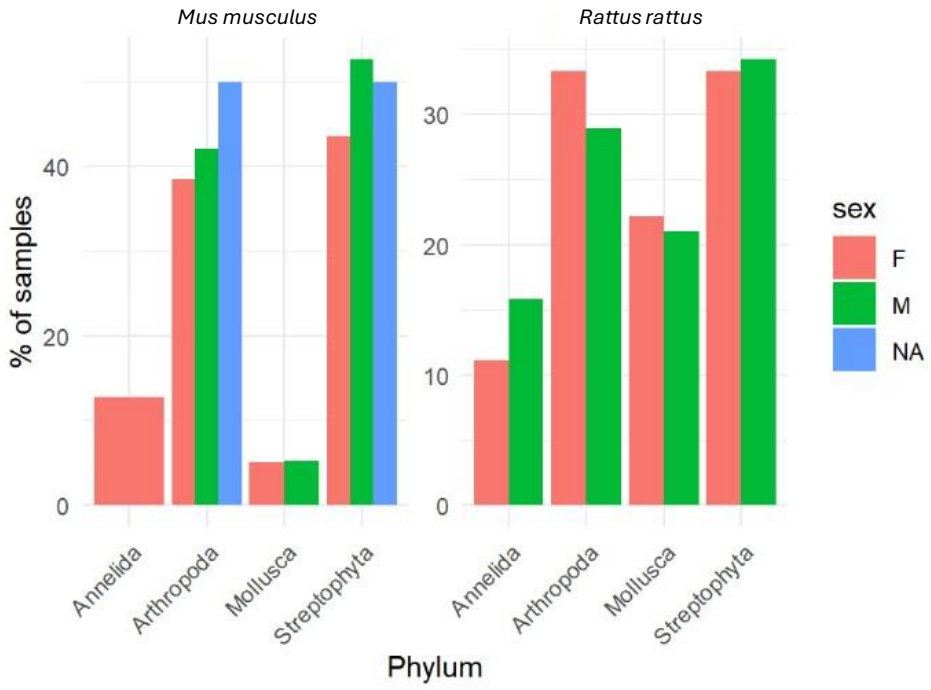

Figure 11. Diet composition by phylum in *Mus musculus* and *Rattus rattus*, separated by sex (F = female, M = male, NA = undetermined). Bars show the percentage of samples in which each phylum was detected.

3.10 - Differences in diet composition between predator species

The NMDS plot (Figure 12) shows a tight cluster with a large overlap between *Mus musculus* and *Rattus rattus*, meaning their diets were very similar. Only small differences were visible, with a weak tendency for some *Rattus* samples to appear slightly higher on the NMDS2 axis. Two extreme outliers were removed because they distorted the scaling: SNI24.1747 (*Rattus*, dominated by *Acacia longifolia*) and SNI24.1755 (*Rattus*, dominated by *Pittosporum undulatum*). After removing these samples, no clear separation between the two predators was evident. The PERMANOVA test still detected a small but statistically significant difference between species ($R^2 = 0.043$, $F = 2.11$, $p = 0.002$), while the dispersion test was not significant ($F = 1.15$, $p = 0.288$).

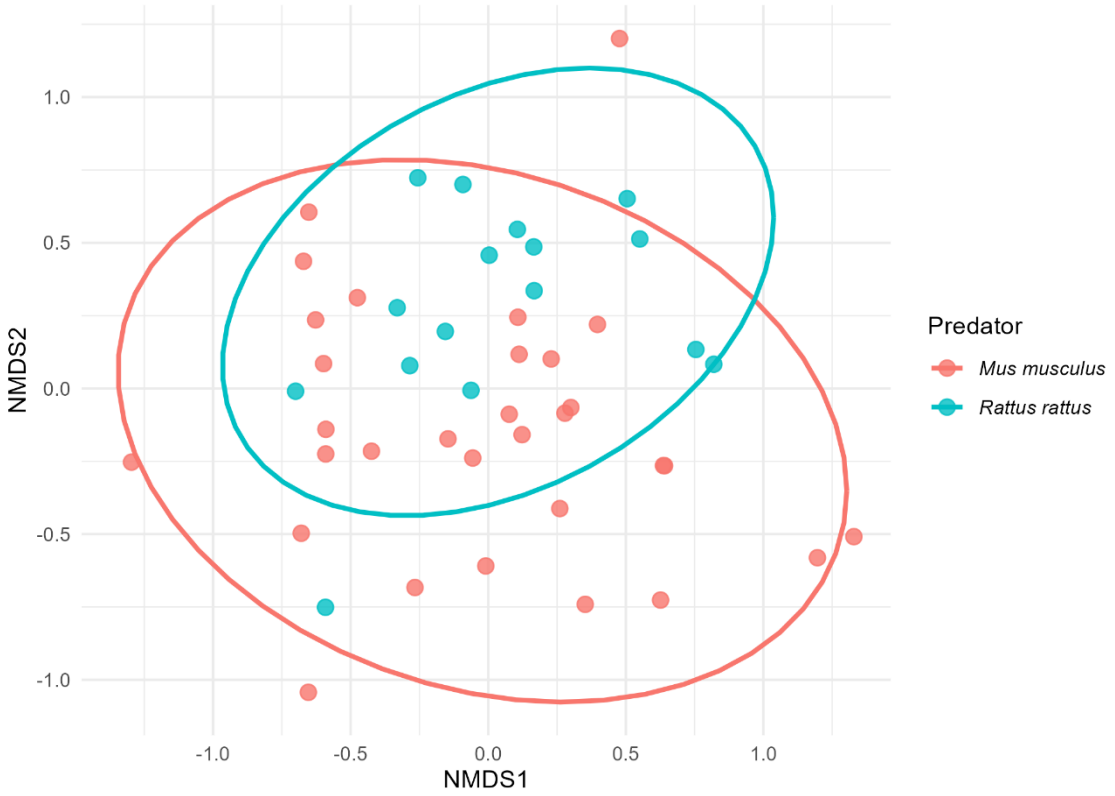

Figure 12. NMDS plot of diet composition in *Mus musculus* and *Rattus rattus* based on Jaccard presence/absence distances. Colours represent the two predator species.

3.11 - Dietary items contributing to dissimilarity between predator species

The SIMPER analysis (Jaccard: Figure 13) identified a small set of prey taxa that explain most of the dietary dissimilarity between *Mus musculus* and *Rattus rattus*. The largest incidence differences were consistently associated with *R. rattus*, including *Calliphora vicina* (50.0% vs. 6.5%), *Andryala integrifolia* (37.5% vs. 3.2%), *Lumbricus terrestris* (31.2% vs. 6.5%), *Drosophila suzukii* (18.8% vs. 3.2%), *Arion intermedius* (18.8% vs. 3.2%), *Deroceras invadens* (12.5% vs. 0%), and *Silene gallica* (12.5% vs. 0%). All these contrasts were statistically significant (permutation test, $p < 0.05$).

In contrast, several plant taxa showed higher incidence in *M. musculus*, including *Briza maxima* (38.7% vs. 6.2%), *Agrostis castellana* (29.0% vs. 0%), *Geranium robertianum* (16.1% vs. 0%), and *Plantago lanceolata* (19.4% vs. 6.2%). However, these differences were not statistically significant and are therefore not represented as drivers of dissimilarity in the SIMPER plot.

Overall, the separation between predator species is mainly driven by a limited number of prey items strongly associated with *R. rattus*, whereas prey items more frequently consumed by *M. musculus* do not contribute significantly to between-group dissimilarity despite large absolute differences in occurrence.

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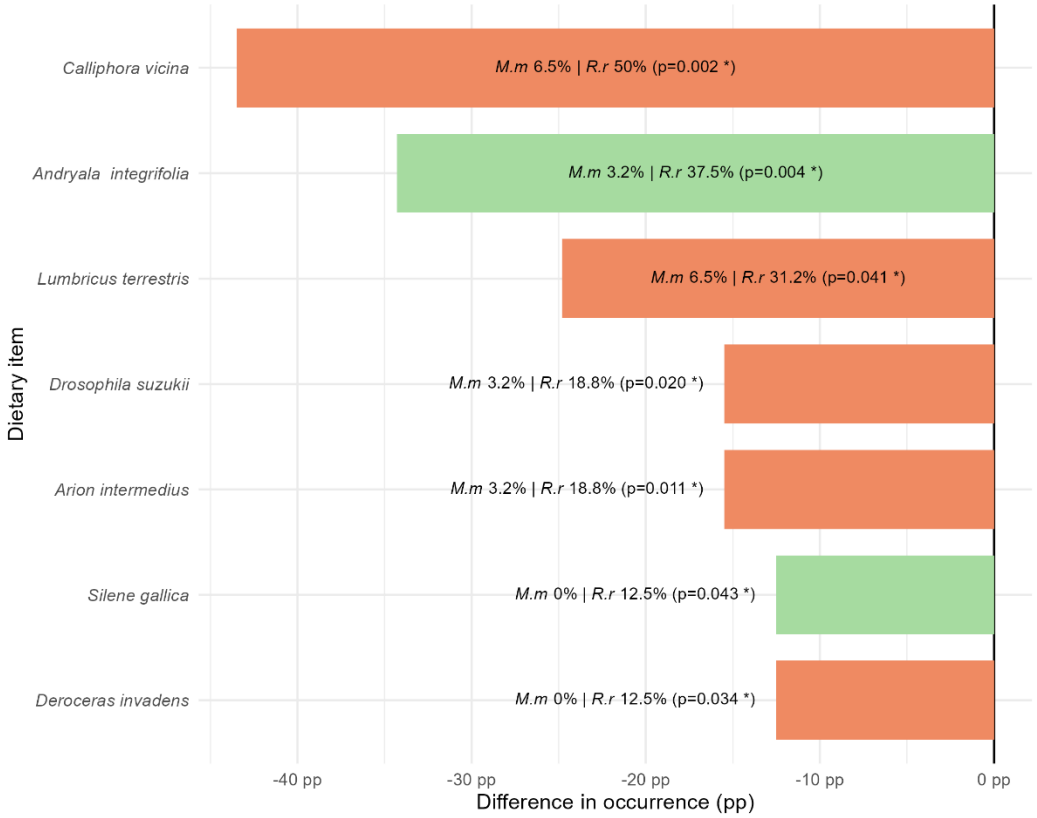

Figure 13. Bars show the significant difference in incidence (percentage points) between *Mus musculus* and *Rattus rattus*. Positive values (green bars) indicate higher occurrence in *Mus musculus*, and negative values (orange bars) indicate higher occurrence in *Rattus rattus* (permutation test, $p < 0.05$).

Chapter IV - Discussion

The main goal of this study was to examine the trophic interactions of the invasive rodents *Mus musculus* and *Rattus rattus* in Madeira and to evaluate their potential impacts on native biodiversity. This is the first detailed investigation of their diet on the island, and the first study to apply DNA metabarcoding to these species in this region.

Previous work in the Ecological Park of Funchal analysed rodent diet using morphological identification of stomach contents (Nunes et al., 2010), reporting a generalist diet for both *Mus musculus* and *Rattus rattus*, mainly composed of vegetation, fruits, and invertebrates. The present study confirms this generalist feeding behaviour but reveals important differences in dietary detail and taxonomic resolution. By applying DNA metabarcoding, a higher diversity of taxa was detected, including endemic and exotic plants and a wider range of invertebrates, particularly soft-bodied taxa such as dipterans, annelids, and gastropods, which were absent or poorly represented in the morphological study. This suggests a methodological bias in Nunes et al. (2010) towards prey with hard, identifiable structures, which are more likely to be preserved during digestion.

As in Nunes et al. (2010), no vertebrates were detected in the diet. However, this result should be interpreted cautiously. In the present study, vertebrates were not specifically targeted, as the primer sets used were designed primarily for plants and invertebrates. Although the COI marker can theoretically amplify vertebrate DNA, detection efficiency is low when using invertebrate-biased primers, and vertebrate prey may therefore remain undetected (Pompanon et al., 2012; Deagle et al., 2013). Consequently, the absence of vertebrate DNA does not exclude occasional predation events. Indeed, field observations in the study area have documented egg predation and chicks showing signs of rat attacks, indicating that birds may be affected even if this was not detected in the metabarcoding data. The use of vertebrate specific markers in future studies could provide a more complete assessment of the role of rodents as predators of vertebrates in the Ecological Park of Funchal.

From 49 faecal samples, 78 dietary species were identified, belonging to 60 families and 78 genera. Of these, 61 species were detected in *Mus musculus* and 47 in *Rattus rattus*, confirming that both are highly generalist and feed on a wide variety of plants and invertebrates, including endemic *taxa* of conservation concern and introduced species with invasive potential. Since no previous dietary studies had been conducted for these

rodents in Madeira, this work provides the first baseline information on their trophic ecology. Comparable studies in Hawai‘i (Gabrielson et al., 2024), the Canary Islands (Pomeda-Gutiérrez et al., 2021) and the Azores (Ceia et al., 2017) have also reported broad and opportunistic feeding in invasive rodents. Other metabarcoding studies revealed similar patterns while noting methodological limitations (Pinho et al., 2022). Generalist feeding has also been widely documented in continental rodents (Zhang et al., 2022), and small mammal diets have been used as indicators of local biodiversity (Schuette et al., 2022). Altogether, the patterns observed here are consistent with broader ecological trends in island and continental ecosystems.

4.1 - General diet composition

Both *M. musculus* and *R. rattus* consumed multiple groups of invertebrates (diplopods, gastropods, dipterans, lepidopterans, coleopterans) together with plants from families such as Poaceae, Rosaceae and Asteraceae. The frequent detection of the millipede *Ommatoiulus moreletii*, eaten by both species, suggests that abundant resources are quickly incorporated in their diets. The presence of endemic plants such as *Rubus bollei* and *Tolpis succulenta*, together with exotic species like *Acacia melanoxylon* and *Ageratina adenophora*, shows their ability to exploit a wide range of resources.

Similar patterns were reported in other islands. In the Canary Islands, black rats consumed both plants and invertebrates, including native *taxa* (Pomeda-Gutiérrez et al., 2021). In Hawai‘i, *R. rattus* had a very diverse diet including arthropods, plants and fungi (Gabrielson et al., 2024). In the Azores, both species fed mainly on invertebrates but also on plants, especially exotic ones (Ceia et al., 2017). In Mediterranean islands, mice showed flexible diets dominated by lepidopterans and plants (Gallozzi, 2025). These comparisons confirm that invasive rodents are generalists able to exploit locally available resources, a flexibility that increases their ecological impact.

4.2 - Consumption of endemic, native and introduced *taxa*

Several plant taxa of high conservation value were detected in the diet, including endemic species such as *Tolpis succulenta*, *Erica platycodon*, *Vaccinium padifolium* and *Melanoselinum decipiens*. In addition, native plant species such as *Rubus bollei* and

Phyllis nobla were also consumed, indicating that native flora of conservation concern present in the study area is exposed to rodent feeding.

Introduced plant species were also recorded in the diet, including *Hypochaeris radicata*, *Briza maxima*, *Agrostis castellana*, *Acacia melanoxylon* and *Ageratina adenophora*. The detection of taxa such as *Acacia melanoxylon* is particularly relevant, as acacias are among the most problematic introduced plants in the Ecological Park of Funchal and other areas of Madeira, where they strongly alter soil properties, nutrient cycling and native plant communities (Fontinha et al., 2014). By consuming both native and introduced plants, rodents may contribute to the persistence and potential spread of introduced species, either through seed predation or dispersal, as reported for invasive rodents in other island ecosystems (Traveset et al., 2009; Ceia et al., 2017; Shiels & Drake, 2011). This opportunistic feeding behaviour indicates that rodents exploit both rare and widespread resources and are able to adapt easily to different habitat conditions, potentially reinforcing the impacts of biological invasions in disturbed and semi-natural habitats (Russell & Kueffer, 2019).

A wide range of invertebrates was also consumed, including millipedes, isopods, lepidopterans and dipterans. Detected taxa comprised endemic species, such as the Madeiran grasshopper *Calliptamus madeirae* and the butterfly *Hipparchia maderensis*, native species such as *Antaxius spinibrachius* and *Ctenoplusia limbirena*, and introduced taxa including *Calliphora vicina* and *Deroceras invadens*. By feeding on decomposers (e.g. annelids and detritivorous arthropods) and pollinators (e.g. lepidopterans and dipterans), rodents may influence key ecosystem processes such as nutrient cycling and pollination (Kaiser-Bunbury et al., 2010; St. Clair, 2011; Traveset et al., 2009).

Bird predation was not detected in the metabarcoding data. This absence is not unexpected, as avian prey typically occurs at very low frequencies in rodent diets and predation events are usually opportunistic and concentrated during the breeding season (Towns et al., 2006; Shiels et al., 2013; Zarzoso-Lacoste et al., 2016). Given the sample size, such rare interactions may remain undetected by chance, although they can still be ecologically important when occurring repeatedly across large rodent populations (Carpenter et al., 2025).

4.3 - Niche width differences across predator species

Both *Mus musculus* and *Rattus rattus* exhibited broad diets, confirming their generalist feeding strategies. Although dietary overlap between species was high, the statistical analyses support the existence of **small but consistent differences in diet composition**. These differences were statistically significant but characterised by a low effect size, indicating that most food resources are shared and that niche separation is weak rather than pronounced.

From an ecological perspective, this pattern suggests that niche differentiation between the two rodents is driven not by overall dietary richness, but by the relative importance of a limited number of prey types. *Mus musculus* tended to rely more on plant resources, particularly herbaceous taxa, whereas *Rattus rattus* showed a higher association with animal prey, especially ground-dwelling and soft-bodied invertebrates. Such tendencies are consistent with differences in body size, foraging behaviour and microhabitat use between species.

The absence of significant differences in dietary richness further supports the interpretation of similar niche width between species, despite compositional differences. This combination of broad niche width and subtle dietary divergence is typical of invasive generalist rodents and may facilitate coexistence by reducing direct competition while allowing flexible resource use.

Comparable patterns have been reported in other island systems. In the Azores, dietary differences between rodents were statistically detectable but ecologically modest, reflecting contrasting use of plant and invertebrate resources (Ceia et al., 2017). In the Canary Islands and Mediterranean islands, black rats and mice also show broad dietary overlap with weak but recurrent niche differentiation (Pomeda-Gutiérrez et al., 2021; Gallozzi, 2025). Similarly, in Hawai‘i, *R. rattus* exhibits high dietary plasticity, exploiting a wide range of food resources across habitats (Gabrielson et al., 2024).

Overall, the statistical support for small effect sizes suggests that niche differences between *M. musculus* and *R. rattus* are ecologically meaningful but not strong. Such patterns imply that both species can coexist while simultaneously exerting trophic pressure on native communities. From a conservation perspective, this highlights that management strategies focusing on a single rodent species are unlikely to substantially reduce ecological impacts, given the broad and overlapping niches observed.

4.4 - Plant versus animal consumption

When considering the relative contribution of plant and animal items, *Mus musculus* showed a clear tendency towards a more plant-based diet, whereas *Rattus rattus* exhibited a more balanced consumption of plant and animal resources. This pattern was supported by intraspecific analyses, which indicated that *M. musculus* consumed significantly more plant than animal items, while no such difference was detected for *R. rattus*.

In contrast, the interspecific comparison of the proportion of animal-based dietary items did not reveal a statistically significant difference between the two species.. The substantial overlap between species suggests high inter-individual variability and broadly similar reliance on animal versus plant resources.

Average values further support this interpretation, with *M. musculus* consuming fewer animal items and more plant items per sample than *R. rattus*. Similar patterns have been reported in other island systems, although the balance between plant and animal resources varies according to local environmental conditions and resource availability (Ceia et al., 2017; Pomeda-Gutiérrez et al., 2021; Gabrielson et al., 2024; Gallozzi, 2025).

Overall, these results suggest that *Mus musculus* may interact more frequently with vegetation, while *Rattus rattus* tends to exploit animal prey more regularly. Although the present study does not quantify ecological impacts directly, such dietary tendencies may have different implications for plant and invertebrate communities, particularly in island ecosystems.

4.5 - Conservation implications

By feeding on endemic and native plants, introduced plant species, invertebrates and occasionally birds, invasive rodents contribute to ecological change in the Ecological Park of Funchal. Their impacts are particularly relevant because the area was severely affected by wildfires in 2010 and 2016, which altered vegetation structure and resource availability (Fontinha et al., 2014). The consumption of fire-resilient native species such as *Vaccinium padifolium* suggests that post-fire regeneration of native habitats may be influenced by rodent herbivory.

Rodents may also act as seed dispersers of both native and exotic plants. Although dispersal of native species cannot be excluded, evidence from island ecosystems indicates

that seed dispersal by invasive rodents more frequently facilitates the persistence and spread of exotic plants, particularly in disturbed environments (Traveset et al., 2009; Shiels & Drake, 2011; Gabrielson et al., 2024). In the present study, rodents consumed both native and introduced plant taxa, indicating an opportunistic feeding strategy rather than a clear preference for either group. Consequently, the ecological outcome of seed dispersal is likely context-dependent, but may disproportionately favour exotic species under post-disturbance conditions.

The park also hosts the only monitored Madeiran colony of the Manx shearwater (*Puffinus puffinus*) (Nunes et al., 2010). No bird DNA was detected in the present study, but a chick was found dead with signs consistent with rat predation. These indirect observations should be interpreted cautiously, as predation cannot always be distinguished from necrophagy. Nevertheless, they suggest that even low-frequency interactions may affect this small and vulnerable colony. This concern is consistent with studies from other island systems, where introduced rats have been shown to severely impact burrow-nesting seabirds such as shearwaters (Towns et al., 2011).

4.6 - Study limitations

This study also has limitations. Sampling covered almost a year, but with few samples in summer and winter, so seasonal changes may have been missed. In addition, the strong imbalance between sexes in Rattus rattus (14 males and 4 females) further reduced the statistical power to detect sex-related differences. The total number of captures was relatively low, which may limit the detection of rare dietary items. DNA based analysis cannot quantify biomass and may miss some taxa due to primer bias (Pinho et al., 2022). It also cannot always distinguish between direct and indirect consumption, as some detections may result from secondary ingestion.

Finally, the choice of molecular markers imposes important limitations. The FwhF1–R1 primer amplifies a short COI fragment (~178 bp), which is well suited for degraded DNA and particularly effective at detecting invertebrates, the main strength of this marker (Vamos et al., 2017; Pinho et al., 2022). However, even universal primers can fail to amplify all groups equally, so birds and other vertebrates may be poorly detected or not detected at all. For this reason, absence of DNA does not mean absence of predation, and the use of more than one primer can help to reduce this problem and improve taxonomic

coverage (Pinho et al., 2022; Pomeda-Gutiérrez et al., 2021; Schuette et al., 2022; Zhang et al., 2022). The Uniplant-F–R primer targets the ITS2 region (~200–350 bp), offering good taxonomic resolution for angiosperms and therefore well adapted to describe plant diets in species-rich insular habitats (Moorhouse-Gann et al., 2018; Zhang et al., 2022). Still, its length variation can reduce efficiency in some taxa (Zhang et al., 2022). Although neither marker is universal, their combined use provides a complementary and cost-effective approach, capturing both animal and plant components of the diet, and remains one of the most reliable metabarcoding strategies for baseline studies in island ecosystems (Pinho et al., 2022).

Conclusion

This study represents the first DNA metabarcoding assessment of the diet of invasive rodents on Madeira Island. Prior to this work, rodent diet in the Ecological Park of Funchal had only been investigated using morphological analysis, which described a generalist diet composed mainly of plants, fruits and invertebrates. The present study confirms this generalist feeding behaviour for *Rattus rattus* and *Mus musculus*, but provides a higher taxonomic resolution through the use of molecular methods. Endemic plants of conservation concern, exotic plants with invasive potential, and a wide range of invertebrate groups were detected.

Some differences were observed between the two species. *Mus musculus* tended to consume a higher proportion of plant material, whereas *R. rattus* included more invertebrates and showed higher occurrence of certain prey taxa. Although these differences were weak and associated with small effect sizes, they are consistent with patterns reported from other island systems, where mice generally rely more on plant resources and rats exploit a broader range of animal prey. Both species consumed native plants of conservation interest, including *Rubus bollei* and *Vaccinium padifolium*, as well as exotic plants such as *Acacia melanoxylon* and *Ageratina adenophora*, which may be dispersed by rodents. By feeding on decomposers such as millipedes and on potential pollinators, rodents may also influence ecosystem processes such as nutrient cycling and pollination.

These impacts are particularly relevant in the Ecological Park of Funchal, an area severely affected by wildfires in 2010 and 2016. The consumption of fire-resilient native species such as *Vaccinium padifolium* suggests that post-fire habitat recovery may be negatively affected by continued rodent herbivory.

Despite some limitations, including the inability to quantify consumed biomass or to clearly distinguish between direct and secondary ingestion, this study provides the first molecular baseline of rodent diet in Madeira. Future studies should combine DNA metabarcoding with complementary approaches, such as seed germination experiments to test whether seeds remain viable after gut passage, as applied by Gabrielson et al. (2024), seasonal dietary monitoring, or stable isotope analyses, in order to better evaluate the ecological impacts of invasive rodents. Comparative studies with other Macaronesian islands would also help to place the results from Madeira in a broader regional context.

Overall, the results indicate that invasive rodents in Madeira exploit a wide range of resources, including endemic plants of conservation concern, exotic plants with invasive potential, and diverse invertebrate taxa. These patterns suggest that rodents may contribute to ecological change, as observed in other island ecosystems, and that similar processes may occur in Madeira. This reinforces the need for conservation strategies aimed at reducing risks to sensitive species and habitats.

Future rodent control programmes should be adapted to the local context. Although anticoagulant rodenticides may still be used, they require caution, as prolonged application can select for resistant individuals and reduce effectiveness. An integrated management approach combining traditional methods with automatic toxin-free traps and continuous monitoring may be more effective. In addition, specific action plans should be developed to provide targeted protection for the most vulnerable species and habitats.

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