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

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

Browett et al. (2021) optimised a dual primer approach for the DNA metabarcoding of bat diet using DNA previously extracted from non-invasively collected faeces, previously identified to species, sex and individual level using real-time PCR and microsatellite genotyping (Harrington 2018; Harrington et al. 2019). This approach uses proven good quality and quantity DNA, and excludes low quality samples, and facilitates the inclusion of questions related to sex and individual level dietary preferences. Such questions were previously only addressed in studies that captured bats and placed them in cloth bags to facilitate the collection of faeces (Mata et al. 2016; Galan et al. 2017; 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, Hymenoptera, 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* (R²: 0.00273-0.0236, Pr(>F): >0.05). However, roost location was found to be a statistically significant factor impacting the *R. hipposideros* diet (R²: 0.26115-0.3276, Pr(>F): <0.01) (Table 2). The R² 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 PERMANOVA as sex differences were not found to influence dietary composition. Statistic R values for sex ranged from \(-2.11 \times 10^{-2}\) to \(1.61 \times 10^{-2}\), 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.
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
in this study combined with an increased sample size to provide more statistically robust insights into sex-biased dietary preferences.

**Ecosystem Services**

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

The most infrequently occurring pest items included *Chamaepsila rosae* (Diptera: Psilidae); *Drepanosiphum platanoidis* (Hemiptera: Aphididae) and *Archips podanus* (Lepidoptera: Tortricidae) each detected in one individual with a total of six reads for the former two species and 12 reads in the later. *Chamaepsila rosae* or carrot fly primarily affects crops such as carrots and parsnips (Collier et al. 2020) and has been described as a major carrot pest within Europe (Szwejda and Wrzodak 2007). *Drepanosiphum platanoidis*, an aphid, is a significant pest of ornamental and amenity trees belonging to the genus Acer, particularly, sycamore trees, and can excrete an abundance of honeydew, providing ideal conditions for the growth of moulds such as *Cryptostroma corticale* causing “sooty bark disease”, resulting in tree mortality (Parry et al. 1989; Binggeli and Rushton 1999; Morecroft et al. 2008). *Archips podanus*, the fruit tree tortrix moth (often referred to as *A. podana*) is polyphagous and is considered to be an important pest of fruit trees including apple, plum, and cherry and reduces the quality of the fruit harvested (Hrudová 2003; Stará and Kocourek 2004). Studies have found that the abundance of this species is not influenced by insecticide use, highlighting the value of bat predation for the suppression of this species (Cross 1996; Stará and Kocourek 2004).
The most frequently occurring pest species included *Plutella xylostella* (Lepidoptera: Plutellidae) and *Tipula oleracea* (Diptera: Tipulidae) detected in four and nine individuals with a total of 47 and 102,224 sequence reads respectively. Considered to be a global and economically important pest species, the Diamondback moth, *P. xylostella* is known to be destructive to brassicaceous crops worldwide (Talekar and Shelton 1993; Zalucki et al. 2012; Li et al. 2016). Control strategies for managing this insect pest are met with difficulty as studies have shown a degree of insecticide resistance by this pest species (Talekar and Shelton 1993; Zalucki et al. 2012; Furlong et al. 2013; Xia et al. 2018). The common crane fly (*T. oleracea*) is found throughout Ireland and Europe (cabi.org 2019; Peck et al. 2006; 2008) and is commonly referred to as an agricultural and horticultural pest of winter cereals, brassicas, clover, strawberries, turnips and several other vegetables and ornamentals (Blackshaw and Coll 1999; Peck et al. 2006; 2008).

**Biodiversity**

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

A number of species, unlikely to be present in Ireland, were identified in this study. *Oricia truncata* identified with 98.7% sequence identity across 223 sequence reads occurs exclusively in Central America (Miller 2009). The next closest species level identification acquired from the MOTU generated for this species was for *Prays rucifeps* and *Homorthodes naverca*, both of which had a lower sequence similarity of 97.4%, making it very difficult to suggest an identification for this MOTU. *Tholera americana*, a native species to North America and not present in Ireland was identified in this study (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)).
The application of DNA metabarcoding here has also allowed for the detection of potential vector organisms that have been implicated in the spread of disease. In this study, several mosquito (Diptera: Culicidae) and midge (Diptera: Ceratopogonidae) species were identified including *Culex pipiens*, *Cx. quinquefasciatus*, *Culiseta annulata*, *Cs. morsitans*, and *Culicoides impunctatus*. The mosquito species *Cx. quinquefasciatus* has not previously been reported in Ireland or Great Britain. Across the British Isles, five *Culex* species have been documented, only one of which has been recorded in Ireland, *Cx. pipiens* (Ashe et al. 1991; Folly et al. 2020). Here, *Cx. quinquefasciatus* was detected in two individuals with 328 sequence reads and implies that Irish mosquito species are potentially underestimated and *Culex* species may be more diverse than previously thought. The potential occurrence of this species in Ireland poses a risk for future arthropod-borne disease outbreaks e.g. West Nile Virus and highlights the need for effective and multidisciplinary surveillance methods of vector organisms. However, the sequence region used in this study was very short and often much longer and additional gene regions, such as the second internal transcribed spacer (ITS2) of the ribosomal RNA, are required for accurate species identification and differentiation of the *Culex* complex (e.g. Laurito et al. 2013). In addition, care has to be taken that the originally deposited sequence was also accurately identified. However, the approach of using a predator diet to indirectly survey potential airborne vectors has shown great promise in this study and has the potential to be a powerful surveillance tool.

Additionally, this study adds further records of two Lepidoptera species (*Bactra lacteana* and *Prays ruficeps*) which were recently observed in Ireland (Bond et al. 2017; Bond 2018). Five *Bactra* spp. have been recorded, *B. furfurana* (also recorded in this study), *B. lancealana*, *B. robustana*, and *B. vanosana* (a migrant species). *Bactra lacteana* and *B. lancealana* are said to be highly morphologically similar species. But, in this case *B. lacteana* was identified within the diet of four *R. hipposideros*, with a total of 5725 sequence reads, with 100% sequence similarity and 100% sequence query cover and the species is well represented on the GenBank database. However, no reference DNA barcode exists for *B. lancealana*. *Prays rucifeps*, a micromoth, was detected in one individual with 10,685 sequence reads 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.
to suppress with the use of insecticidal methods, which are also known to be detrimental to wider insect diversity. Our work suggests that promoting and conserving bats and their associated habitats, particularly in areas of crop production, would benefit food producers, bat conservation and insect 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

Figure 1: Distribution of (A) *R. hipposideros* in Ireland [MO: Co. Mayo, GY: Co. Galway, CE: Co. Clare, KY: Co. Kerry, LK: Co. Limerick, CO: Co. Cork] and (B) the roosts sampled for *R. hipposideros* faecal samples for this study.

Figure 2: Donut chart representing the orders (inner circle), families (middle circle), and genera (outer circle) of the identified arthropods in the *R. hipposideros* diet. The numbers in the outer circle refer to the number of species identified within that genus.

Figure 3: Stacked bar plots showing the relative abundance (%) of all orders detected in the diet of *R. hipposideros* across the six roosts sampled (1: Co. Mayo, 2: Co. Limerick, 3: Co. Kerry, 4: Co. Cork, 5: Co. Galway, 6: Co. Clare) and sex (female and male) [Black lines represent the relative read abundance for each MOTU within the respective order].

Figure 4: NMDS plots of samples according to the variable location when MOTUs are agglomerated to order, family, genus, and species (Roost 1 = Co. Mayo, Location 2 = Co. Limerick, Location 3 = Co. Kerry, Location 4 = Co. Cork, Location 5 = Co. Galway, Location 6 = Co. Clare).

Figure 5: Heatmap showing the read abundance of five pest species detected from the *R. hipposideros* diet that are known to be significant pests to the agriculture sector (ID in the sample legend refers to the individual bat sample).
| Order      | Species                  | Abundance |
|-----------|--------------------------|-----------|
| Diptera   | Chamaepsila rosae        | 65536     |
| Diptera   | Tipula oleracea          | 4096      |
| Hemiptera | Drepanosiphum platanoidis| 256       |
| Lepidoptera | Archips podanus     | 16        |
| Lepidoptera | Plutella xylostella   | 1         |
List of Tables

Table 1: The numbers of families, genera, and species identified within each order (via GenBank and 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** |

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

| Sex          | PERMANOVA  | ANOVA  | PERMUTEST | ANOSIM |
|--------------|------------|--------|------------|--------|
|              | $R^2$      | $Pr(>F)$ | $Pr(>F)$ | $Pr(>F)$ | Statistic R | Significance |
| Order        | $2.73 \times 10^{-3}$ | 0.97 | 0.97 | 0.96 | $-2.11 \times 10^{-2}$ | 0.83 |
| Family       | $1.92 \times 10^{-2}$ | 0.62 | 0.82 | 0.81 | $1.61 \times 10^{-2}$ | 0.27 |
| Genus        | $2.26 \times 10^{-2}$ | 0.47 | 0.65 | 0.64 | $1.61 \times 10^{-2}$ | 0.27 |
| Species      | $2.36 \times 10^{-2}$ | 0.39 | 0.62 | 0.61 | $1.61 \times 10^{-2}$ | 0.27 |

| Roost Location | PERMANOVA  | ANOVA  | PERMUTEST | ANOSIM |
|----------------|------------|--------|------------|--------|
| Order          | $9.999 \times 10^{-5}$ | 0.12 | 0.13 | 0.19 | $2 \times 10^{-4}$ |
| Family         | $2.73 \times 10^{-1}$ | 0.66 | 0.67 | 0.40 | $1 \times 10^{-4}$ |
| Genus          | $2.64 \times 10^{-1}$ | 0.94 | 0.93 | 0.40 | $1 \times 10^{-4}$ |
| Species        | $2.61 \times 10^{-1}$ | 0.91 | 0.92 | 0.40 | $1 \times 10^{-4}$ |
Table 3: List of 38 potential pest species identified in the diet of *R. hipposideros* in the west of Ireland via comparison to Baroja et al. (2019) [1], Tournayre et al. (2021) [2], and the Arthemis database [3].

Host plant ranges including native, horticultural and crop species were identified using the Arthemis database.

| Order    | Species                   | Host Plant Range                                                                 |
|----------|---------------------------|---------------------------------------------------------------------------------|
| Lepidoptera | *Acleris schalleriana*²  | *Populus tremula*, *Viburnum lantana*, *Viburnum opulus*                          |
| Lepidoptera | *Agonopterix conterminella*³ | *Salix*, *Anthriscus cerefolium*, *Apium graveolens*, *Daucus carota sativus*, *Pastinaca sativa*, *Pimpinella anisum* |
| Lepidoptera | *Agonopterix nervosa*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, Antheischus cerefolium, Betula, Caltha palustris, Chrysanthemum, Cirsium, Spirea, 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, Daectylis*                                                           |
| Lepidoptera | *Clepsis spectrana*³     | *Arundo donax, Centaurea, Cyclamen, Euphorbia, Iris, Lilium, Rosa, Rumex acetosa, Spirea, 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 |                                                                                   |
| Lepidoptera | *Epinotia tedella*³      | *Acer campestre, Acer monspessulanum, Acer platanoides, Acer pseudoplatanus*     |
| Lepidoptera | *Epinotia tenerana*²     | *Picea*                                                                         |
| Lepidoptera | *Eupsilia transversa*²   | *Alnus, Betula, Corylus*                                                        |
| Lepidoptera | *Exoteleia dodecella*¹   | *Populus*                                                                        |
| Lepidoptera | Hosts                                                                 |
|------------|----------------------------------------------------------------------|
| **Hedya nubiferana** | Alnus, Betula, Crataegus, Fraxinus, Fraxinus excelsior, Malus, Prunus, Prunus armeniaca, Prunus domestica, Prunus dulcis, Prunus persica, Pyrus communis, Quercus, Ribes uva-crispa, Rosa, Salix, Sorbus |
| **Hedya pruniana** | Crataegus, Malus, Prunus, Prunus cerasus, Prunus domestica, Pyrus communis, Salix, Sorbus |
| **Hepialus humuli** | 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 |
| **Hydriomena furcata** | Abies balsamea, Corylus avellana, Picea sitchensis, Populus, Salix, Salix caprea |
| **Lomaspilis marginata** | Betula pendula, Corylus avellana, Populus, Populus nigra, Populus tremula, Salix, Salix aurita, Salix caprea |
| **Lozotaenia forsterana** | Campanula, Hedera, Lonicera, Prunus laurocerasus |
| **Notocelia trimaculana** | Crataegus |
| **Odontopera bidentata** | 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 |
| **Orthotaenia undulana** | Acer, Alnus, Betula, Hippophae rhamnoides, Juniperus, Lonicera, Pinus, Salix, Ulmus |
| **Pandemis cerasana** | Acer, Acer pseudoplatanus, Betula, Crataegus, Fraxinus, Prunus, Pyrus communis, Quercus, Rhamnus, Ribes, Rosa, Sorbus, Sorbus aucuparia, Tilia |
| **Pandemis heparana** | Betula, Forsythia, Lonicera, Malus, Populus, Prunus, Prunus cerasus, Prunus domestica, Prunus persica, Pyrus communis, Salix, Tilia |
| **Parornix devoniella** | Corylus |
| **Phyllonorycter maestingella** | Fagus sylvatica, Wisteria floribunda |
| **Phyllonorycter quercifoliella** | Quercus |
| **Phyllonorycter salicicolella** | Salix |
| **Plutella xylostella** | Brassica napus, Brassica oleracea, Brassica oleracea var. botrytis, Brassicaceae, Capparis spinosa, Cicer arietinum, Fragaria, Matthiola incana, Papaver, Raphanus, Reseda, Tropaeolum |
| **Prays fraxinella** | Fraxinus excelsior |
| Insecta     | Species            | Hosts                                      |
|------------|--------------------|--------------------------------------------|
| Lepidoptera| *Rhopobota naevana* | Crataegus, *Erica aquifolium*, *Malus*, *Prunus domestica*, *Pyrus communis*, *Rhamnus*, *Sorbus*, *Vaccinium myrtillus* |
| Diptera    | *Tipula oleracea*  |                                            |
| Lepidoptera| *Tortrix viridana* | *Populus*, *Quercus*, *Quercus robur*      |
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