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Broad ecological threats of an invasive hornet revealed through a deep sequencing approach

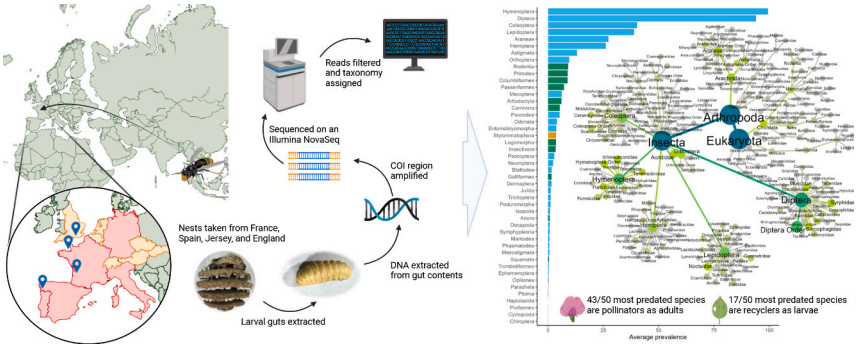
Siffreya Pedersen [a,*], Peter J. Kennedy [b], Thomas A. O'Shea-Wheller [b], Juliette Poidatz [b,1], Alastair Christie [c], Juliet L. Osborne [b,2], Charles R. Tyler [a,*,2]

[a] Biosciences, Geoffrey Pope Building, University of Exeter, Stocker Road, Exeter EX4 4QD, UK
[b] Environment and Sustainability Institute, University of Exeter, Penryn TR10 9FE, UK
[c] Department for Infrastructure and Environment, Government of Jersey, Howard Davis Farm, La Route de la Trinite, JE3 5JP, Jersey

HIGHLIGHTS

- A highly diverse range of invertebrates are predated on by *Vespa velutina*
- Considerable spatiotemporal dietary variation implies a highly flexible predator
- *Apis mellifera* is the most frequently predated species
- The functional groups most likely at risk are wild pollinators and decomposers

GRAPHICAL ABSTRACT



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ABSTRACT

Most terrestrial invertebrates are in considerable decline, and the range expansion of the invasive hornet, *Vespa velutina nigrithorax*, poses an additional threat. Although now found in much of western Europe, the full extent of the hornet's predatory activity remains unexplored. While impacts on honey bees are well-documented, evidence of a wider dietary spectrum is emerging, indicating potentially broad ecological ramifications. Here, we conduct the first large-scale study of the diet of *V. velutina*, utilising deep sequencing to characterise the larval gut contents of over 1500 samples from Jersey, France, Spain, and the UK. Our results indicate that *V. velutina* is a highly flexible predator, enabling its continued range expansion capacity. Analyses detected 1449 taxa, with greater prey richness in samples from southern latitudes, and considerable spatiotemporal variation in dietary composition. Hymenoptera, Diptera, Hemiptera, Coleoptera, Lepidoptera, and Araneae were the most frequently occurring orders predated, each characterised by high taxonomic diversity. The honey bee *Apis mellifera* was the most abundant species, being found in all sampled nests and showing greater relative read numbers with increasing apiary density and proximity, supporting concerns for the impact of *V. velutina* on apiculture. Notably, 43 of the 50 most commonly predated invertebrates were also flower visitors, including 4 common bumblebee

* Corresponding authors.
E-mail addresses: s.pedersen@exeter.ac.uk (S. Pedersen), c.r.tyler@exeter.ac.uk (C.R. Tyler).
[1] Present address: Cirad-Bios-Umr-Pvbmt, Station Ligne Paradis – Pole de Protection des plantes, 7 chemin de l'IRAT, 97410 St Pierre, La Réunion.
[2] Juliet L. Osborne and Charles R. Tyler should be considered joint senior author.

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species, indicating potentially substantial risks to wild pollinators. These data provide wide and deep evidence to support risk evaluation of this species and its potential environmental impact as it spreads across Europe.

1. Introduction

Most terrestrial invertebrate populations are declining in Europe and globally (Dirzo et al., 2014; Hallmann et al., 2017; van Klink et al., 2020) with potentially considerable costs to ecosystem functions and services they provide including pollination, decomposition, and pest control (Eisenhauer et al., 2023). The drivers of the decline, although not fully understood, are largely attributed to land-use change, agriculture, and chemical pollution. Invasive species, however, can also add to invertebrate population pressures and the spread of *Vespa velutina nigrithorax,* commonly known as the Asian hornet or Yellow-legged hornet, a voracious invasive predator, poses an additional threat to the invertebrate species it predates on. Originating from Southeast Asia, *V. velutina* has already become an established invasive species in South Korea (Choi et al., 2012), Japan (Takahashi et al., 2019), and most of Western Europe where its range is still expanding (Apiservice, 2023; Goldarazena et al., 2015; Government of Jersey, 2019; Grosso-Silva and Maia, 2012; Haxaire et al., 2006; Jones et al., 2020; Porporato et al., 2014; Ries et al., 2021; Rome et al., 2012; Schorkopf et al., 2024; Smit et al., 2018; States of Guernsey, 2022; Villemant et al., 2006; Witt, 2015). Nests have also recently been found in Central Europe and North America (Hoebeke et al., 2024; Márta and Vas, 2023; Purkart et al., 2024; Walter et al., 2024). Understanding of the diet of the *V. velutina* in their new range is fundamental to assess the level of risk(s) to native invertebrates.

Vespa velutina predates upon social Hymenoptera, and actively targets the Western honey bee, *Apis mellifera*, leading to major economic concerns for the beekeeping sector (Monceau et al., 2014; Requier et al., 2023). The hornets hunt *A. mellifera* by "hawking" outside of hive entrances and intercepting returning workers, or by targeting individual foragers at floral patches. Unlike the Eastern honey bee, *Apis cerana*, which has evolved defensive mechanisms such as killing the hornets through thermal shock (bee balling) (Ken et al., 2005), *A. mellifera* has no effective defence against *V. velutina*. Furthermore, attacks on *A. mellifera* hives can reduce foraging effort to a point that threatens colonies with starvation, termed "foraging paralysis" (Arca et al., 2014). A recent study modelling the effects of the hornets on apiculture in France estimated that hornet-related honey bee colony losses could reach 29.2 % in some cases, resulting in a national annual economic cost of € 30.8 million (Requier et al., 2023). Moreover, some studies have indicated that *V. velutina* has a broad invertebrate prey spectrum (Perrard et al., 2009; Rome et al., 2021), and negatively impacts pollination efficacy in native plants (Rojas-Nossa et al., 2023; Rojas-Nossa and Calviño-Cancela, 2020), suggesting that many wild insects could be at risk, and thus implying a wider ecological impact profile for this invasive predator.

To date, there have been no large-scale investigations into the diet of *V. velutina* in Europe that capture geographical and seasonal differences. Indeed, those studies that have been undertaken thus far have been limited both spatially and temporally. Most work in Europe has been conducted in France (Perrard et al., 2009; Rome et al., 2021; Villemant et al., 2011), with single studies also carried out in Spain (Rojas-Nossa and Calviño-Cancela, 2020), Portugal (Verdasca et al., 2022), and the UK (Stainton et al., 2023); all being restricted to the summer months (July onwards). Notably, these omit the period when primary nests are being established in the spring, and thus miss a key embryonic stage in the life-cycle of *V. velutina* colonies (Perrard et al., 2009; Rojas-Nossa and Calviño-Cancela, 2020; Rome et al., 2021). This is pertinent, as embryo nests are founded and cared for by single queens until workers emerge, while secondary nests are built later in the year (sometimes following relocation) to house its growing population (Rome et al., 2015) and might have different predatory habits. Furthermore, predation on other social insects during the early stages of their colony development has the potential to have a far greater impact on prey colony development and populations than predation in summer or autumn when abundance is already high. Consequently, to fully assess the potential risks posed by *V. velutina* to invertebrate biodiversity and associated ecosystem services, large-scale studies including multiple invaded regions, and spanning the full colony life-cycle, are needed.

Previous studies conducted on the diet of *V. velutina* have generally used morphological methods to identify prey pellets taken from hornets as they return to the nest and are thus limited in accuracy and comprehensiveness (Perrard et al., 2009; Rome et al., 2021; Villemant et al., 2011). In one such study, almost a third of the pellets could not be identified beyond the rank of (invertebrate) order, and less than a third of soft-bodied insects and pieces of vertebrate flesh were identifiable, potentially introducing bias to their results (Villemant et al., 2011). Molecular techniques can provide a cost and time effective alternative to these traditional morphological methodologies, while mitigating for errors in sample detection and identification. Molecular sequencing, however, confers its own set of limitations such as primer biases, incomplete databases and mislabelled reference sequences for sequence identification (Keck et al., 2023; Krehenwinkel et al., 2017). Despite this, previous usage in the context of dietary analyses has shown great promise, with Rome et al. (Rome et al., 2021) finding that 99 % of sequenced prey pellets that produced barcodes could be identified to the species level. Molecular sequencing can also identify prey items from digested or partially digested material, allowing for the detection of prey from gut contents (Hagler et al., 2013; Saqib et al., 2021; Stainton et al., 2023; Unruh et al., 2016) or faeces (Lefort et al., 2020). Prey are collected by foraging adult *V. velutina* to feed the larvae within their nests to provide their requirement for protein and other essential nutritional components. Throughout the larval stage, larvae are fed by nest-resident adults and the larval gut is emptied only when fecal waste (the meconium) is ejected by the last larval instar just before pupation. Consequently, analysis of DNA in the larval gut has the potential to reveal the diversity of prey consumed by a larva from emergence until the point of collection. Applying larval gut sequencing analysis can therefore more readily reveals the full diversity of prey entering a nest over a longer period than would be measurable from sampling individual prey pellets returned by foragers to the nest at individual timepoints.

Through deep sequencing of gut samples from >1500 *V. velutina* larvae originating from 103 nests, the aim of this study was to provide the first large-scale dietary analysis of *V. velutina* across European regions. Data encompassed southwest France, northwest Spain, the island of Jersey, and the UK, and spanned the entire hornet colony life-cycle, thus supporting a more comprehensive assessment of the potential ecosystem-level impacts of this invasive species. To this end, we sought to establish how the predation of *V. velutina* on invertebrates varied geographically and seasonally, and how this in turn related to the surrounding landscapes, allowing for assessments of which species are potentially most at risk, and if any are species of conservation concern (determined using the IUCN Red List [IUCN, 2023a]). We further aimed to assess the relative impact of *V. velutina* specifically on *A. mellifera* and on particular invertebrate functional groups: pollinating insects, recyclers (decomposers), and pest species. We evidence *V. velutina* as a highly adaptable predator with an incredibly wide array of invertebrate prey, spanning the orders Hymenoptera, Diptera, Hemiptera, Coleoptera, Lepidoptera, and Araneae with considerable dietary species variation across seasons and geographical regions. Our analyses of larval gut contents additionally highlight *V. velutina* as a potentially serious threat to wild insect pollinators, and possibly to 'recycling' insects (flies and

wasps) across Europe as well as to apiculture.

2. Methods

2.1. Sample collection and preparation

2.1.1. Study area and nests

Larvae were sampled from as wide a range of nests from four regions and across the hornet's flight season (April – November) as possible (Supplementary Table 1). As part of national containment or eradication strategies, nests were reported to authorities or researchers in each of the four regions sampled: Jersey – a self-governing British crown dependency off the coast of north-west France and largest of the Channel Islands; Aquitaine – the administrative region of Nouvelle-Aquitaine in south-west France, in which *V. velutina* were first reported in Europe approximately 20 years ago; Galicia – an autonomous region in north-west Spain; and the UK – the United Kingdom, with the two nests analysed in this study stemming from southern England.

The nests used in this study were collected between 2020 and 2022, separately or in conjunction with the authors, by local partners (Government of Jersey and Jersey Asian Hornet Group; INRAe Nouvelle-Aquitaine Bordeaux; University of Vigo – both Vigo and Ourense campuses; and Animal and Plant Health Agency) and their beekeeping contacts. Depending on accessibility and colony stage/size, nests were either collected with adults alive but contained, subdued by anaesthetic (chilling or carbon dioxide) or by treatment with an authorised insecticide. On collection, the nests were quickly transported to a facility where they were frozen (−20 °C) or the larvae were collected fresh, within 48 h of treatment (with the exception of 72 h for the two UK nests). The date and location (region, department/parish, site descriptor, latitude & longitude, elevation) at which nests were collected as well as attachment points, approximate height, and a brief habitat descriptor of nest surroundings were recorded on location whenever possible.

Nests were dissected in partner facilities in the region of collection. Details concerning nest dimensions were recorded, enabling them to be categorised as embryo, small, or large nests. Dissections did not routinely determine whether the nests were primary nests (incorporating the queen's original embryo nest) or secondary nests (following expansion in size and population, sometimes after relocation and abandonment of the primary nest). Consequently, embryo nests were defined as those solely cared for by the queen (absence of workers; 1–2 combs; and < 8 cm in diameter); small nests were defined by presence of workers, typically 2–4 combs, < 32 cm diameter, and < 25 cm length; and large nests with workers and typically 4–9 combs, > 25 cm diameter, and > 25 cm in length. Overall, 11 embryo, 44 small and 37 large nests were defined; 11 nests could not be assigned a category due to insufficient nest parameters being collected.

2.1.2. Larval collection and preparation

Larvae, from unsealed cells, were collected from dispersed areas of nests to minimise sampling larvae that may have been fed by the same worker during a feeding event. The degree to which this was possible differed with the size of the nest and the extent to which larvae were distributed across multiple combs. Typically, 20–25 larvae were collected from a nest, when available (range 3 to 37 larvae per nest). Only larvae visually in good condition were sampled (larvae collected from nests not frozen were typically alive despite any nest treatment; in frozen nests, larvae with creamy-white colouration and clear segmentation were judged as of suitable condition). The intention was to sample a range of instars whenever possible, but in practice 1st & 2nd instars were prone to being damaged during collection and less likely to yield sufficient prey DNA for analysis such that sampling focussed on 3rd to 5th instar larvae. Larvae were removed with clean forceps and placed immediately in separate 2–5 ml vials filled with >98 % ethanol. These were held at room temperature until ready for shipment to the Environment and Sustainability Institute, University of Exeter, Penryn campus.

Each larva was then weighed, the head capsule removed, and its width measured via microscopy using an eyepiece graticule to determine its instar. The body of the larva was opened longitudinally with a sterile scalpel along a dorsal line following the direction of the digestive tract to reveal the ventriculus, which was then carefully removed while scraping as much fat off the ventriculus surface as possible. The ventriculus was placed in a clean vial with fresh >98 % molecular-grade ethanol before being transported to the Biosciences department, University of Exeter, Streatham campus, for DNA extraction.

2.2. DNA extraction and library preparation

Trialling multiple DNA extraction procedures (including the Zymo research Quick-DNA Miniprep plus kit Zymo Quick-DNA Fecal/Soil Microbe Miniprep kit, GeneAll ExGene Stool mini kit, and in-house CTAB and Chelex protocols), Qiagen's Blood and Tissue kit was found to provide the most consistent yield of DNA that was amplifiable by PCR. DNA extractions of all gut contents were therefore undertaken using Blood and Tissue kits in 96 well-plate format (Qiagen), following the manufacturers protocol with modifications. To allow for the entire gut content to be extracted with the DNeasy kit, therefore providing the most representative sample of DNA from each gut, each sample was weighed and binned into weight categories of 20 mg (the maximum starting material according to the manufacturer) and an additional 180 µl of buffer ATL and 20 µl of proteinase K were added for each category (i.e. 180 µl buffer ATL and 20 µl proteinase K was added to samples weighing 20 mg or less, 360 µl buffer ATL and 40 µl proteinase K added to samples weighing over 20 mg but equal to or under 40 mg, etc.). To reduce the amount of host DNA extracted, the gut was slit lengthwise with a bleach sterilised scalpel and the gut lining was removed with bleach sterilised forceps while shaking the gut contents into the lysis buffer. The samples were bead beaten at 30 Hz for 40 s in buffer ATL with 0.1 g of 1.4 mm ceramic beads and 0.1 g garnet matrix prior to proteinase K addition and incubation in an oven at 56 °C for 3 h under constant rotation. Lysis of the gut contents was undertaken in batches of 2–4 nests at a time, and a blank extraction was included with every batch. After incubation with proteinase K, the lysates were stored at room temperature (for no longer than 6 months, as advised by the manufacturer, Qiagen) until a suitable number were prepared to be purified with the 96 well-plate kit. At that time, the lysates were centrifuged to pellet undissolved particles, and 200 µl of clear supernatant was transferred to the Qiagen collection tubes for DNA extraction from tissue in 96 well-plate format, following the manufacturer's protocol for extraction from tissue. Two wells of each plate were used for a no-template PCR control and a positive PCR control of a mock community mixed sample of 13 DNA extracts of likely hornet prey taxa (*A. mellifera, Bombus hortorum, Episyrphus balteatus, Eristalis arbustorum, Lucilia ampullacea, Sarcophaga variegata, Bombyx mori, Tribolium castaneum, Typhaeus typhoeus, Euthrix potatoria, Araneus diadematus, Eratigena atrica*, and *Leiobunum rotundum*). DNA was eluted into 50 µl AE buffer, with 5 min of incubation at 56 °C.

A short fragment of the cytochrome *c* oxidase I gene was chosen as the target sequence for identifying the prey species in the larval guts, that was amplified with the primers mlCOIintF (Leray et al., 2013) and HCO2198 (Folmer et al., 1994). This primer pair has been shown to amplify a wide range of arthropods (Leray et al., 2013) and successfully amplified all 13 species in the mock community we established for this study. A nested barcode approach was used to tag individual samples to allow all of them to be sequenced in a single sequencing run, thus removing possible batch effects from the library preparations and different sequencing runs (Supplementary Figs. 1 and 2). The primers used had 12 nt barcodes taken from Caporaso et al. (2012) on both forward and reverse primers to produce 96 sets with unique dual indices, with a GA spacer between the barcode and primer (Supplementary

Fig. 1). Unique dual indices were used to allow instances of barcode hopping to be detected and removed. Individual samples were thus tagged in the first PCR, which consisted of 25 μl OneTaq 2× master mix with standard buffer, 0.04 mg/ml BSA, 5 ng template DNA, 0.2 μM of each primer, made up to 50 μl with HPLC water. Thermocycling conditions were as follows: initial denaturation at 94 °C for 3 min, 25 cycles of 94 °C for 30 s, 46 °C for 30 s, 68 °C for 30 s, and a final elongation step at 68 °C for 5 min. Each sample was then visualised on a 2 % agarose gel to assess PCR success, cleaned with 0.8× SPRI beads, and quantified with the QuantiFluor ONE dsDNA System (Promega). Each library of a 96 well plate was then pooled in an equimolar manner, and Illumina sequencing adapters and further indexes were ligated to each pool (carried out by the Exeter Sequencing Service). All libraries were subsequently sequenced on a NovaSeq 6000 to generate 250 bp paired-end reads, aiming for 100,000 reads per sample.

2.3. Bioinformatics

Data were received from the Exeter Sequencing Service in a partially demultiplexed state, with each plate demultiplexed with their plate specific indexes. Individual samples from each plate were then demultiplexed with Cutadapt v3.5 (Martin, 2011) in paired-end mode, using anchored adapters and default settings. As the Illumina adapters were added by ligation, the resulting R1 and R2 files contained an even mix of sequences with forward and reverse primers. To reorientate the reads and make the data suitable for DADA2 analysis (Callahan et al., 2016), demultiplexing was completed in two rounds: in round 1 the adapters and input files were given in the standard manner, in round 2 the input files were the unknown sequences produced from round 1, with unknown_R2 being provided as the first input file and unknown_R1 as the second input file. This method produced four files for each sample, with all sequences orientated correctly. Each round of demultiplexed samples was then run through the DADA2 pipeline separately, thus allowing DADA2 to infer error models separately for forward and reverse reads (DADA2 parameters: truncLen = c(180,180), maxEE = 1, pooling = pseudo). Sequences differing in length by more than one base pair from the expected 313 bp were removed. The resultant sequence tables for each round were combined prior to chimera removal with removeBimeraDenovo (method = "pooled"). The actual sequence variants produced by DADA2 were clustered into operational taxonomic units with Swarm v3 (Mahé et al., 2021), using a *d* value of 13 as this has been shown to produce approximate species level OTUs with the CO1 gene (Antich et al., 2021). Taxonomic assignment was carried out in two rounds; in the first round all sequences were assigned to kingdom level using a blastn search against the GenBank database and taking only the first hit. All metazoan sequences were then compared to the MIDORI2 unique CO1 database vGB253 (Leray et al., 2022) with blastn. Any OTUs that could not be identified to at least order level were removed. One OTU that identified to *Episyrphus viridaureus* was manually changed to *Episyrphus balteatus*, based on the frequency of observation and the geographical ranges of these species.

2.4. Data curation

The R package LULU (Frøslev et al., 2017) was used to identify erroneous OTUs and merge them with their parent OTUs. The data was then filtered to remove background noise and very low levels of contamination, based on the results produced from the positive, no template, and blank extraction controls. Any OTUs that had <50 reads in the entire dataset were removed, any read counts that were lower than the maximum read count in a negative control were set to 0, and any read counts that were under 0.2 % of the maximum read count in that sample were also set to 0. Reads identified as *V. velutina* were removed, and any samples with 15,000 reads or less were discarded; this number being derived by inspecting a rarefaction curve (Supplementary Fig. 3) and aiming to remove samples with too few prey reads to obtain reasonable diversity, while minimising sample loss. Where multiple OTUs were assigned to the same species these were agglomerated. Finally, an alignment of all remaining arthropod sequences was inspected for indels, indicative of nuclear mitochondrial pseudogenes (Song et al., 2008), and one OTU that identified to Apidae was removed.

2.5. Data analysis

To mitigate the effects of primer bias, read numbers were transformed into presence/absence. Following this, to account for the different numbers of larvae taken from each nest, and to obtain a quantitative estimate of predation intensity, the data were further transformed to within nest prevalence, using the following formula:

$$\text{Taxon prevalence in nest} = \frac{\text{Number of larvae with the taxon present in a nest}}{\text{Total number of larvae analysed from the nest}}$$

The mean prevalence across all nests was then calculated to assess the taxa most at risk.

Unless otherwise stated, all data analysis was conducted in R version 4.1.0 (R Core Team, 2021). Sequencing data were manipulated using the package phyloseq (McMurdie and Holmes, 2013), the heat tree was produced with the package metacoder (Foster et al., 2017), and all other plots were created with ggplot2 (Wickham, 2016).

2.5.1. Functional groups and species of concern

To assess the potential impact of *V. velutina* on different functional groups of invertebrates, the functional traits of the top 50 most prevalent taxa were collated. For each of these taxa (45 identified to species level; 5 to family level), information on adult food source, larval food source, and ecological function was collated from multiple reliable internet sources (GBIF.org, 2023; inaturalist.org, 2023; naturespot.org.uk, 2023) and Chinery (2012). Food sources were defined as flowers (if visited for nectar and/or pollen), plant tissue (vegetative, sap, fruit), live prey, flesh, carrion, dung, or rotting vegetation and detritus. Ecological function was then defined as potential pollinator, pest, predator, recycler (saprophage or sarcophage), or parasite. Some species had more than one function, and no estimate of quality of their functionality was available.

To explore whether any of the identified arthropods are known to be of conservation concern, all 749 arthropods that were identified to species level were compared to the IUCN Red List (IUCN, 2023a) of threatened European arthropods.

2.5.2. Land cover characterisation

To characterise the land cover surrounding colonies, satellite data were extracted from 500 m radii around each sampled nest, using the 2021 ESA WorldCover (Zanaga et al., 2022) dataset in QGIS (release v. 3.26.3). This provided high fidelity, comprehensive, and temporally relevant land cover data to a resolution of 10 m, utilising a combination of optical and Synthetic-aperture radar (SAR) sensors. The aforementioned distance was selected to best encompass the predicted median

foraging range of colonies (Budge et al., 2017; Poidatz et al., 2018), thus accounting for the majority of possible prey loci. Surrounding land cover could not be measured for 6 nests from Aquitaine as they did not have corresponding GPS coordinates.

Data extraction yielded a total of 8 land classes, with these consisting of tree cover, shrubland, grassland, cropland, built-up areas, bare ground, water bodies, and wetland. These were derived from the full set of 11 possible land classes designated in the ESA WorldCover dataset (Zanaga et al., 2022), specifically excluding the classes snow and ice, mangrove, and moss and lichen, as these were absent from the sampled regions. Following land cover extraction, classes were validated visually using the Esri 2022 satellite imagery WGS84 basemap (Esri, 2022) at a resolution of $\leq$1 m. Prior to incorporation into analyses, all land cover data were centre log-ratio transformed to better represent the relative variation in constituent classes, as is recommended for compositional data (Pawlowsky-Glahn and Egozcue, 2006).

2.5.3. Apiary data collection and analysis

To determine the density and proximity of apiaries surrounding colonies and provide a measure of the availability of *A. mellifera* to foraging *V. velutina*, the Government of Jersey 's 2023 apiary information dataset was utilised. This provided the pseudonymised location of all known apiaries, concordant with the Government of Jersey's requirement for all current beekeepers to be registered ("Diseases of Animals (Bees) (Jersey) Order, 2013," n.d.). The aforementioned data were used to calculate the number of apiaries within 500 m of each colony, and the absolute distance to the nearest apiary. Resultant analyses were restricted to the 68 colonies on the island of Jersey, as it was not possible for the authors to obtain reliable apiary locations from the other sampling regions.

These metrics were then tested for correlation with relative read abundances (RRAs) of honey bee in each of the 968 larvae from Jersey using Kendall's tau-b test for correlation. The RRA of honey bee from individual larvae were used for this analysis rather than nest prevalence, as the nest prevalence was too high and constant to offer any variability. Although the RRA will be affected by primer bias, it has been shown that the relative changes in the RRA of a species in a single study can be indicative of changes in that species' abundance (Elbrecht and Leese, 2015).

2.5.4. Nest attributes relating to prey diversity

A generalised linear mixed model with a Poisson distribution was used to explore which environmental factors (region, month collected, and landcover within 500 m) and nest attributes (stage, and head width as an age proxy for the larvae) affected the diversity of prey items within the larvae. As 14 nests did not have corresponding surrounding land cover and/or nest stage estimates, these were excluded from the model; a total of 1346 larvae from 89 nests were included (Table 1). The R package glmmTMB (Brooks et al., 2017) was first used to fit a full model with the observed richness within each larva as the dependent variable and region, nest stage, year, month, head width, the interaction between nest stage and head width, and all land cover estimates as fixed effects, and nest identity as a random effect. Backward selection was then used to remove predictors that did not improve the model, specifically removing the predictor with the highest AIC value produced by the drop1 function, until the AIC of the model was minimised. The package DHARMa was then used to assess model fit via analysis of the residuals.

Table 1
Sample numbers of the data used in the GLMM, by the factors selected by backward selection. Nest numbers are outside of brackets, and the total number of larvae from those nests within brackets.

		EMBRYO	SMALL	LARGE
2020	Jersey	4 (15)	10 (102)	6 (103)
	Aquitaine	0	9 (166)	9 (166)
	Galicia	0	0	0
	UK	0	0	0
2021	Jersey	3 (42)	13 (236)	15 (289)
	Aquitaine	0	0	0
	Galicia	0	3 (45)	3 (26)
	UK	0	0	2 (50)
2022	Jersey	3 (7)	9 (99)	0
	Aquitaine	0	0	0
	Galicia	0	0	0
	UK	0	0	0

2.5.5. Distance-based redundancy analyses

The effects of environmental factors and nest attributes on the composition of diet were investigated using distance-based redundancy analysis (db-RDA) (Legendre and Anderson, 1999; Mcardle and Anderson, 2001) via the function capscale from the R package vegan (Oksanen et al., 2022). To remove noise from the data and detect changes in the main prey species, only taxa that were found in at least 5 % of the larvae of at least 2 nests were included. Embryo nests were not included, due to the small number of larvae sampled from each nest. Bray-Curtis dissimilarity was used to quantify differences in species composition, calculated from the nest level prevalence. Forward selection was performed to select the best fitting model (vegan function ordistep, 9999 permutations) utilising the full scope of factors including region, nest stage, month collected, year collected, all surrounding land cover estimates, and the interactions between region and month, region and nest stage, and month and nest stage. Significance of the model and of environmental variables was then tested using an ANOVA-like permutation test (function anova.cca, by = "margin"). Where significant interactions were found, the data were then divided into subsets to allow the significance of the main effects to be investigated without the influence of interactions (Legendre and Legendre, 2012, pp. 655–656). To analyse the effect of month, only data from Jersey were used, as this was the only region with samples from the entire hornet activity season of *V. velutina*. Only data from September were used to analyse the effect of region, as it was a month of peak hornet activity and thus had the most even distribution of samples from each region. The function betadisper from the vegan R package was used to assess for heterogeneity of dispersions in all categorical variables selected. Where significant heterogeneity of dispersions occurred, PCoA plots were inspected, and a modified one-way PERMANOVA (Anderson et al., 2017) that is robust to heterogeneity of dispersions in unbalanced designs, was used to assess whether the significance of the db-RDA was due to different average dietary composition, or different dispersions. To visualise how taxa changed with environmental factors, partial db-RDAs were used, with the variable of interest as the constraint, and all other significant variables as conditions.

3. Results

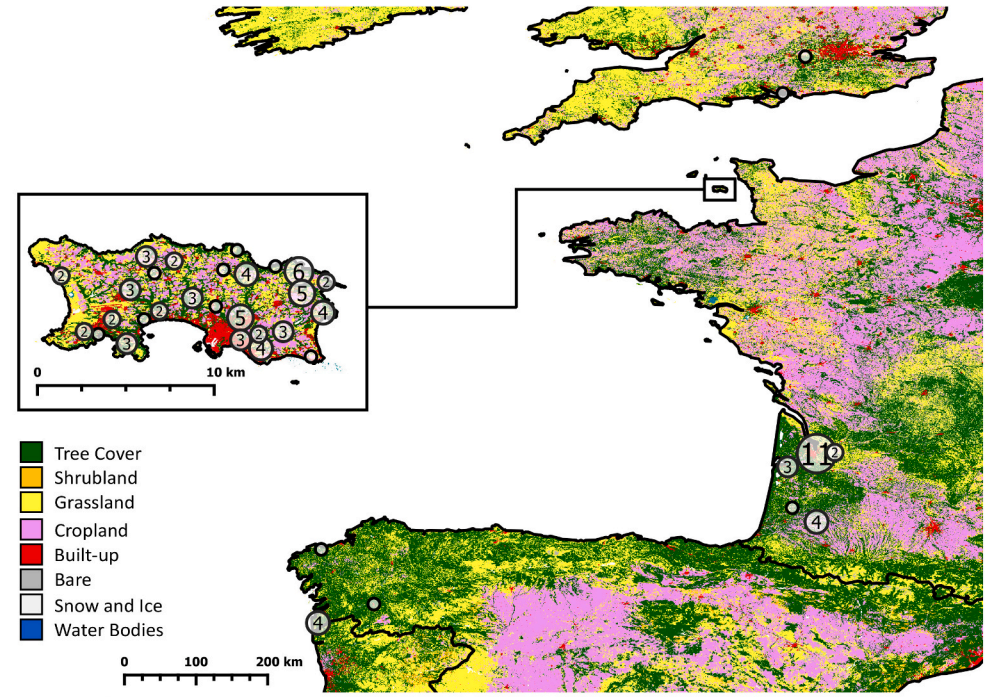
3.1. V. velutina dietary diversity and composition

After all quality filtering steps, 129 million reads were obtained, averaging 84,000 reads per sample. Sufficient quantity and quality of data were obtained from a total of 1545 larval samples from 103 nests: 68 from Jersey, 25 from Aquitaine, 8 from Galicia, and 2 from the UK (Fig. 1). The average number of larvae analysed per nest was 15, although this ranged from 1 to 30 (numbers of larvae analysed per nest are provided in the Supplementary Table 1).

Overall, 1449 operational taxonomic units (OTUs) were detected, 55.1 % of which could be identified to species level (accounting for 86.3 % of reads). All 13 species of the mock community were detected in every positive control with no additional species other than one unidentified mite that was detected in two samples.

Within the 1449 OTUs, 26 orders were detected and all but 51 OTUs detected were Arthropods. Seven orders of Arthropods accounted for 86.7 % of all OTUs detected: Diptera were most diverse with 561 OTUs, Hymenoptera with 252, Lepidoptera with 169, Coleoptera with 137,

a



b

	2020								2021								2022		
	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Apr	May	Jun
Jersey	0	4	3	5	6	3	1	0	0	2	2	2	5	11	10	2	2	5	5
Aquitaine	0	0	2	3	3	6	5	1	0	0	0	2	2	1	0	0	0	0	0
Galicia	0	0	0	0	0	0	0	0	0	1	1	1	0	4	1	0	0	0	0
UK	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0

Fig. 1. Spatial and temporal distribution of the nests analysed. a) Spatial distribution of the nests analysed. The size of and number within circles shows the number of nests analysed from that location. Six nests, 4 from Aquitaine and 2 from Galicia, did not have corresponding GPS data, and so are not included in the map. b) The number of nests analysed from each region, by year and month.

Hemiptera with 84, Orthoptera with 70, and Araneae with 53 OTUs (Fig. 2). Many detected OTUs identified as taxa that were unlikely to be predated on directly by *V. velutina*, such as microscopic mites within Astigmata, and multiple vertebrate orders including Primates (all of which was human, see discussion).

The most abundant orders (which were also among the most diverse) were Hymenoptera, occurring with an average prevalence of 99.5 %, Diptera at 94.0 %, Coleoptera at 40.3 %, Lepidoptera at 38.9 %, Araneae at 27.3 %, and Hemiptera at 26.1 % (Fig. 3). Although there was a high diversity of Orthoptera, the average prevalence of this order was substantially lower at 9.5 %.

At the species level, the honey bee, *Apis mellifera,* was the most frequently predated insect, occurring in every nest with an average prevalence of 98.1 %, followed by the common wasp, *Vespula vulgaris,* found in 75.7 % of nests with an average prevalence of 52.2 %, and the blow fly, *Calliphora vicina,* found in 77.7 % of nests with an average prevalence of 51.7 % (Fig. 4).

Aside from *A. mellifera* which had consistently high prevalence throughout the active flying period of *V. velutina*, the prevalence of most OTUs followed seasonal trends for those species, which often varied between geographical regions (Fig. 4).

3.2. Impact on functional groups and species of concern

Out of the top 50 most commonly occurring prey species, 43 were potential pollinators (feeding on nectar or pollen as adults) and this included 3 of the most dominant European crop pollinators – *A. mellifera*, *Bombus terrestris* and *Bombus lapidarius* (Kleijn et al., 2015). In fact, all of the top 10 species by prevalence were flower visitors. Also included in the top 50 prey species were 4 well-known crop plant pests - *Tipula paludosa* (European cranefly), *Merodon equestris* (Narcissus bulb fly), *Drosophila suzukii* (spotted wing fruit fly), *Delia platura* (seedcorn maggot); and one OTU identified to the family of click beetles Elateridae, which contains several plant pests. When considering their feeding habits as larvae, the top 50 prey species displayed greater functional diversity, with 7 feeding on pollen, 17 classed as recyclers (mostly flies and wasps feeding on dung, carrion, and/or vegetation, 3 of which also feed on live animal wounds), 10 phytophagous (not including pollen feeders), 9 predators, 1 parasite, and 4 that could not be assigned due to low taxonomic resolution.

Of all identified species, there were 20 matches with the IUCN red list (IUCN, 2023a). Of these, 2 were classed as near threatened, one as data deficient, and the remaining 17 classed as least concern. The two near threatened species were *Epeolus cruciger* and *Platycheirus fasciculatus* and were detected only in 1 and 3 larval guts, respectively.

3.3. Effect of nest attributes on dietary richness

The richness of OTUs detected in the larval guts ranged widely, from 1 to 71, with a mode of 15 and mean of 16.4. Galicia and Aquitaine both had higher larval gut richness than Jersey and the UK; additionally, the year 2021 had higher prey richness than 2020 and 2022 (Supplementary Table 2). Subsetting the data to Jersey or Aquitaine (the only regions with data over multiple years) showed year to be a significant factor in Jersey, but not in Aquitaine. Specifically, the year 2021 in Jersey had a higher and earlier peak in species richness than 2020 (Supplementary Fig. 11). An interaction was found between nest stage and larval head width: namely that richness increased with head width faster in embryo nests than in small or large nests (Supplementary Fig. 12). While the overall larval gut richness in small and large nests was not significantly

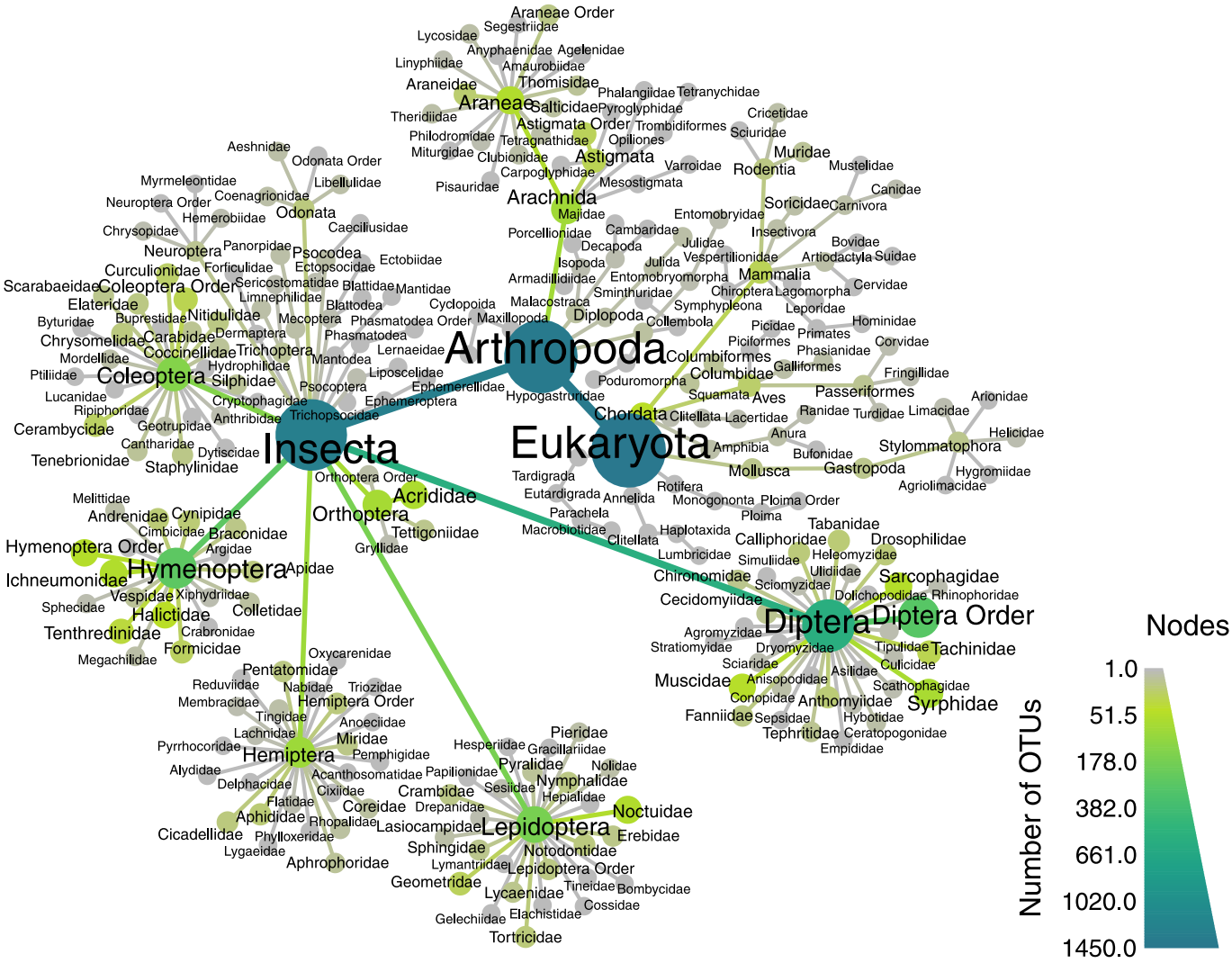


Fig. 2. Overall diversity of taxa detected in the larval guts of *V. velutina*. The 1449 taxa across 26 orders are shown to family level for readability. Both node size and colour relate to the number of OTUs that were assigned to the displayed taxon.

different, both had significantly higher richness than embryo nests, with this difference decreasing as larval head width increased (Supplementary Table 2 and Supplementary Fig. 12). Coefficients, standard errors, and *p*-values are listed in Supplementary Table 2.

3.4. Differences in species eaten by V. velutina across geographical regions and for different nest stages

Geographical region, month of nest collection, year of nest collection, and the proportion of land classed as 'built up' (defined as land covered in man-made structures such as roads, buildings, and railroads, not including urban green spaces or waste deposits) within a 500 m radius of the nest were all found to affect the composition of the diet. A significant interaction between region and month of collection was also present (Table 2). Other than the area defined as 'built up' no other land cover estimates were significant (for full descriptions of each land cover classification, see the WorldCover Product User Manual V2.0 [De Kerchove Ruben, 2020]).

All categorical variables found to significantly affect the dietary composition of larvae were also found to have significantly different dispersions. Inspection of plots of the betadisper objects (Supplementary Figs. 13–15), and modified one-way PERMANOVAs indicated significantly different dietary compositions in all cases (Table 1).

Taxa that varied most by geographical region were largely Dipterans, with two Syrphids, *Volucella zonaria* and a Syrphidae_Seq30, found more commonly in Aquitaine and Jersey than Galicia; the soldier fly *Hermetia illucens* detected mostly from Aquitaine; and one crop pest *Drosophila suzukii* detected primarily from Galicia followed by Aquitaine (Fig. 5a). Several species of Calliphoridae (genera *Lucilia* and *Calliphora*) varied between regions, with most associated with Jersey—except for *Lucilia sericata* that was associated with Aquitaine. A single species of Muscidae, *Muscina stabulans*, varied strongly with region and was detected most frequently from Galicia. Among the Hymeonpterans, the common wasp and two species of wild Apidae were found to differ between regions; *Vespula vulgaris* was found most commonly in samples from Jersey, the sweat bee *Lasioglossum malachurum* was detected more from Galicia, and the mining bee *Colletes succinctus* was detected more from Aquitaine (Fig. 5a).

Species that varied the most between seasons were also almost entirely Dipterans (Fig. 5b). The syrphids that varied by time of the year occurred primarily in the early season (*Volucella bombylans*) or mid-season (*Volucella zonaria*). The Calliphorids (all of which were from the genus *Lucilia*) occurred in the mid to late season. A variety of Muscidae flies were found to vary strongly with season, occurring either in the early season or late season, with none being predated on specifically in the mid-season. Aside from Dipterans, the early bumblebee, *Bombus pratorum*, and the sap beetle, *Cryptarcha strigata*, were found in the early season; while the social wasps were found in the mid and late

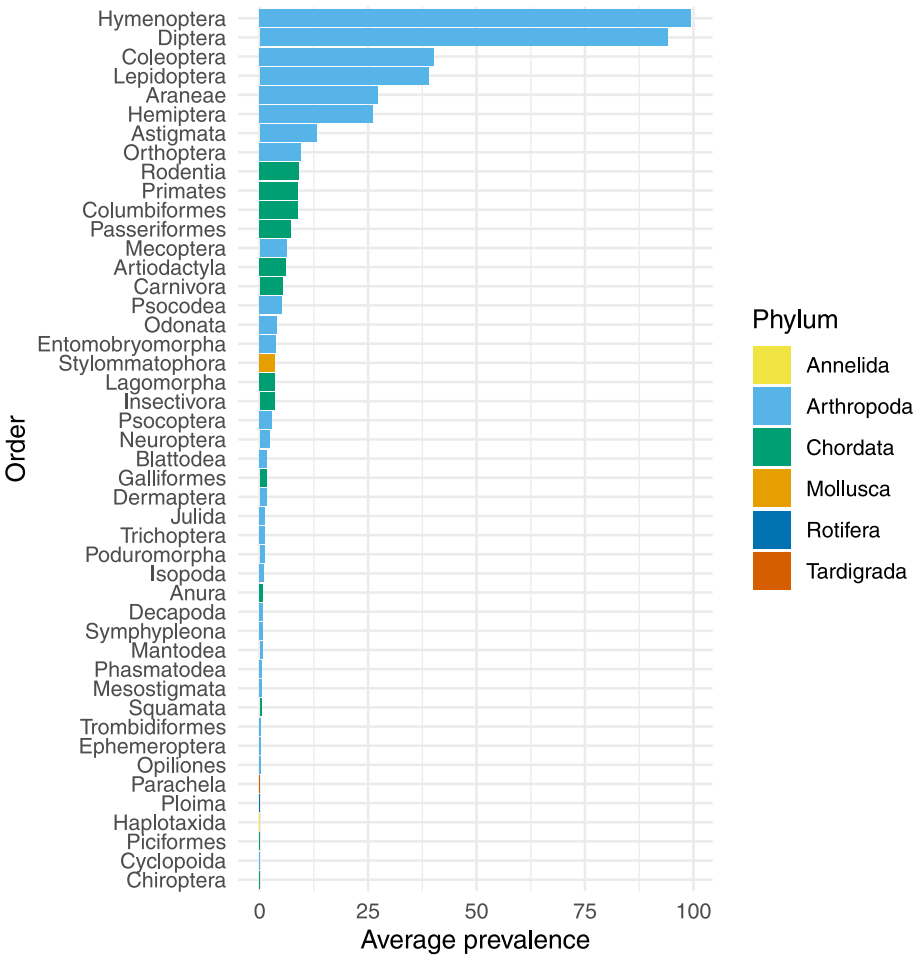


Fig. 3. The average prevalence of all animal orders detected. Average prevalence is the percentage of larvae from each nest that contained any taxa from that order, calculated as a mean across all nests studied (68 nests from Jersey, 25 from Aquitaine, 8 from Galicia, and 2 from the UK).

(*V. vulgaris*) and late (*V. germanica*) seasons. Two Dipteran species, *Polietes lardarius* and *Scathophaga stercoraria*, were found primarily in the early and late seasons, with lower occurrence in the mid season.

Changes in dietary composition were also found between years. Subsetting the data to include Jersey or Aquitaine only (these being the regions with nests collected over multiple years) showed that the difference between years was significant for Jersey, but not Aquitaine. Concurring with the higher species richness found in Jersey in 2021, most species that varied between years were detected more in 2021 than 2020 or 2022. These were largely dipterans, including multiple species of the hoverfly family, as well as the orb-web spider, *Araneus diadematus*. On the other hand, in 2022 a species of click beetle of the family Elateridae, a weevil of the family Curculionidae, and a scorpionfly (*Panorpa communis*) were found more frequently, along with a nonspecific Dipteran (*Dolichopus* sp.) and the hoverfly *Merodon equestris*. In 2020 *Vespula vulgaris* was found more frequently, as was the Dipteran *Panzeria ampelus* (Supplementary Fig. 16).

Differences in dietary composition were also found depending on the percentage of land cover classed as built-up surrounding nests. Few species were associated with increased built-up land cover, including *Vespula vulgaris* and *Colletes succinctus*. Many more were negatively associated with this land cover class, including the flesh fly *Sarcophaga variegata*, multiple species of Calliphoridae and Muscidae, the cranefly *Tipula paludosa,* the red-tailed bumblebee *Bombus lapidarius,* the orb-web spider *Araneus diadematus,* and the sap beetle *Cryptarcha strigata* (Supplementary Fig. 17).

3.5. Honey bee predation levels relative to apiary locations

Significant correlations with the RRAs of the honey bee were found for both apiary metrics (Fig. 6). The strongest correlation was with the number of apiaries within 500 m (tau = 0.145), while the correlation with distance to the nearest apiary was comparatively weak but significant (tau = 0.07).

4. Discussion

4.1. DNA metabarcoding reveals high dietary variation and adaptability of V. velutina

Applying deep sequencing, we uncover a much broader prey spectrum of *V. velutina* than identified in any previous study, with concordant implications for European ecosystems. We show that the primary predated taxa were in the orders Hymenoptera and Diptera, with Hymenoptera being the most frequently preyed upon, and Diptera the most diverse—consistent with previous studies. Other targeted taxa included the Coleoptera, Lepidoptera, Araneae, Hemiptera, and Orthoptera; each of which have also been reported from observations in Europe, but not to the extent or diversity that we identify here (Perrard et al., 2009; Rojas-Nossa and Calviño-Cancela, 2020; Rome et al., 2021; Stainton et al., 2023; Verdasca et al., 2022; Villemant et al., 2011).

The DNA metabarcoding approach used here identified a far greater range of *V. velutina* prey species than previously known, many of which could not have been detected via morphological analyses. It is important to emphasise, however, that the universal primers used in DNA

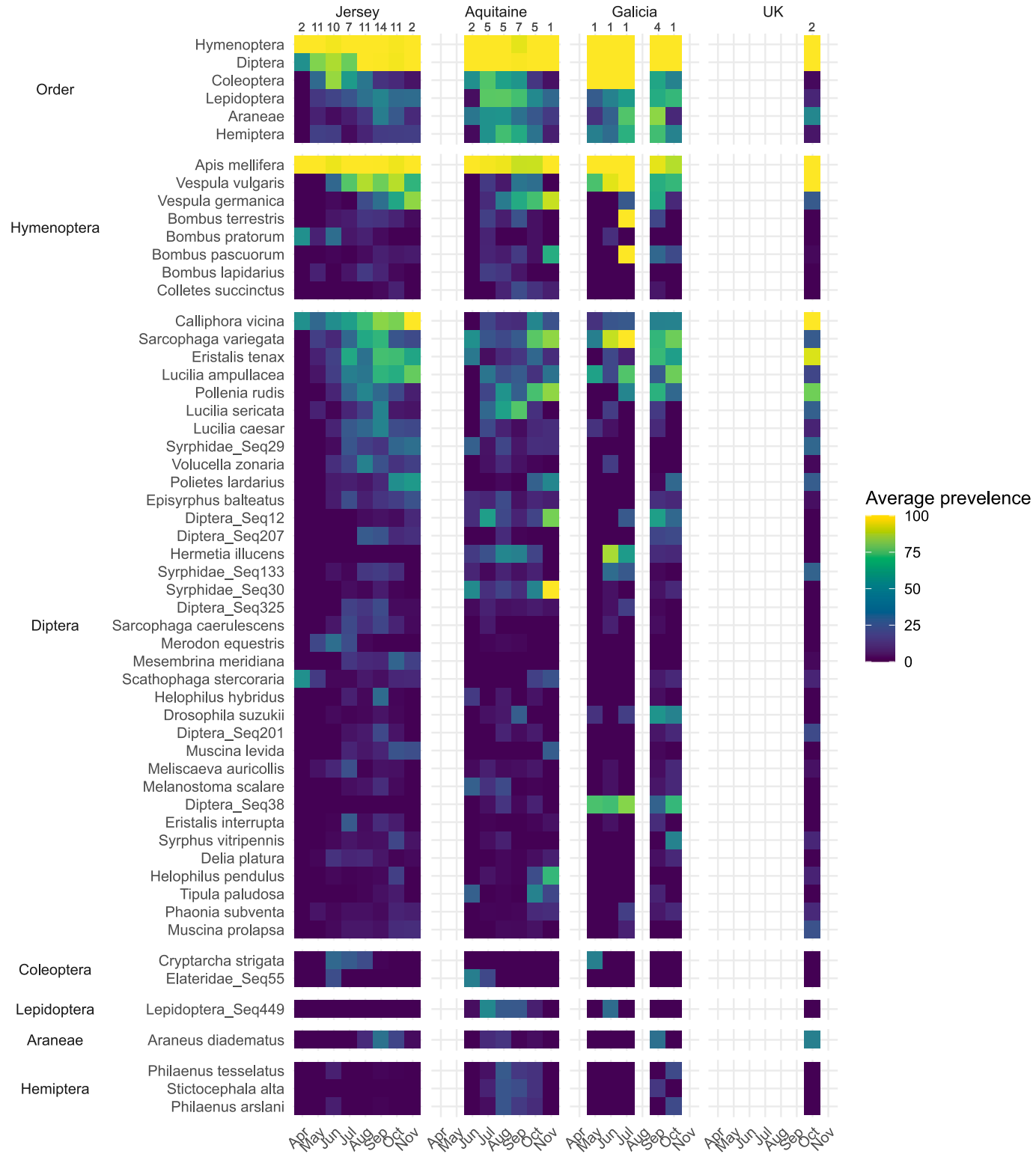


Fig. 4. Average prevalence of the top 50 most frequently occurring taxa from the top 6 most prevalent orders, split by geographical region and month. The number of nests analysed for each geographical region/month is indicated at the top of the heatmap. The top ribbon shows prevalence at the order level, where 100 % prevalence indicates that 100 % of the larvae contained at least one taxon from that order. The lower ribbons provide OTU level prevalence. OTUs that were not assigned to species level are labelled with the highest taxonomic resolution achieved and a suffix with the sequence number created in the bioinformatic pipeline. The average prevalence of every OTU detected can be found in Supplementary Figs 4–10.

metabarcoding have biases, and thus the absence of a species from the data presented here cannot be used to definitively conclude that it is absent from the hornet's diet. Primer biases can also complicate the interpretation of read numbers when estimating relative abundance of prey items. We mitigate for this in our study by using prey presence/absence and estimating abundance only through prevalence within a nest, thus avoiding the assumption that RRA is directly related to prey abundance. This does however, mean that a nest containing very low

Table 2
Results of distance-based RDA and modified PERMANOVA.

	db-RDA		Modified PERMANOVA	
	Pseudo F_1	*P*-value	Pseudo F_2	*P*-value
MONTH:REGION	1.4482	0.0001	*NA*	*NA*
YEAR	1.9681	0.0001	3.7372	0.0001
BUILT UP COVER	1.5461	0.028	*NA*	*NA*
MONTH[1]	2.0667	0.0001	2.8158	0.0001
REGION[2]	1.9759	0.0018	3.3713	0.0001

[1] Data were subset to include only larvae collected from Jersey to investigate the effect of month, removing the interaction between region and month.
[2] Data were subset to include only the month of September to investigate the effect of region, removing the interaction between region and month.

amounts of DNA of a particular species throughout the larvae studied would appear the same as another nest in which every larva contained a large amount of that DNA. Furthermore, observations of *V. velutina* worker activity at a nest in captivity have shown workers feeding small clusters of larvae with a single prey pellet (Isaacs and de Carteret, 2024), meaning that the occurrence of a prey species in multiple larvae does not necessarily mean that multiple predation events occurred. This was taken into consideration in our study, by asking sample collectors to take larvae from different combs/areas of the nest. However, it was often not possible to do so for the smallest nests—that typically occur early in the season—due to the low number of available larvae. Indeed, it is possible that all larvae from the smallest nests were fed from the same single food pellets on each return visit of the queen (or workers if present).

A final salient point regarding the application of DNA metabarcoding to studies of the nature we present here, is that intentional predation events are not easily separated from those that are accidental or incidental consumption events. The high prevalence of Astigmata in the present study supports this - mites are ubiquitous in nature and accidental consumption of them is highly likely. The large diversity of vertebrate taxa detected may be explained similarly. *Vespa velutina* are known to scavenge on carrion and human food (Rome et al., 2021; Williams, 1988), but accidental consumption of animal faeces while hunting coprophagous insects could also result in the detection of vertebrate DNA in samples. This may explain the high frequency of *Canis lupus,* likely the domestic dog (the Leray fragment cannot differentiate between the domestic dog and Eurasian wolf) found in 34 nests across all geographical regions in our study. Similarly, frequent predation on necrophagous insects such as *Calliphora vicina*, or other predatory species such as *V. vulgaris*, could result in secondary predation that cannot be distinguished from intentional predation by DNA metabarcoding. Finally, the relatively high occurrence of human DNA found, while possibly resulting from contamination from the researchers involved in the collection and/or processing of the larval material, could also have arisen through predation by *V. velutina* on hematophagous or coprophagous insects that had fed on human blood and faeces respectively, or through accidental consumption of human saliva while scavenging on waste human food.

Accepting the above limitations, the overall picture obtained from our DNA metabarcoding approach is that *V. velutina* has an extremely broad dietary spectrum and is a generalist and opportunistic predator. The predation patterns of *V. velutina* suggest that it has a preference for honey bees, other social hymenopterans (primarily wasps), and species that can be found in relatively high local densities such as the blowfly *Calliphora vicina*. However, the extremely broad spectrum of other taxa found in the larval guts, with certain taxa occurring only in particular seasons and regions, indicate opportunistic behaviour, predating on any suitable species that is locally abundant. This indicates a high degree of flexibility and adaptability to new climates and surrounding ecosystems, consequently enhancing this species' invasive success. Differences in the

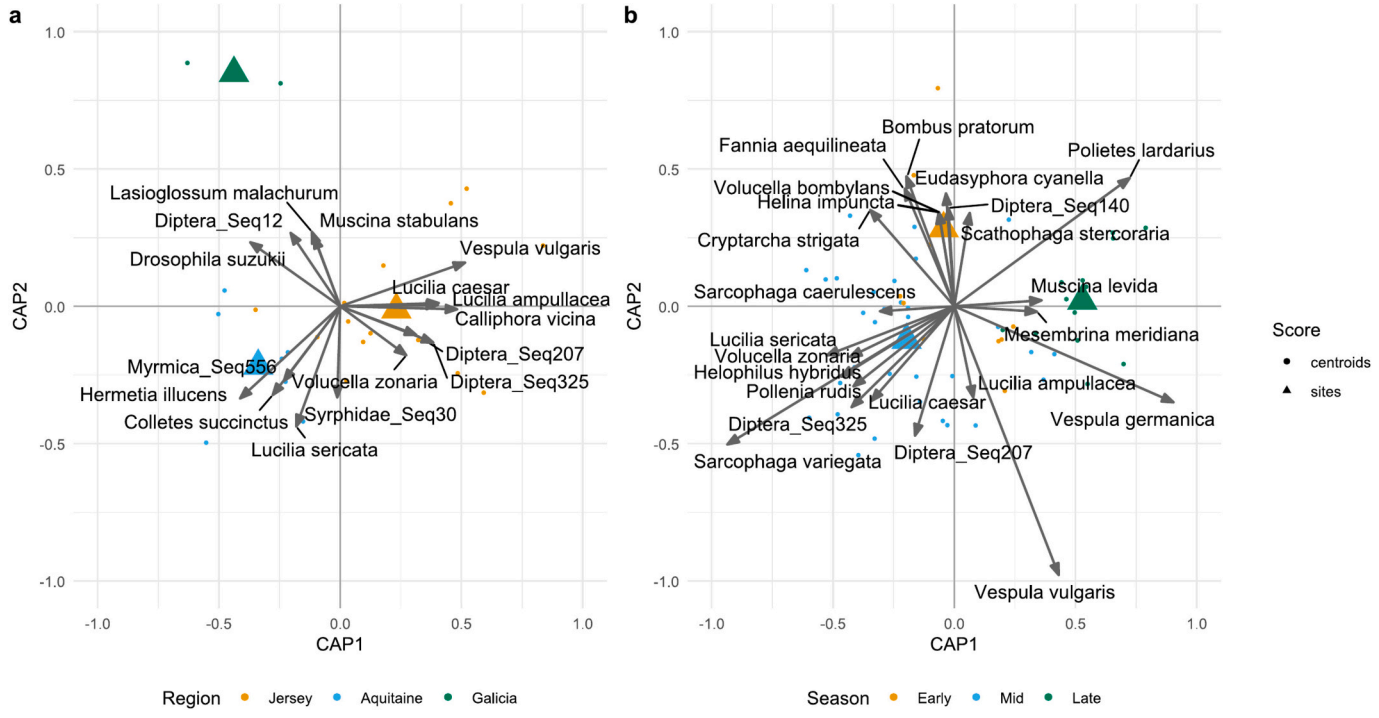


Fig. 5. Triplots of partial db-RDAs showing species scores (arrow heads), weighted average (WA) scores for nests (coloured dots), and centroids of the constraining variable (coloured triangles) with symmetric scaling. Filled triangles represent the centroids of each group. Correlations between species and region/season can be deduced by the direction and length of the species arrow in relation to the position of each centroid. Length of the arrows corresponds to the intensity of variation in relation to the environmental variable. a) Data from September only was used to explore differences between regions and included 14 nests from Jersey, 7 nests from Aquitaine, 4 nests from Galicia, and none from the UK. Only species with scores over 0.25 are displayed. b) Data from Jersey only was used to explore differences between seasons and included 13 nests from the early season (April, May, and June), 32 nests from the mid season (July, August, and September), and 13 nests from the late season (October and November). Only species with scores over 0.30 are displayed.

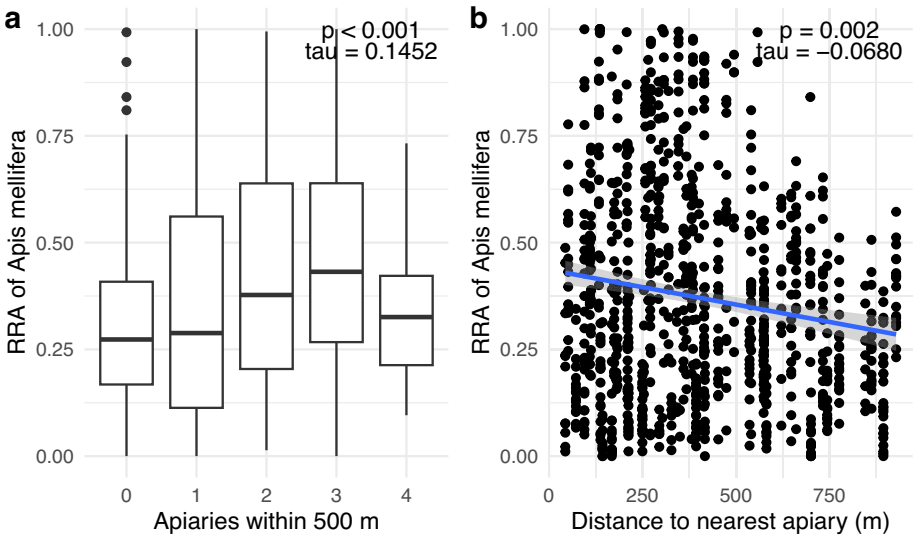


Fig. 6. Relative read abundance of *A. mellifera* in relation to the number of apiaries within 500 m of the nest (a), and the distance to the nearest apiary (b).

composition of the diet between regions and seasons as indicated by the db-RDA also suggest that the hornets display high adaptability to new climates and surrounding ecosystems.

The increasing species richness seen within the larval gut as latitude decreased could be due to a higher species diversity in the south of Europe. Although there are no comparable insect biodiversity estimates to confirm that there is higher diversity in southern regions, this pattern is consistent with the latitudinal diversity gradient (Hillebrand, 2004).

The observed changes in species richness and composition between years in Jersey were unexpected and could be the result of the hornets encountering novel food sources following their recent invasion and continued expansion. A lack of data covering multiple years from Aquitaine and Galicia, however, prevents the comparison of year-on-year dietary changes between the more recently invaded region of Jersey, and regions where *V. velutina* has been established for two decades. An alternative explanation could be differences in the availability and diversity of insects in Jersey between these years. While there is no insect monitoring scheme in Jersey that could provide suitable data to compare with the hornet's diet, early results from the UK Pollinator Monitoring Scheme did record a lower diversity of hoverflies, and to a lesser extent bees, in 2020 compared to 2021, which concurs with the finding that *V. velutina* fed more on hoverflies in 2021 (UK Pollinator Monitoring Scheme, 2023).

4.2. Potential impacts on apiculture

The very high prevalence of *A. mellifera* detected in this study concurs with previous research indicating that honey bees are often the single most frequently targeted prey (Perrard et al., 2009; Rome et al., 2021; Stainton et al., 2023; Villemant et al., 2011), and further validates concerns that the hornets may pose a serious risk to apiculture. This trend is likely in part due to the high abundance of *A. mellifera* in Europe as a consequence of human management and exacerbated by the fact that the studied nests originated from well-populated areas where hive numbers are also high. Nevertheless, the consistency of detection of *A. mellifera*, including during the spring months, suggests that it forms a major basis of the diet of *V. velutina*, with apiaries providing a food source when other insects are scarce. Further, the correlation between the relative read abundance of *A. mellifera* and the number of, and proximity to, apiaries in an area also indicates preferential predation on the honey bee where it is more abundant.

4.3. Potential impacts on other species of ecological concern

Pollinator diversity is essential for protecting food security and mitigating biodiversity loss (Kleijn et al., 2015) and this has been a major concern in the spread of *V. velutina*. Previous studies have found pollination of native plants to be disturbed by *V. velutina*, both through direct predation of insect pollinators, and by reducing their flower visitation durations or presence at flower patches (Rojas-Nossa et al., 2023; Rojas-Nossa and Calviño-Cancela, 2020). The high frequency of potential pollinators that we observe in *V. velutina*'s diet has further implications for the breadth of such impacts, with 43 of the top 50 prey taxa being flower visitors as adults, including two of the most important wild European crop pollinators.

Beyond the context of pollination, the prevalence of Diptera in the hornets' diet, and the fact that 17 of the top 50 species are known to feed on carrion, dung or rotting vegetation, also indicates that there may be impacts on species with key decomposition and recycling roles in the environment – a topic worthy of further investigation.

Although we found little evidence of *V. velutina* predating on species of conservation concern as defined by the IUCN Red List, it remains possible that the hornets are threatening rare species not yet evaluated by the IUCN. As of 2023, only 1.2 % of known insects have been evaluated by the IUCN, and of these 25.9 % are data deficient (IUCN, 2023b). This resulted in only 20 out of the 749 species that we searched for having a match within the Red List. Furthermore, those insects that have been evaluated tend to be the larger, widely distributed, and easily identifiable; while those from hyper-diverse clades or narrow distribution ranges are more likely to be excluded (Cardoso et al., 2011). This is notable, as the opportunistic nature of *V. velutina*'s diet suggests that insects that are globally scarce but locally abundant—such as endemic species—may be at risk.

4.4. Potential impacts on bumble bees

Only very low levels of predation on bumblebees have so far been reported (Perrard et al., 2009; Rome et al., 2021; Verdasca et al., 2022). Furthermore, while predation attempts *on Bombus terrestris* by *V. velutina* have been recorded in Europe, none of the predation attempts observed in two recent studies were successful (O'Shea-Wheller et al., 2023; Rojas-Nossa et al., 2023). In the case of *B. terrestris*, despite frequent attacks by *V. velutina* at bumblebee nest entrance, the defensive behaviour of workers (dropping to the ground, rolling onto their backs and raising their sting) has been observed to thwart the hornets' predation attempts, allowing the bees to escape (O'Shea-Wheller et al.,

2023). Despite this, our study has found bumblebee DNA frequently in the gut of *V. velutina* larvae, with four species of *Bombus* in the top 50 most prevalent species. This discrepancy with other metabarcoding studies to date could be due to the use of different primers, resulting in divergent biases affecting the results.

Studies recording predation attempts have focussed either solely at the entrances to *B. terrestris* colonies (O'Shea-Wheller et al., 2023), or at flower patches where *B. terrestris* were present (Rojas-Nossa et al., 2023). It is possible that smaller bumblebees, or bumblebees that are weakened by cold or disease, are more easily predated on by *V. velutina*. Another possibility is that bumblebee DNA is deposited on the mandibles of a hunting hornet after an unsuccessful predation attempt and is then transferred to the larvae along with other food. Finally, hornets have been observed entering and leaving *B. terrestris* colonies in the field (O'Shea-Wheller, personal observation), suggesting that they may be foraging on pollen, nectar, or detritus from bumblebee nests. While such scenarios may explain occurrences where the RRA of bumblebees was very low, there were also instances where bumblebee reads accounted for over 10 % of the total RRA, indicating that there was focused and successful predation of bumblebees in these cases.

4.5. Implications for management

The wide and broad diet illustrated in this study confirms the high adaptability of the *V. velutina* such that we suggest that when modelling areas suitable for its establishment as part of contingency planning, food availability for this species is unlikely to be a limiting factor. Identifying the major food sources of *V. velutina* can also be used to indicate their main foraging habitats and in turn assist monitoring efforts, especially in areas with lower apiary densities. Demonstrating the breadth and adaptability of the diet of *V. velutina* also provides important evidence to improve the risk assessment for this non-native species. The current UK non-native species Risk Assessment (https://www.nonnativespecies.org/assets/Risk-assessment-Asian-hornet-Vespa-velutina.pdf) identifies 'moderate uncertainty' for the environmental impact of *V. velutina* based, in part, on lack of evidence as to which wild species are most likely to be affected. As such, the evidence provided here of which functional groups are most at risk is key to supporting and updating such risk assessments, both in the UK and across Europe. This is particularly important if countries start to consider scaling back on *V.velutina* management: essentially it is not just a beekeepers' problem and the wider environmental footprint needs equal consideration.

5. Conclusion

Here we have provided the most comprehensive view of the diet of *V. velutina* in Europe to date, highlighting an extremely broad and flexible range of potential prey. We have shown that dietary composition varies between geographical regions and across the hornets' activity season, indicating high adaptability to new ecosystems; driving both the invasive success of *V. velutina*, and the risk that it poses to a broad spectrum of native invertebrate fauna. The elevated dietary prevalence of *A. mellifera* concurs with previous studies and reports from beekeepers, supporting concerns over the risk to apiculture. Our results also reveal a further potential ecosystem-level pressure as we find that *V. velutina* demonstrates a high predation frequency on wild pollinators and recyclers, implying a threat to pollinator and recycler diversity and resultant ecosystem services.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2025.178978.

CRediT authorship contribution statement

Siffreya Pedersen: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Peter J. Kennedy:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Thomas A. O'Shea-Wheller:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Juliette Poidatz:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Alastair Christie:** Writing – review & editing, Data curation. **Juliet L. Osborne:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Charles R. Tyler:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Contributions

J.L.O, C.R.T and P.J.K. conceived the study; P.J.K and J.P coordinated the collection of samples; P.J.K, J.P. and T.A.O.-W dissected and measured the larval samples; A.C provided apiary data; T.A.O.-W calculated land cover estimates and apiary proximity and density; S·P conducted the molecular analyses, bioinformatics and statistical analyses. S.P. drafted the manuscript with subsequent contributions and editing from all other co-authors. J.L.O and C.R.T obtained the project funding.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The metabarcoding data generated in this study has been deposited in the NCBI's Sequence Read Archive under the BioProject number PRJNA1155314 and the associated metadata can be found in Supplementary Table 1.

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