One Bat's Waste is Another Man's Treasure: A DNA Metabarcoding Approach for the Assessment of Biodiversity and Ecosystem Services Using Bat Faeces

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One bat's waste is another man's treasure: A DNA metabarcoding approach for the assessment of biodiversity and ecosystem services using bat faeces

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Abstract

Arthropod populations are constantly changing due to changes in climate and the globalisation of trade and travel. Effective and diverse monitoring techniques are required to understand these changes. DNA metabarcoding has facilitated the development of a broad monitoring method to sample arthropod diversity from environmental and faecal samples. In this study, we applied DNA metabarcoding to DNA extracted from bat faecal pellets of *Rhinolophus hipposideros,* the lesser horseshoe bat in Ireland, a highly protected bat species of conservation concern in Europe. From as few as 24 bat faecal pellets, we detected 161 arthropod species, spanning 11 orders, including 38 pest species of which five were determined to be priority pests, highlighting important ecosystem services. We also report the identification 14 species not previously reported in Ireland, but upon further investigation found that many of these are likely misidentified due to inadequacies in the genetic reference database. For the first time, we were able to use non-invasively collected bat samples to examine the role of sex in the diet of bats and found that the male and female diets did not differ significantly. However, sampling location did explain variation within the diet, highlighting how landscape features influence arthropod composition and diversity. We discuss the current limitations of the methodology in Ireland, how these can be overcome in future studies, and how this data can be used for biodiversity monitoring and informing conservation management of protected bat species.

Keywords: Arthropod diversity; Dietary analysis; Non-invasive genetics; *Rhinolophus hipposideros*

Introduction:

Biodiversity plays a globally important role in the successful functioning of healthy ecosystems, vital for human health, wellbeing and food production, collectively known as ecosystem services (Díaz et al. 2019; Dainese et al. 2019). Declines in biodiversity are associated with habitat loss caused by agricultural intensification, urbanisation, globalisation of trade and climate change (Hallmann et al. 2017). Reduced biodiversity can lead to weakened ecosystem resilience, resulting in the loss of economically important species such as pollinators, while promoting the establishment and subsequent spread of invasive species, pests, and disease vectors, through the simplification of landscapes and the creation of favourable habitats to enable their establishment (Clare 2014; Isbell et al. 2018; Dainese et al. 2019; Browett et al. 2020).

Projections of the Paris Agreement on Climate Change show that up to 40% of global insect diversity is in decline and at risk of extinction, and despite some uncertainty regarding the magnitude of this crisis, scientists collectively agree that a decline is occurring (Warren et al. 2018; Komonen et al. 2019; Sanchez-Bayo and Wyckhuks 2019, Thomas et al. 2019). Of ten major taxonomic orders, 37% of species are in decline, and 18% of mainly agricultural and nuisance pest species, are increasing in population numbers (Sanchez-Bayo and Wyckhuks (2021). Butterfly populations in the United Kingdom (UK) and the Netherlands have declined by around 50% between 1976 and 1990 (Warren et al. 2021). Some of the biggest challenges surrounding biodiversity and vector/ pest monitoring is the labour-intensive work that is required for sampling, morphological identification, and the counting of individual species. This work is vital for the generation of robust surveillance data but requires intensive field sampling and taxonomic expertise making large-scale longitudinal surveys expensive and difficult (Pataki et al. 2021). Traditional approaches inadequately account for the importance of trophic interactions between species within a habitat, hindering the effectiveness of subsequent management strategies. Indirect monitoring of biodiversity via environmental sources or the diet of a predator, such as insectivorous bat species, can provide data regarding the composition and

interactions of species within a community (de Sousa et al. 2019), providing a more holistic approach to assessment.

Bats are described as indicators of diversity and can be studied relatively easily across landscapes using well-established surveillance methods (Jones et al. 2009; Park 2015; Russo and Jones 2015; Russo et al. 2018; Harrington et al. 2019). Bats are considered specialised hunters, with different species seeking areas of open, narrow and edge space habitats to hunt (Denzinger and Schnitzler 2013; Heim et al. 2016). In Europe, *Pipistrellus* spp. and *Nyctalus* spp. forage over open arable and pasture landscapes, but more variable habitat mosaics containing trees promote activity of species such as *Myotis*, *Plecotus*, and *Rhinolophus* spp., offering a broader suite of ecosystem services, vital for the overall functioning of healthy ecosystems (Heim et al. 2015). Bats can also suppress crop pests and potential vectors of disease relevant to human and animal health (Maine and Boyles 2015; Ancillotto et al. 2017; Taylor et al. 2018; Baroja et al. 2019).

Traditional dietary analysis of faecal samples involves hard-part analysis, a labour-intensive process which is limited by time constraints, an inability to detect soft-bodied prey and low taxonomic resolution (Clare 2014; Tournayre et al. 2020), thus reducing the ability to carry out informative studies across broad geographical areas. The advent of Next Generation Sequencing (NGS) technology has revolutionised our capability to gain greater dietary resolution and insights from faecal material (Deagle et al. 2019; de Sousa et al 2019; Browett et al. 2020). DNA metabarcoding can be described as the simultaneous and parallel identification of multiple taxa using a standardised region of DNA. It is a useful technique not only to address questions related to the diet of a species, but it can also be used as an ecosystem approach to detect and track trophic interactions at spatio-temporal scales (Bohmann et al. 2014) with significant developments in the analysis of mammalian diets being made over the last decade (Pompanon et al. 2012; Shokralla et al. 2014; Tournayre et al. 2020; Browett et al. 2020, 2021; Tournayre et al. 2021).

89 Across Europe, several studies have applied DNA metabarcoding to bat faeces to understand bat
90 trophic niches and the insect communities that they predate upon (Arrizabalaga-Escudero et al. 2019;
91 Galan et al. 2018; Swift et al. 2018). DNA metabarcoding of the lesser horseshoe bat (*Rhinolophus*
92 *hipposideros*) diet within a vineyard-dominated Mediterranean agroecosystem showed that the
93 species is a natural suppressor of many insect pests that negatively impact agriculture (Baroja et al.
94 2019) and consumption of pest species by *R. hipposideros* was higher than for other bat species
95 (Baroja et al. 2021). Such evidence can support the establishment of management programmes
96 favouring population growth of bats, thereby benefiting insect diversity and the wider agricultural
97 community via the suppression of pest species.

98 An investigation into the diet of the greater horseshoe bat (*R. ferrumequinum*) in France found that
99 the core diet consisted of a small number (n=15 common prey species) of preferred taxa (25% of all
100 occurrences), and a secondary diet (75%) consisted of rare prey that varied between sampling
101 occasions and colonies. Demonstrating that high dietary plasticity might enable adaptation to
102 changing environments and habitats (Tournayre et al. 2021). A degree of functional flexibility was also
103 evident within the trophic niche of *R. euryale*, as it consumed a wide range Lepidoptera which varied
104 in their energy content throughout the season (Arrizabalaga-Escudero et al. 2019).

105 Browett et al. (2021) optimised a dual primer approach for the DNA metabarcoding of bat diet using
106 DNA previously extracted from non-invasively collected faeces, previously identified to species, sex
107 and individual level using real-time PCR and microsatellite genotyping (Harrington 2018; Harrington
108 et al. 2019). This approach uses proven good quality and quantity DNA, and excludes low quality
109 samples, and facilitates the inclusion of questions related to sex and individual level dietary
110 preferences. Such questions were previously only addressed in studies that captured bats and placed
111 them in cloth bags to facilitate the collection of faeces (Mata et al. 2016; Galan et al. 2017;
112 Arrizabalaga-Escudero et al. 2019), but species such as *R. hipposideros* are sensitive to disturbance

(Weinberger et al. 2009) and best studied using a non-invasive approach (Harrington 2018; Baroja et al. 2021).

Rhinolophus hipposideros is the only horseshoe bat species that occurs in Ireland, and has a restricted range, occurring in parts of six counties along the western coast (Fig. 1), with the next closest population occurring in Wales, Britain. resulting in its isolation from all other European populations (Carden et al. 2010; Roche et al. 2015; Dool et al. 2016; Harrington 2018). The most recent Article 17 conservation and population assessment (required under the European Habitats Directive) reported that the species is increasing in range, but numbers are declining (NPWS 2019), and genetic studies have shown that populations are becoming increasingly fragmented and isolated, a risk for future extinction (Dool et al. 2016; Harrington 2018).

Building on the work of Browett et al. (2021), the aim of this study is to further explore the diet of *R. hipposideros* to describe the overall arthropod diversity present within the species' diet and demonstrate the ecosystem services provided through the identification of insect pest species that can negatively impact agriculture and those implicated in the spread of disease. For the first time, we were also able to investigate differences in diet between sexes and populations using non-invasively collected samples. Based on our findings, we make recommendations on how the technology can be used to its full potential as a tool for assessing and surveying arthropod biodiversity across spatio-temporal scales.

Methods

The methodology surrounding the collection and processing of the *R. hipposideros* samples (n = 24) used in this study was fully described in Harrington (2018) and Browett et al. (2021). Briefly, the faecal pellets of *R. hipposideros* were non-invasively collected by Harrington (2018) at six roosts within the distribution of the species in the west of Ireland (Fig. 1) under license from NPWS (licence number DER/BAT 2016-29). Each DNA extract was identified to species and sex using real-time PCR assays (Harrington 2018; Harrington et al. 2019) and identified to individual level using a panel of seven microsatellite markers originally designed by Puechmaille et al. (2005) and re-designed and optimised by Harrington (2018). Twenty-four *R. hipposideros* samples were used as part of this DNA metabarcoding work and evenly represented sex (n = 12 for male and female samples) and location (n = 4 samples from each of the six roosts with sex evenly represented at each roost).

DNA was amplified using the primers designed by Zeale et al. (2011) and Gillet et al. (2015) that targeted 157 bp and 133 bp fragments of the Cytochrome C Oxidase Subunit 1 (COI) gene, respectively. Using a combination of COI primers aids in maximising amplification and assessment of diversity within the diet. Extended details regarding PCR reaction mixes, multiplexing, thermocycling conditions, library preparation, sequencing, and bioinformatic steps required to generate Molecular Operational Taxonomic Units (MOTUs) are provided in Browett et al. (2021).

Taxonomic Assignment

Taxonomic assignment was made by assigning MOTUs generated to species level with a minimum identity of 98% requiring at least 90% coverage using the GenBank and BOLD databases, the latter of which was used to confirm identification when MOTUs presented more than one possible species-level identification and were removed from the dataset when more than one species was assigned to the same MOTU (Supplementary Information 1,2,3). If multiple MOTUs were assigned to the same species, they were agglomerated together using the sum of their sequence reads.

Dietary Diversity Measures

Using the R packages *ggplot2*, *tidyverse*, and *knitR* a "donut chart" was constructed to graphically present taxonomic data for each MOTU detected within the *R. hipposideros* diet (donut chart script source at https://github.com/ShrewlockHolmes/Taxa_Donut_Chart_Visual). The donut chart was separated into three levels, each representing a different taxonomic rank i.e. order, family and genus. The outermost level also contained a number providing an indication of the number of species within that genus that was identified.

Associations between dietary composition at the levels of sex and location were assessed using multiple statistical measures. The data were transformed into relative read abundance (RRA) using the *transform_sample_counts* function within the R package *phyloseq* to provide an indication of how common or rare certain taxa are in relation to other taxonomic groups. Stacked bar plots were constructed in the R package *ggplot2* using the RRA for each order.

Using RRA, a distance matrix was created using the Bray-Curtis dissimilarity method. Permutational multivariate analysis of variance (PERMANOVA) was performed using the *adonis2* function in the R package *vegan* (Oksanen et al., 2019) with 10,000 permutations to determine compositional difference in the prey taxa identified within the *R. hipposideros* diet by sex and location. To ensure that the homogeneity of variance within the groups was not affecting the compositional differences, the function *betadisper()* was used to measure the multivariate distance of samples to the group centroid. All diversity measures described here were repeated with MOTUs agglomerated to order, family, genus, and species taxonomic ranks. The data were then visualised using a non-metric multidimensional scaling (NMDS) ordination plot (R scripts available at: https://github.com/ShrewlockHolmes/Browett_and_Curran_et_al_2021_Mam_Biol). Analysis of similarities (ANOSIM), a non-parametric measure, was used to determine differences between two or more groups (i.e. six locations and two sexes) compared to the mean of ranked dissimilarity within groups (Clarke and Green 1988; Chiarucci et al. 2019). This was performed in R using function *anosim*

in the package *vegan* with 9,999 permutations to calculate the difference between the dietary dataset for a given factor, i.e. sex and location (R scripts available at: https://jkzorz.github.io/2019/06/11/ANOSIM-test.html). The ANOSIM provides two measures, statistic R and significance. The statistic R is a measure that compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups. Statistic R values indicate similarities and differences within and between groups. Values close to zero indicate an even distribution, and no difference between groups. Positive values suggest that similarity is occurring more within groups instead of between groups (McCoy 2020; Chiarucci et al. 2019). Values less than 0.05 are considered statistically significant.

To explore the potential ecosystem services provided by *R. hipposideros* in Ireland, the dietary species identified were compared to previously published works by Baroja et al. (2019) and Tournayre et al. (2021) as both studies identified agriculturally important pest species occurring in the Mediterranean and Continental European diets of *R. hipposideros* and *R. ferrumequinum*. The species identified were also compared to the Arthemis database (http://arthemisdb.supagro.inra.fr), which contains a repository of 2,185 known arthropod pest species in France (Tournayre et al. 2021). The Arthemis database contains information about the host plant range that the arthropods affect. Using the *plot_heatmap* function in the R package *phyloseq* and *ggplot2*, a heatmap indicating the abundance of pest species that were identified as posing potential agricultural and economic burden within the *R. hipposideros* diet was constructed.

The overall list of identified species from this study was compared to records of arthropod diversity documented within Ireland using several established record repositories including Biodiversity Ireland https://biodiversityireland.ie/, Moths Ireland http://www.mothsireland.com/, the Irish Biogeographical Society, and the Natural History collections of the National Museum of Ireland.

Results

Dietary composition

A total of 8,967,124 sequence reads were obtained from the MiSeq sequencing run, as outlined in Browett et al. (2021). A threshold of 98% for sequence clustering was applied for downstream analysis. This threshold has been applied in several studies involving the use of the COI genetic region for invertebrate identification (e.g. Alberdi et al. 2018; Browett et al. 2021). This threshold, coupled with robust species-level confirmation using GenBank and BOLD databases, amounted to the generation of 348 MOTUs (164 MOTUs identified using primers designed by Gillet et al. [2015], and 184 MOTUs identified using primers designed by Zeale et al. [2011]) from 24 *R. hipposideros* faecal pellets (Supplementary Information 1,2, and 3).

These 348 MOTUs represented ten arthropod orders (Araneae, Coleoptera, Diptera, Glomerida, Hemiptera, Hymenoptera, Isopoda, Lepidoptera, Neuroptera, and Trichoptera), and one Annelida order (Opisthopora: Crassiclitellata); consisting of 60 families, 120 genera, and 161 species (Fig. 2). The most dominant order in the diet was Lepidoptera, followed by Diptera (Table 1), which accounted for 55.23% and 18.01% of species in the diet, respectively. The orders Araneae, Hymeoptera, and Trichoptera occurred less frequently in the diet and accounted for six, seven, and fifteen of the identified species respectively (17.4% of the overall species level diet) (Table 1). Species identified within rarely occurring orders / suborders, such as Coleoptera (1.24%), Crassiclitellata (1.24%), and Glomerida (0.62%) contributed marginally to the overall diet of *R. hipposideros*. Furthermore, several species were recorded in this study that have not previously been documented in Ireland (see discussion and Supplementary Information 4 for further details).

Barplots were constructed based on RRA to represent the variations of *R. hipposideros* diet according to roost site location and sex (Fig. 3). At the roost level, Lepidoptera and Diptera were found to be the most dominant orders overall with the exception of roost 3 (Co. Kerry), where the order Hymenoptera was dominant. When diet was investigated by sex, Lepidoptera and Diptera were again the dominant

orders. Female *R. hipposideros* tended to consume more Lepidoptera than males. Less frequently occurring orders including Neuroptera, Trichoptera and Hymenoptera were also more common in the female diet, with Trichoptera only occurring in the female diet and Neuroptera and Hymenoptera rarely occurring in males.

The PERMANOVA showed that sex did not have a statistically significant effect on the diet of *R. hipposideros* (R^2: 0.00273-0.0236, Pr(>F): >0.05). However, roost location was found to be a statistically significant factor impacting the *R. hipposideros* diet (R^2: 0.26115-0.3276, Pr(>F): <0.01) (Table 2). The R^2 values showed that between 26% and 32% of distance variation (depending on the taxonomic rank assessed) was caused by the roost location. This data, at each taxonomic rank, was also visualised using NMDS plots (Fig. 4). The NMDS plots showed that at order level there was an overlap in most of the roost locations, with slight variation. However, roost 3 (Co. Kerry) formed its own cluster outside of the other locations. This pattern can be seen at all taxonomic ranks, where some overlap of each roost was observed, with slight variation, except for roost 3, showing that the diet of *R. hipposideros* at this roost differed to the others.

The Permutest and Tukey analysis showed that sample homogeneity did not influence the compositional difference detected via PERMANOVA as all p-values at both sex and roost for all taxonomic ranks were >0.05.

The ANOSIM results also corroborated the trend observed via PERMONVA as sex differences were not found to influence dietary composition. Statistic R values for sex ranged from -2.11×10^{-2} to 1.61×10^{-2}, and significance at all taxonomic ranks was >0.05 showing that sex did not significantly impact diet. However, roost location was again found to have a statistically significant effect on the diet of *R. hipposideros*, with statistic R ranging from 0.19 to 0.40, and significance values for all taxonomic ranks <0.01.

Identification of pest species

A total of 38 potential pest species were identified, representing almost 24% of the overall species identified in the diet (Table 3). Pest species were mostly Lepidopteran species, with 35 of the 38 (~92%) pest species identified as Lepidoptera. The rest of the potential pest species identified consisted of two Diptera species (~5%) and one Hemiptera species (~2%) (Supplementary Information 5).

Of the 38 species listed in Table 3, five species were identified as posing a significantly negative environmental impact. These were two Lepidoptera species: *Archips podanus* and *Plutella xylostella*, two Diptera species: *Tipula oleracea* and *Chamaepsila rosae,* and one Hemiptera species *Drepanosiphum platanoidis*. A heatmap of the read abundance of these five species within each of the *R. hipposideros* samples included in this study (n =24) was constructed (Fig. 5). From the heatmap, *Tipula oleracea* was the most commonly occurring pest species across each of the bat samples, followed by *Plutella xylostella*. *Chaempsila rosae*, *Drepanosiphum platanoidis* and *Archips podanus* were only found to occur within the diet of one *R. hipposideros* individual each.

Discussion

In this study, we expanded upon our earlier work (Browett et al. 2021) where we developed a dual primer DNA metabarcoding approach to study the diet of insectivorous mammals. Here, we further explored the diet of a bat species, *R. hipposideros*, and described the range of arthropods found in its diet, with a particular focus on the effects of roost location and sex and explored the ecosystem services provided by the species in the form of pest species consumption. This and the earlier work by Browett et al. (2021) are the first studies in Ireland or Great Britain to use a DNA metabarcoding approach to examine the diet of *R. hipposideros*. McAney and Fairley (1989) used traditional hard-part analysis to identify the remains of insects predated upon by *R. hipposideros* and reported eight arthropod orders occurring within the diet from 630 faecal pellets, but here, DNA metabarcoding allowed for the detection of 11 orders from as few as 24 faecal pellets. Of the 11 orders detected here, three are not typical constituents of bat diet (i.e. Annelida order [Opisthopora: Crassiclitellata], Glomerida, and Isopoda). It is likely that these detections are a result of exposure to environmental contamination during sample collection rather than actual dietary constituents (Aldasaro et al. 2019; Browett et al. 2021). In McAney and Fairley (1989), arthropods were only identified to family level, whereas here we have been able to identify arthropod species predated upon by *R. hipposideros*, something not normally achievable via hard-part analysis. This highlights the sensitivity of the DNA metabarcoding approach over traditional hard-part methods and the resolution of the data generated.

Location- and Sex-based Dietary Variation

Roost location was found to be the most informative variable to explain dietary differences across the dataset, which was also found to be the case in *R. ferrumequinum* when studied in France (Tournayre et al. 2021). Here, the diet of *R. hipposideros* was dominated by Diptera and Lepidoptera, but their frequencies and composition varied according to location. The order Hymenoptera was relatively abundant at roost 3 (Co. Kerry) and was also detected at roost 1 (Co. Mayo), but at a lower abundance.

Some less frequently occurring orders were also identified, including Araneae, Coleoptera, Crassiclitellata, Neuroptera, and Trichoptera. Araneae, Coleoptera, and Trichoptera were all identified in Co. Kerry. Dietary variation, particularly for the Kerry site, was evident in Fig. 4, where the points around the group centroid for the Kerry samples clustered separately to the other five locations. Even though the other roosts are located near woodland areas, most are in agriculture-dominated areas, whereas the Kerry site is located in the centre of a heavily wooded area, considered as ideal habitat for *R. hipposideros* in Ireland. The site in Co. Kerry is of international interest as it is a Special Area of Conservation (SAC) for a range of priority habitats listed on Annex I and II of the European Habitats Directive. This suggests that *R. hipposideros* diet is representative of what arthropods are present at the time of sampling (i.e. opportunistic foraging) and that variable habitats play a role in influencing bat diet. This is a factor which should be considered for future studies intending to use DNA metabarcoding as a tool to investigate arthropod diversity and presence/absence of target organisms/groups (Thomsen and Willerslev 2015).

Our analysis showed that the sex of the bat did not significantly impact their diet, with both male and female *R. hipposideros* having a heavy Dipteran and Lepidopteran based diet, but again at varying frequencies, but were not statistically significant. Females appeared to prefer Lepidoptera over Diptera, while males predated more often on Diptera (Fig. 3). The female diet was also found to include less frequently occurring orders (i.e. Hymenoptera, Neuroptera, and Trichoptera). Similar observations have been made in other studies, such as a hard part analysis study of the wrinkle-lipped free-tailed bat (*Tadarida plicata*), where females predated on more Lepidoptera and Coleoptera and fewer Odonatathan than males (Leelapaibul et al. 2005), and a DNA metabarcoding study showed that female European free-tailed bat (*Tadarida teniotis*) predated upon larger and more migratory species than males (Mata et al. 2016). Female bats have high energy requirements during breeding, pregnancy, and lactation (Racey and Entwistle 2000), which may influence their hunting strategies to focus on larger prey items with a higher energy content to support their nutritional demands. These subtle but important differences could be further investigated using the molecular approach outlined

317 in this study combined with an increased sample size to provide more statistically robust insights into
318 sex-biased dietary preferences.

319 **Ecosystem Services**

320 A total of 38 potential pest species were detected in this study, but the magnitude of the risk posed
321 by each of these species in Ireland is not well known, as the species were identified by comparing the
322 data generated from this study with studies from Spain and France (Baroja et al. 2019; Tournayre et
323 al. 2021) and the Arthemis Database based in France. However, some of the more well recognised
324 pest species that we explored using the heatmap (Fig. 5) showed how the diet of the bat can be used
325 to detect and monitor the distribution of pest species, in addition to providing a natural method for
326 pest removal. Five known agricultural pests identified were further investigated due to their
327 recognised economic, societal, and environmental impacts.

328 The most infrequently occurring pest items included *Chamaepsila rosae* (Diptera: Psilidae);
329 *Drepanosiphum platanoidis* (Hemiptera: Aphididae) and *Archips podanus* (Lepidoptera: Tortricidae)
330 each detected in one individual with a total of six reads for the former two species and 12 reads in the
331 later. *Chamaepsila rosae* or carrot fly primarily affects crops such as carrots and parsnips (Collier et
332 al. 2020) and has been described as a major carrot pest within Europe (Szwejda and Wrzodak 2007).
333 *Drepanosiphum platanoidis,* an aphid*,* is a significant pest of ornamental and amenity trees belonging
334 to the genus Acer, particularly, sycamore trees, and can excrete an abundance of honeydew, providing
335 ideal conditions for the growth of moulds such as *Cryptostroma corticale* causing "sooty bark disease",
336 resulting in tree mortality (Parry et al. 1989; Binggeli and Rushton 1999; Morecroft et al. 2008).
337 *Archips podanus*, the fruit tree tortrix moth (often referred to as *A. podana*) is polyphagous and is
338 considered to be an important pest of fruit trees including apple, plum, and cherry and reduces the
339 quality of the fruit harvested (Hrudová 2003; Stará and Kocourek 2004). Studies have found that the
340 abundance of this species is not influenced by insecticide use, highlighting the value of bat predation
341 for the suppression of this species (Cross 1996; Stará and Kocourek 2004).

342 The most frequently occurring pest species included *Plutella xylostella* (Lepidoptera: Plutellidae) and
343 *Tipula oleracea* (Diptera: Tipulidae) detected in detected in four and nine individuals with a total of 47
344 and 102,224 sequence reads respectively. Considered to be a global and economically important pest
345 species, the Diamondback moth, *P. xylostella* is known to be destructive to brassicaceous crops
346 worldwide (Talekar and Shelton 1993; Zalucki et al. 2012; Li et al. 2016). Control strategies for
347 managing this insect pest are met with difficulty as studies have shown a degree of insecticide
348 resistance by this pest species (Talekar and Shelton 1993; Zalucki et al. 2012; Furlong et al. 2013; Xia
349 et al. 2018). The common crane fly (*T. oleracea*) is found throughout Ireland and Europe (cabi.org
350 2019; Peck et al. 2006; 2008) and is commonly referred to as an agricultural and horticultural pest of
351 winter cereals, brassicas, clover, strawberries, turnips and several other vegetables and ornamentals
352 (Blackshaw and Coll 1999; Peck et al. 2006; 2008).

353 **Biodiversity**

354 The dataset generated here suggested the presence of 14 arthropod species not previously reported
355 in Ireland (Figure S4). However, further investigation revealed uncertainties that these identifications
356 were truly new, and more likely caused by an inadequate reference database. A little over 10.5% of
357 the species level identifications generated from this study provided inconclusive results, despite using
358 internationally accepted thresholds for identification (Alberdi et al. 2018; Alberdi et al. 2020; Browett
359 et al. 2021).

360 A number of species, unlikely to be present in Ireland, were identified in this study. *Oricia truncata*
361 identified with 98.7% sequence identity across 223 sequence reads occurs exclusively in Central
362 America (Miller 2009). The next closest species level identification acquired from the MOTU generated
363 for this species was for *Prays rucifeps* and *Homorthodes naverca*, both of which had a lower sequence
364 similarity of 97.4%, making it very difficult to suggest an identification for this MOTU. *Tholera*
365 *americana,* a native species to North America and not present in Ireland was identified in this study
366 (98.7%) from two individuals with sequence reads ranging from 26 to 67. However, it is likely that we

have identified one of two other *Tholera* species documented in Ireland, *T. cespitis* and *T. decimalis* (both listed as critically endangered on Moths Ireland) (Bond and O'Connor 2012), but not present on the genetic reference database. *Tholera cespitis* and *T. decimalis* have a limited and localised distribution, found in parts of the west of Ireland such as the Burren in Co. Clare and parts of west Cork, Kerry, and Galway (Bond and Gittings 2008), overlapping with sampling locations used for this study, suggesting that it is likely that one of those species were identified. Further DNA barcoding and the generation of a morphologically identified reference database would be an invaluable resource to enable more accurate identifications.

Five cranefly species were identified as potential new species records for Ireland in this study, *Tipula banffiana* (99.4% identity [1096 sequence reads across eight individuals]), *Tipula coleana* (98.5% identity [six sequence reads from one individual]), *Tipula luridorostris* (99.2% identity [353 sequence reads across five individuals]), *Tipula platymera* (99.2% identity [108 sequence reads across six individuals]) and *Metalimnobia triocellata* (98.1% [371 reads from one individual]). Previous bat dietary studies have reported a number of *Tipula* spp. predated upon and this arthropod group appears to be a common feature within the diet (Andriollo et al. 2019). Other *Tipula* species identified in this study and previously recorded in Ireland include *T. oleracea* and *T. varipennis*. Craneflies are well documented in Ireland via the "Craneflies of Ireland" database, but the vast majority of the species have not been DNA barcoded, and we cannot accurately identify the sequences to species level without the generation of an accurate genetic reference database. Similar identification difficulties were experienced in relation to *Mesochorus suomiensis*, a parasitoid wasp in the family Ichneumonidae. The MOTU for this species was identified with 98.8% similarity and was recorded in two individuals with sequence reads ranging from eight to 601, but this particular genus of hyperparasitoids and other ichneumonids have been described as being poorly understood in respect to taxonomy (O'Connor et al. (2007).

391 The application of DNA metabarcoding here has also allowed for the detection of potential vector
392 organisms that have been implicated in the spread of disease. In this study, several mosquito (Diptera:
393 Culicidae) and midge (Diptera: Ceratopogonidae) species were identified including *Culex pipiens*, *Cx.*
394 *quinquefasciatus*, *Culiseta annulata*, *Cs. morsitans*, and *Culicoides impunctatus*. The mosquito species
395 *Cx. quinquefasciatus* has not previously been reported in Ireland or Great Britain. Across the British
396 Isles, five *Culex* species have been documented, only one of which has been recorded in Ireland, *Cx.*
397 *pipiens* (Ashe et al. 1991; Folly et al. 2020). Here, *Cx. quinquefasciatus* was detected in two individuals
398 with 328 sequence reads and implies that Irish mosquito species are potentially underestimated and
399 *Culex* species may be more diverse than previously thought. The potential occurrence of this species
400 in Ireland poses a risk for future arthropod-borne disease outbreaks e.g. West Nile Virus and highlights
401 the need for effective and multidisciplinary surveillance methods of vector organisms. However, the
402 sequence region used in this study was very short and often much longer and additional gene regions,
403 such as the second internal transcribed spacer (ITS2) of the ribosomal RNA, are required for accurate
404 species identification and differentiation of the *Culex* complex (e.g. Laurito et al. 2013). In addition,
405 care has to be taken that the originally deposited sequence was also accurately identified. However,
406 the approach of using a predator diet to indirectly survey potential airborne vectors has shown great
407 promise in this study and has the potential to be a powerful surveillance tool.

408 Additionally, this study adds further records of two Lepidoptera species (*Bactra lacteana* and *Prays*
409 *ruficeps*) which were recently observed in Ireland (Bond et al. 2017; Bond 2018). Five *Bactra* spp. have
410 been recorded, *B. furfurana* (also recorded in this study), *B. lancealana*, *B. robustana*, and *B. vanosana*
411 (a migrant species). *Bactra lacteana* and *B. lancealana* are said to be highly morphologically similar
412 species. But, in this case *B. lacteana* was identified within the diet of four *R. hipposideros*, with a total
413 of 5725 sequence reads, with 100% sequence similarity and 100% sequence query cover and the
414 species is well represented on the GenBank database. However, no reference DNA barcode exists for
415 *B. lancealana*. *Prays rucifeps,* a micromoth*,* was detected in one individual with 10,685 sequence reads
416 and with 100% identity. The species was first recorded in Ireland in 2000 (Moths Ireland) but was not

reported in Bond and O'Connor (2012), and only two recordings for *P. rucifeps* exist, both of which have been in the east of Ireland. However, a closely related species *P. faxinella* is present in Ireland and *P. rucifeps* was formerly considered to be a dark variant of this species, but DNA barcoding has enabled the distinction between these two species. When the MOTU generated in this study was compared to *P. faxinella* it was found to only be 97% similar, providing good confidence that both *P. rucifeps* and *P. faxinella* are present in Ireland as has been recognised in Britain (Barnett 2017), and that it is more common and widespread than previously thought.

Conclusion

In this study, DNA metabarcoding of relatively few bat faecal pellets provided a large arthropod dataset. We found that the location of the bat roost was an important factor to explain dietary variation in *R. hipposideros*, a finding which could be adapted in future studies aiming to investigate the impact of land use on biodiversity. Our findings were not limited by the methodology we employed, but by the lack of available DNA sequences present on reference databases to compare Irish insect diversity. Our study was relatively small in scale but as a result, we were in a position to robustly critique our identifications and are consequently provide recommendations to further expand this work to better use the technology for future applications which include monitoring of biodiversity, bat diet, ecosystem services and even as early warning systems for the tracking of pests and vectors. Future studies could include the development of a reference arthropod library using malaise traps, morphological identification, and DNA barcoding to generate more robust datasets for biodiversity recording (e.g. deWaard et al. 2019). In addition, DNA barcoding of target species such as those collected by Moths Ireland and those submitted to the National Museum of Ireland and Biodiversity Ireland could be DNA barcoded to generate genetic references or DNA barcodes for morphologically identified species. Indeed, our findings have similar and relevant implications for other geographically remote and isolated regions. Our work has shown that *R. hipposideros* provides an important economic role in the suppression of influential crop pests, many of which prove difficult

442 to suppress with the use of insecticidal methods, which are also known to be detrimental to wider
443 insect diversity. Our work suggests that promoting and conserving bats and their associated habitats,
444 particularly in areas of crop production, would benefit food producers, bat conservation and insect
445 diversity.

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Statements & Declarations

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Competing Interests

The authors have no competing interests to declare.

Author Contributions

ADM, TGC, SSB, and DBO'M conceived and designed the study. Bat faecal sampling was part of APH, D'ON, DBO'M and CORs project on non-invasive genetic monitoring of lesser horseshoe bats. TGC and SSB performed the laboratory work and bioinformatics associated with the DNA metabarcoding. TGC and DBO'M analysed the data, AO'H helped with entomological identifications. TGC and DBO'M wrote the paper, with all authors contributing to editing, discussions, and approval of the final manuscript.

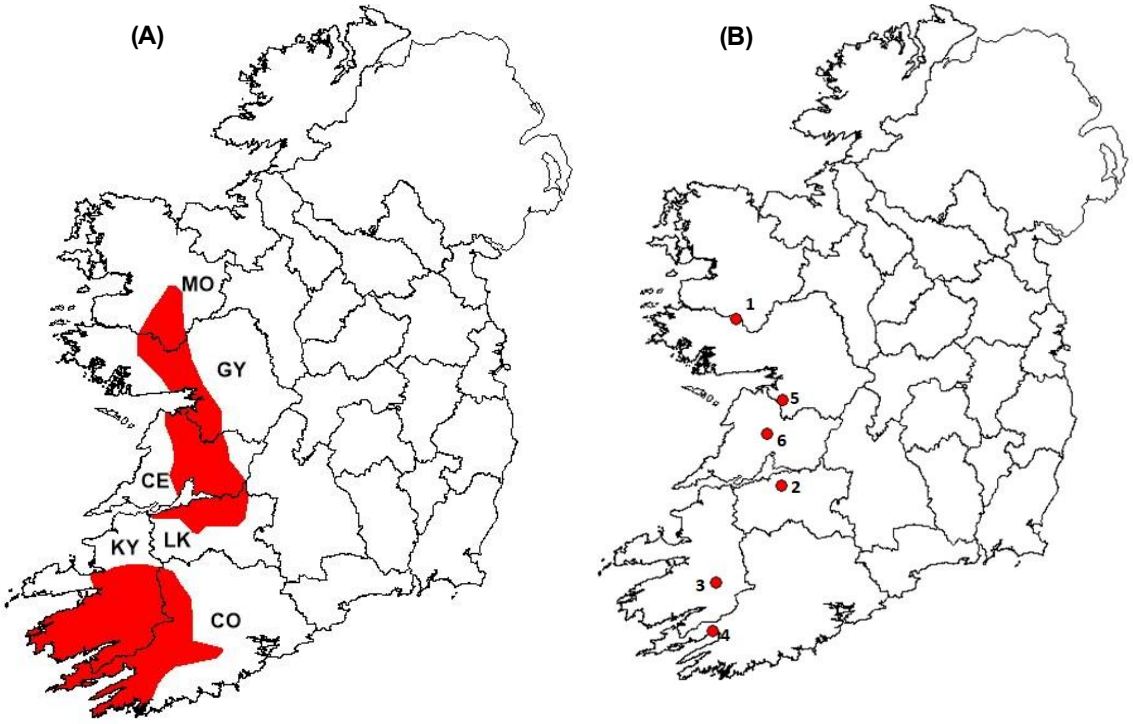
Data Availability

The dataset generated and analysed in this study is available in the Supplementary Information.

Compliance with Ethical Standards

Rhinolophus hipposideros faecal pellets were non-invasively collected by APH at six roosts within the distribution of the species in the west of Ireland under license from NPWS (licence number DER/BAT 2016-29)

List of Figures



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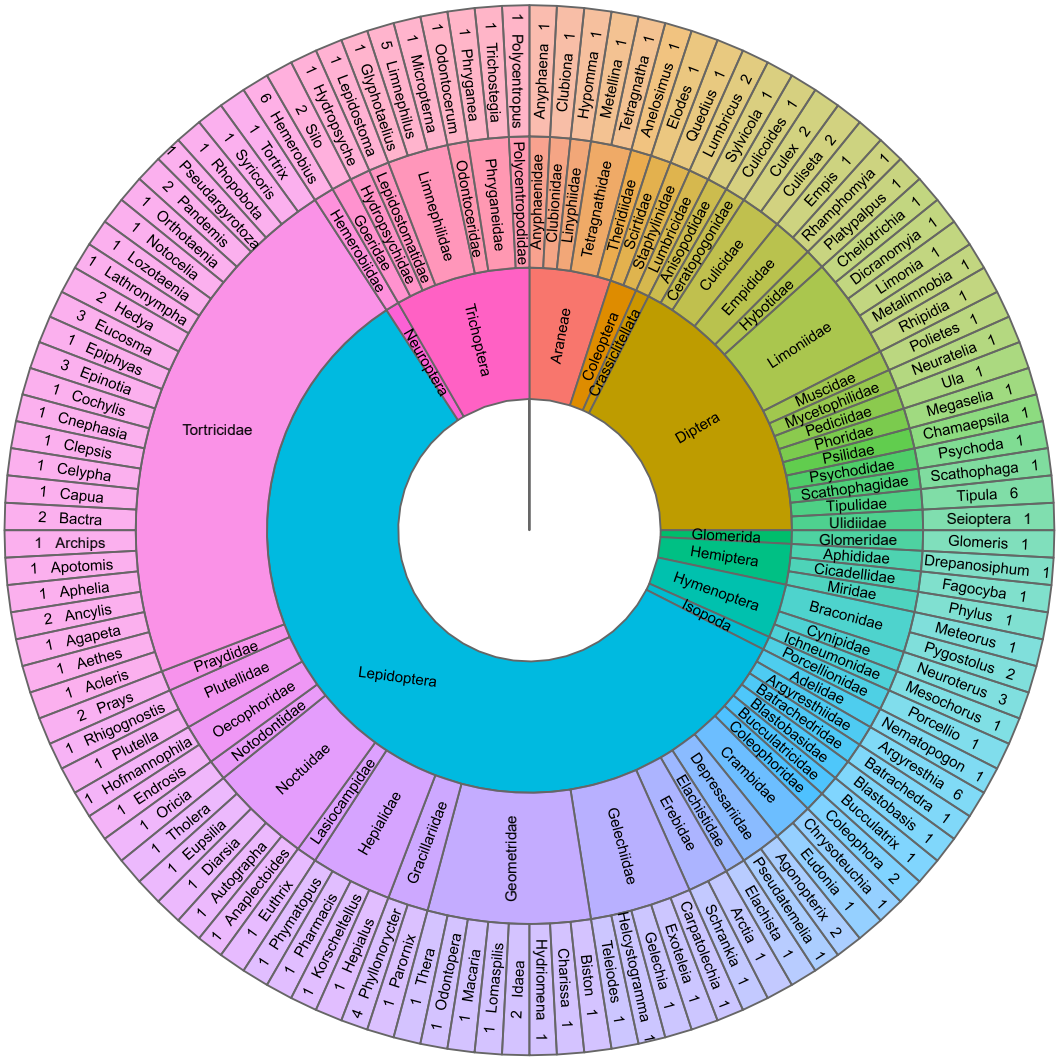
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Neuroptera
Glomerida
Hemiptera
Hymenoptera
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Tortricidae
Praydidae
Plutellidae
Oecophoridae
Notodontidae
Noctuidae
Lasiocampidae
Hepialidae
Gracillariidae
Geometridae
Gelechiidae
Erebidae
Elachistidae
Depressariidae
Crambidae
Coleophoridae
Bucculatricidae
Blastobasidae
Batrachedridae
Argyresthiidae
Adelidae
Ichneumonidae
Porcellionidae
Cynipidae
Braconidae
Miridae
Cicadellidae
Aphididae
Glomeridae
Ulidiidae
Tipulidae
Scathophagidae
Psychodidae
Psilidae
Phoridae
Pediciidae
Mycetophilidae
Muscidae
Limoniidae
Hybotidae
Empididae
Culicidae
Ceratopogonidae
Anisopodidae
Limnophilidae
Lepidostomatidae
Hydropsychidae
Goeridae
Hermerobiidae
Polycentropodidae
Phryganeidae
Odontoceridae
Anyphaenidae
Clubionidae
Linyphiidae
Tetragnathidae
Theridiidae
Scintidae
Staphylinidae
Limbricidae
1 Polycentropus
1 Trichostegia
1 Phryganea
1 Odontocerum
1 Micropterna
5 Limnephilus
1 Glyphotaelius
1 Lepidostoma
1 Hydropsyche
2 Silo
1 Hydroptila
6 Hemerobius
1 Tortrix
1 Syricoris
1 Rhopobota
1 Pseudargyrotoza
2 Pandemis
1 Orthotaenia
1 Notocelia
1 Lozotaenia
1 Lathronympha
2 Hedya
3 Eucosma
1 Epiphyas
3 Epinotia
1 Cochylis
1 Cnephasia
1 Clepsis
1 Celypha
1 Capua
2 Bactra
1 Archips
1 Apotomis
1 Aphelia
2 Ancylis
1 Agapeta
1 Aethes
1 Acleris
2 Prays
1 Rhigognostis
1 Plutella
1 Hofmannophila
1 Endrosis
1 Oricia
1 Tholera
1 Eupsilia
1 Diarsia
1 Autographa
1 Anaplectoides
1 Euthrix
1 Phymatopus
1 Pharmacis
1 Korscheltellus
1 Hepialus
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1 Paromix
1 Thera
1 Odontopera
1 Macaria
1 Lomaspilis
2 Idaea
1 Hydriomena
1 Charissa
1 Biston
1 Teleiodes
1 Helcystogramma
1 Gelechia
1 Exoteleia
1 Carpatolechia
1 Schrankia
1 Arctia
1 Elachista
1 Pseudatemelia
2 Agonopterix
1 Eudonia
1 Chrysoteuchia
2 Coleophora
1 Bucculatrix
1 Blastobasis
1 Batrachedra
6 Argyresthia
1 Nematopogon
1 Porcellio
1 Mesochorus
2 Pygostolus
3 Neuroterus
1 Meteorus
1 Phylus
1 Fagocyba
1 Drepanosiphum
1 Glomeris
1 Seioptera
6 Tipula
1 Scathophaga
1 Psychoda
1 Megaselia
1 Chamaepsila
1 Ula
1 Neuratelia
1 Polietes
1 Rhipidia
1 Metalimnobia
1 Limonia
1 Dicranomyia
1 Cheilotrichia
1 Platypalpus
1 Rhamphomyia
1 Empis
2 Culiseta
1 Culex
1 Culicoides
1 Sylvicola
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1 Metellina
1 Hypomma
1 Clubiona
1 Anyphaena

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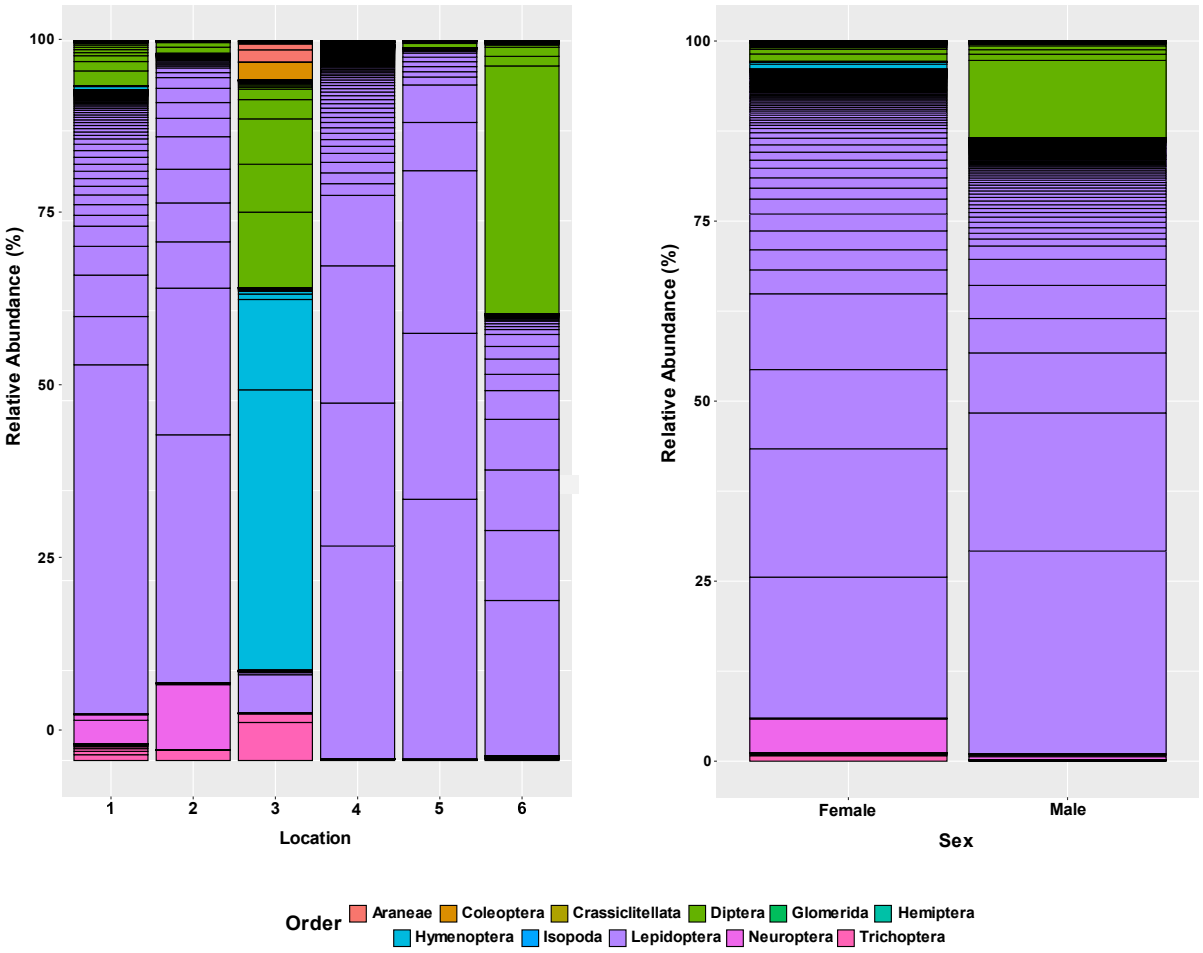
Relative Abundance (%)
100
75
50
25
0
1
2
3
4
5
6
Location
Female
Male
Sex
Order
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Coleoptera
Crassiclitellata
Diptera
Glomerida
Hemiptera
Hymenoptera
Isopoda
Lepidoptera
Neuroptera
Trichoptera

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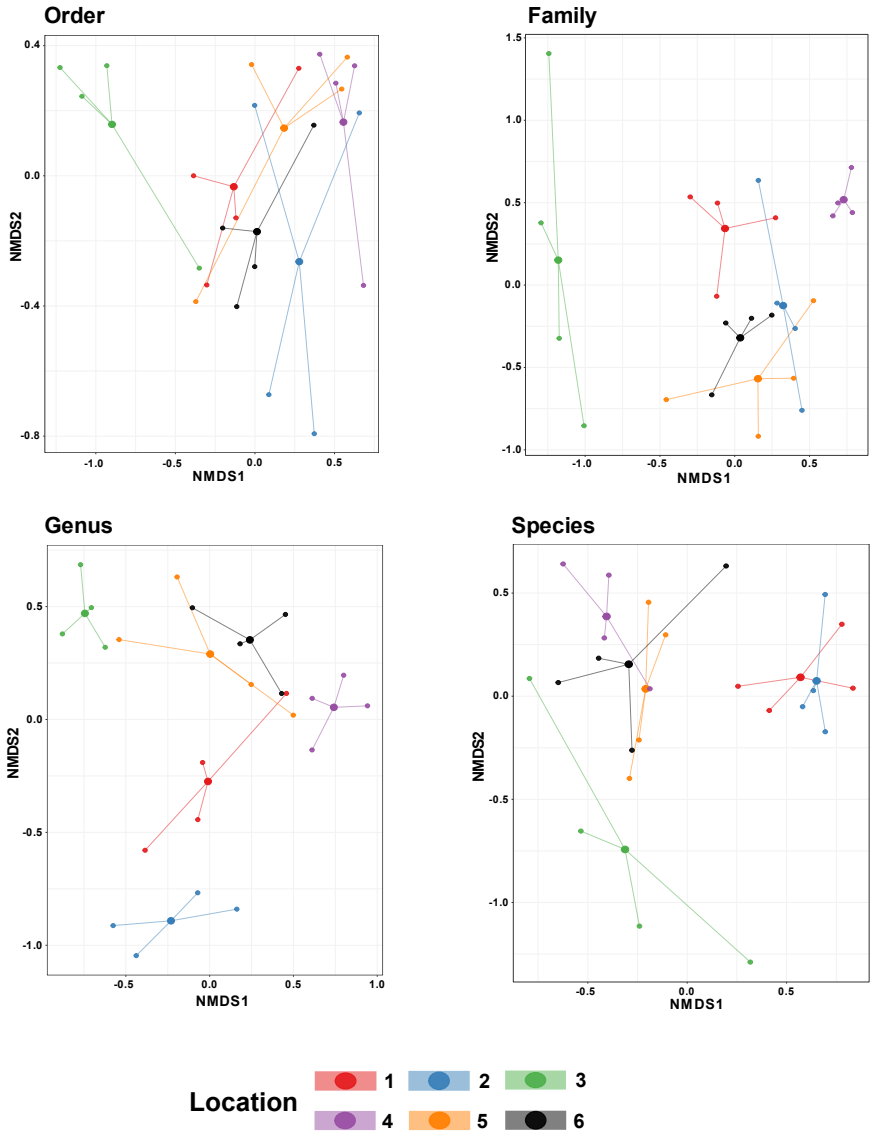

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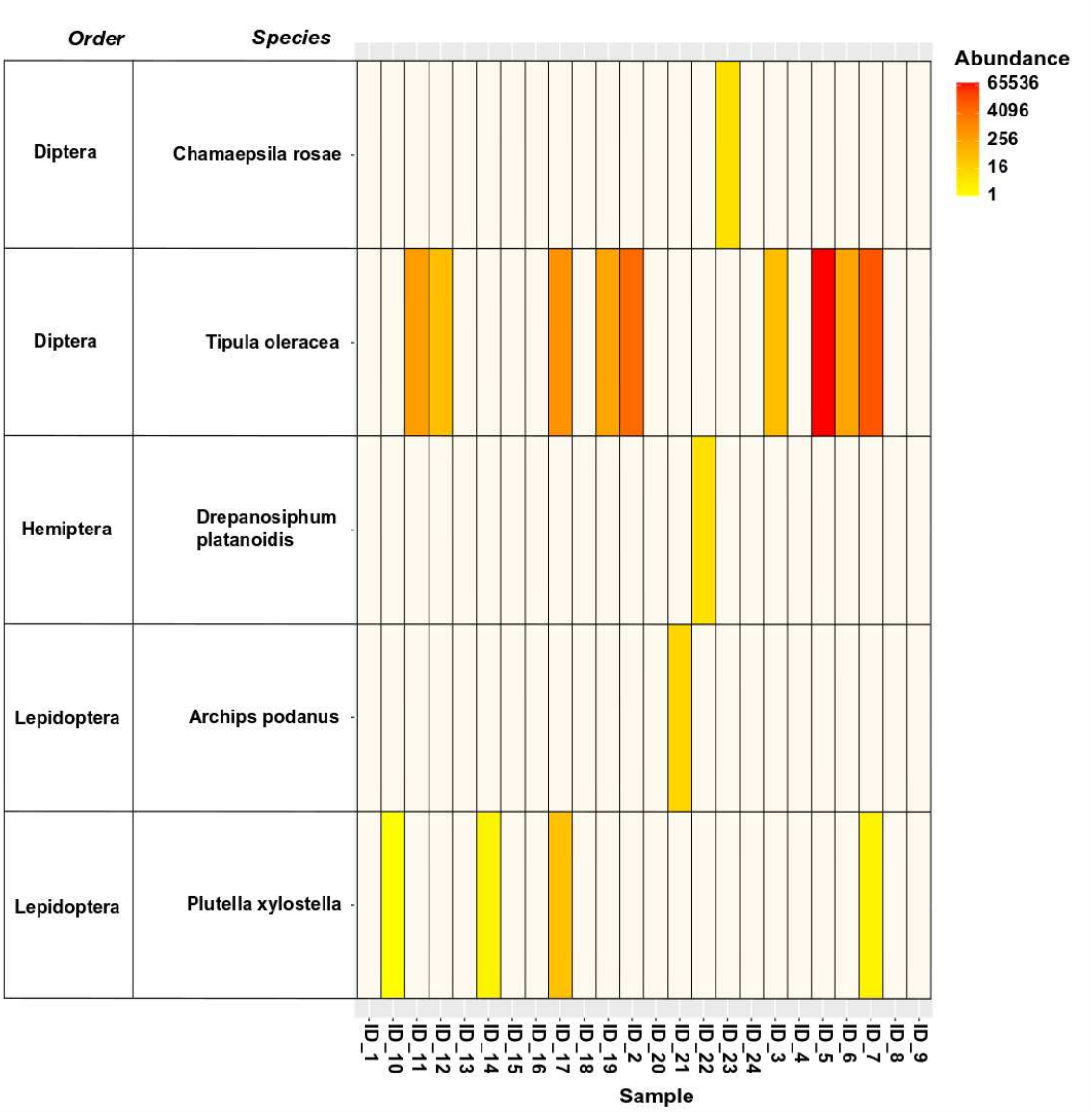

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744 **List of Tables**

745 Table 1: The numbers of families, genera, and species identified within each order (via GenBank and
746 BOLD) that contributed to the overall diet of *R. hipposideros.*

Order	Families	Genera	Species
Lepidoptera	21	70	90
Diptera	15	21	28
Trichoptera	7	10	15
Hymenoptera	3	4	7
Araneae	5	6	6
Neuroptera	1	1	6
Hemiptera	3	3	3
Coleoptera	2	2	2
Crassiclitellata	1	1	2
Glomerida	1	1	1
Isopoda	1	1	1
Total	**60**	**120**	**161**

747

748 Table 2: Statistical analyses (PERMANOVA, ANOVA, PERMUTEST and ANOSIM) performed on the *R.*
749 *hipposideros* diet at order, family, genus, and species taxonomic ranks to understand the influence of
750 roost and sex on the diet.

	Sex					
	PERMANOVA		**ANOVA**	**PERMUTEST**	**ANOSIM**	
	R^2	**Pr(>F)**	**Pr(>F)**	**Pr(>F)**	**Statistic R**	**Significance**
Order	2.73×10^{-3}	0.97	0.97	0.96	-2.11×10^{-2}	0.83
Family	1.92×10^{-2}	0.62	0.82	0.81	1.61×10^{-2}	0.27
Genus	2.26×10^{-2}	0.47	0.65	0.64	1.61×10^{-2}	0.27
Species	2.36×10^{-2}	0.39	0.62	0.61	1.61×10^{-2}	0.27
	Roost Location					
	PERMANOVA		**ANOVA**	**PERMUTEST**	**ANOSIM**	
	R^2	**Pr(>F)**	**Pr(>F)**	**Pr(>F)**	**Statistic R**	**Significance**
Order	3.30×10^{-1}	2×10^{-4}	0.12	0.13	0.19	2×10^{-4}
Family	2.73×10^{-1}	9.999×10^{-5}	0.66	0.67	0.40	1×10^{-4}
Genus	2.64×10^{-1}	9.999×10^{-5}	0.94	0.93	0.40	1×10^{-4}
Species	2.61×10^{-1}	9.999×10^{-5}	0.91	0.92	0.40	1×10^{-4}

751

752 Table 3: List of 38 potential pest species identified in the diet of *R. hipposideros* in the west of Ireland
753 via comparison to Baroja et al. (2019) [1], Tournayre et al. (2021) [2], and the Arthemis database [3].
754 Host plant ranges including native, horticultural and crop species were identified using the Arthemis
755 database.

Order	Species	Host Plant Range
Lepidoptera	*Acleris schalleriana*[1]	*Populus tremula, Viburnum lantana, Viburnum opulus*
Lepidoptera	*Agonopterix conterminella*[3]	*Salix*
Lepidoptera	*Agonopterix nervosa*[3]	*Anthriscus cerefolium, Apium graveolens, Daucus carota sativus, Pastinaca sativa, Pimpinella anisum*
Lepidoptera	*Archips podanus* ***(A. podana)***[3]	*Abies, Alnus, Betula, Citrus, Clematis, Cornus mas, Corylus, Crataegus, Cydonia oblonga, Euonymus japonicus, Fagus, Fraxinus, Heracleum, Juglans, Lonicera, Malus, Picea, Populus, Primula, Prunus, Prunus cerasus, Prunus domestica, Prunus persica, Pyrus communis, Rhododendron, Ribes, Rosa, Salix, Sorbus, Tilia, Trifolium, Vaccinium myrtillus, Vitis vinifera*
Lepidoptera	*Arctia villica*[3]	*Achillea, Centaurea, Cynara scolymus, Erysimum cheiri (hyb.), Fragaria, Lamium, Plantago, Rubus, Taraxacum, Urtica, Vitis vinifera*
Lepidoptera	*Argyresthia conjugella*[3]	*Crataegus, Fraxinus, Malus, Prunus padus, Sorbus, Sorbus aucuparia*
Lepidoptera	*Argyresthia laevigatella*[3]	*Larix, Larix decidua, Larix kaempferi*
Lepidoptera	*Argyresthia spinosella*[1]	
Lepidoptera	*Celypha lacunana*[2]	*Fragaria, Larix, Ligustrum, Mentha, Myosotis, Picea, Primula, Quercus, Ranunculus, Rubus, Salix, Agrimonia, Anthriscus cerefolium, Betula, Caltha palustris, Chrysanthemum, Cirsium, Spiraea, Ulmus, Urtica, Viola*
Diptera	*Chamaepsila rosae* ***(Psila rosae)***[3]	*Apium graveolens, Carum carvi, Daucus carota sativus, Pastinaca sativa, Petroselinum crispum*
Lepidoptera	*Chrysoteuchia culmella*[2]	*Agrostis, Dactylis*
Lepidoptera	*Clepsis spectrana*[3]	*Arundo donax, Centaurea, Cyclamen, Euphorbia, Iris, Lilium, Rosa, Rumex acetosa, Spiraea, Urtica, Viola, Vitis vinifera*
Lepidoptera	*Cnephasia incertana*[1]	*Aster, Centaurea, Chrysanthemum, Cirsium, Dianthus, Fragaria, Lotus, Medicago, Primula, Saxifraga,* *Vicia faba, Vitis vinifera*
Hemiptera	*Drepanosiphum platanoidis*[3]	*Acer campestre, Acer monspessulanum, Acer platanoides, Acer pseudoplatanus*
Lepidoptera	*Epinotia tedella*[3]	*Picea*
Lepidoptera	*Epinotia tenerana*[2]	*Alnus, Betula, Corylus*
Lepidoptera	*Eupsilia transversa*[3]	*Populus*
Lepidoptera	*Exoteleia dodecella*[1]	

Lepidoptera	*Hedya nubiferana*[1/2]	*Alnus, Betula, Crataegus, Fraxinus, Fraxinus excelsior, Malus, Prunus, Prunus armeniaca, Prunus cerasus, Prunus domestica, Prunus dulcis, Prunus persica, Pyrus communis, Quercus, Ribes uva-crispa, Rosa, Salix, Sorbus*
Lepidoptera	*Hedya pruniana*[1/2]	*Crataegus, Malus, Prunus, Prunus cerasus, Prunus domestica, Pyrus communis, Salix, Sorbus*
Lepidoptera	*Hepialus humuli*[2]	*Anemone, Asparagus, Asparagus officinalis, Aster, Beta vulgaris, Brassica napus var. napobrassica, Brassica rapa, Campanula, Cannabis sativa, Chrysanthemum, Convallaria majalis, Cynara scolymus, Dahlia, Daucus carota sativus, Delphinium, Fragaria, Fungi, Gladiolus, Helianthus tuberosus, Humulus lupulus, Iris, Lactuca sativa, Lupinus, Narcissus, Paeonia, Pastinaca, Phaseolus, Phlox, Pisum sativum, Rumex, Solanum tuberosum, Taraxacum*
Lepidoptera	*Hydriomena furcata*[3]	*Abies balsamea, Corylus avellana, Picea sitchensis, Populus, Salix, Salix caprea*
Lepidoptera	*Lomaspilis marginata*[2]	*Betula pendula, Corylus avellana, Populus, Populus nigra, Populus tremula, Salix, Salix aurita, Salix caprea*
Lepidoptera	*Lozotaenia forsterana*[2]	*Campanula, Hedera, Lonicera, Prunus laurocerasus*
Lepidoptera	*Notocelia trimaculana*[3]	*Crataegus*
Lepidoptera	*Odontopera bidentata*[3]	*Abies, Betula, Fagus, Fraxinus excelsior, Larix, Larix decidua, Malus, Picea abies, Pinus sylvestris, Populus alba, Populus nigra betulifolia, Prunus domestica, Quercus, Ribes uva-crispa, Salix, Sorbus aucuparia, Tilia, Tilia platyphyllos, Trifolium pratense, Vaccinium myrtillus*
Lepidoptera	*Orthotaenia undulana*[1/2]	*Acer, Alnus, Betula, Hippophae rhamnoides, Juniperus, Lonicera, Pinus, Salix, Ulmus*
Lepidoptera	*Pandemis cerasana*[2]	*Acer, Acer pseudoplatanus, Betula, Crataegus, Fraxinus, Prunus, Pyrus communis, Quercus, Rhamnus, Ribes, Rosa, Sorbus, Sorbus aucuparia, Tilia*
Lepidoptera	*Pandemis heparana*[2]	*Betula, Forsythia, Lonicera, Malus, Populus, Prunus, Prunus cerasus, Prunus domestica, Prunus persica, Pyrus communis, Salix, Tilia*
Lepidoptera	*Parornix devoniella*[1]	*Corylus*
Lepidoptera	*Phyllonorycter maestingella*[3]	*Fagus sylvatica, Wisteria floribunda*
Lepidoptera	*Phyllonorycter quercifoliella*[3]	*Quercus*
Lepidoptera	*Phyllonorycter salicicolella*[3]	*Salix*
Lepidoptera	*Plutella xylostella*[1/2]	*Brassica napus, Brassica oleracea, Brassica oleracea var. botrytis, Brassicaceae, Capparis spinosa, Cicer arietinum, Fragaria, Matthiola incana, Papaver, Raphanus, Reseda, Tropaeolum*
Lepidoptera	*Prays fraxinella*[2]	*Fraxinus excelsior*

Lepidoptera	*Rhopobota naevana*[3]	*Crataegus, EricaIlex aquifolium, Malus, Prunus domestica, Pyrus communis, Rhamnus, Sorbus, Vaccinium myrtillus*
Diptera	*Tipula oleracea*[1/2]	
Lepidoptera	*Tortrix viridana*[2]	*Populus, Quercus, Quercus robur*

756

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