Predator-prey traits and foraging habitat shape the diet of a common insectivorous bat

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ABSTRACT

Individual and species traits may determine the functional relationships between predators and their prey, with clear consequences for the ecosystem services potentially associated with predation. Bats are well-known potential deliverers of ecosystem services in agroecosystems through predation of pest arthropods that may affect crop production. Here we test the role of individual and prey traits in shaping the dietary habits of the Savi's pipistrelle (Hypsugo savii), a common bat species that frequently occurs in agroecosystems across the Mediterranean area, assessing diet composition and trait-based biases through molecular tools and multivariate testing techniques. DNA metabarcoding identified 173 distinct prey taxa in bats sampled in the Vesuvius National Park (Southern Italy). The diet was strongly dominated by pest insects associated with agricultural areas, which covered 57% of the overall prey diversity, some representing major pests of high economic relevance. The multivariate analyses detected strong relationships between diet composition, sampling date, and individual bat body conditions. Larger bats consumed larger prey items, and prey consumed later in the season (August) were smaller than those eaten in early summer (July) and were typical of less natural habitats. Hypsugo savii is potentially an important pest suppressor in the agroecosystems of the Mediterranean region and the functional relationships between the traits of individual bats and their prey set the basis for a quantitative assessment of the associated ecosystem service. We also remark on the value of bat diet studies in aiding the surveillance of arthropod species relevant to agriculture, human health, and biodiversity conservation.

1. Introduction

Bats are key providers of ecosystem services globally, and their role as pest suppressors in agroecosystems and forestry is being increasingly recognized and quantified (Boyles et al., 2011; Kolkert et al., 2020; Rodríguez-San Pedro et al., 2020). Understanding the relationships between bats and their prey, particularly pest arthropods, is a key asset to assessing quantitatively the ecosystem service insectivorous bats deliver, besides clarifying the detailed interconnections between ecosystem elements. This is crucial to fostering a better understanding of species' ecological requirements and role in trophic networks

(Hemprich-Bennett et al., 2021; Russo et al., 2018). Recent advances in molecular tools have greatly improved the taxonomic resolution of prey identification and favoured analyses of wildlife's dietary preferences in unprecedented detail (Dahl et al., 2017; Epp et al., 2012; Zeale et al., 2011). Obtaining diet information at a high degree of taxonomical resolution is, in fact, important to unveil fine-grained functional relationships between predator and prey, and to understand mechanisms underlying prey selection that arise from individual and species-specific life traits (Bolnick et al., 2003). Predators optimize predation according to a cost/benefit balance that depends on the predator's ability to detect, catch, and consume prey, as well as the latter's nutritional value,

defensive abilities and palatability (Cyriac and Kodandaramaiah, 2019; Evans, 1983; Skelhorn and Rowe, 2006).

Bats intertwine complex networks of relationships in their habitats, catching hundreds of insects per night from a diverse range of species (Aizpurua et al., 2018; Arrizabalaga-Escudero et al., 2018; Mata et al., 2016; Razgour et al., 2011), showing seasonal and individual-based dietary differences, and sometimes covering long distances to reach their foraging sites (e.g., Kipson et al., 2018). As such, trait-based approaches may provide an in-depth insight into the prey-predator relationship between bats and their prey (Arrizabalaga-Escudero et al., 2019; Spitz et al., 2014).

Here we explore the diet of a generalist bat, assessing trait-based diet habits to identify prey and individual bat characteristics that are linked to the consumption of arthropods by bats. We focus on a common bat species, the Savi's pipistrelle (*Hypsugo savii*), which based on its abundance (Ancillotto et al., 2021; Bosso et al., 2018), is likely an important deliverer of economically significant ecosystem services (Boyles et al., 2011) across its wide Mediterranean distribution (Smeraldo et al., 2021). A radiotracking study on the species in the same study area (Ancillotto et al., 2018) revealed *H. savii*'s preference for foraging in non-intensive farmland within the mosaic of natural and human habitats present in the area. As such, when investigating the species' dietary habits, we specifically tested the following hypotheses and predictions.

1. *Hypsugo savii* is a generalist bat with a preference for foraging in non-intensive farmland (Ancillotto et al., 2018), so we hypothesize that prey from different habitat types will be disproportionately represented in the diet of bats and predict that most preyed taxa will be associated to farmland.
2. Bats usually feature sex- and period-biased dietary habits (e.g., Mata et al., 2016) that may arise from different patterns of spatial use according to e.g., phenological phase. We, therefore, predict that male and female *H. savii* will show differences in diet composition reflecting different mobility patterns, and that prey composition will also change according to the period of sampling.
3. Different prey types may represent a differently profitable resource to bats (Arrizabalaga-Escudero et al., 2019; Zahn et al., 2007), so we hypothesize that prey traits such as size will influence individual bat body conditions and predict that bats preying upon larger taxa will show a better body condition.

2. Materials and methods

2.1. Study area and bat sampling

We conducted our study in the Vesuvius National Park and its surrounding territories, in the municipality of Naples, southern Italy (40.81 N, 14.42 E; elevation: 200–1200 m a.s.l.). The area is characterized by a rich mosaic of natural and anthropogenic habitat types, including coniferous and broadleaved forests, Mediterranean scrubland, and lava-rocky areas, as well as large portions of urban areas and non-intensive farmland. In July–August 2019 we mistnetted *H. savii* at an artificial pond (4 capture sessions), the only permanent water source within the Park apart from private pools in human settlements. We concentrated sampling in midsummer for two main reasons: 1) bats in Mediterranean areas have high energy demands due to pregnancy/lactation (females) and/or water scarcity (both sexes) so their habitat selection and – consequently – dietary preferences are a key aspect of individual survival; 2) most crops in the area ripe in summer, so that pests may represent a particularly significant threat and are presumably more abundant at this time of the year, thus potentially highlighting an even more important role of bats as pest predators. At capture, each bat was sexed, its age class (adult vs. juvenile) was estimated, and forearm length (mm) and body mass (g) were measured respectively with a digital precision calliper and a balance scale to the nearest 0.1 mm and 0.1 g. For each bat, we also calculated the scaled mass index (Peig and Green, 2009) as a proxy of individual fitness.

2.2. Diet analysis and prey traits

Captured bats were individually placed in clean cotton bags, from which faecal pellets were collected after 10–20 min from capture, stored in tubes containing 98% ethanol, and kept at 4 °C until DNA extraction. DNA was extracted from 3 bat droppings per sample (n = 38) using PowerSoil® DNA Isolation Kit (MoBio, CA, USA), following the manufacturer's protocol. To better cover prey taxonomic diversity, we used two primer sets targeting COI (LCO1490/COI-CFMRa) and 16 S rRNA (Coleop_16Sc/Coleop_16Sd) mitochondrial markers, respectively (Epp et al., 2012; Jusino et al., 2019). These primers are widely considered highly efficient in detecting a broad spectrum of arthropod prey from bat droppings (Jusino et al., 2019) with some bias reported for beetles – which are occasionally preyed upon by *H. savii* (Kipson et al., 2022). The PCR conditions for the COI region amplification were 60 s at 94 °C for; 5 cycles of 60 s at 94 °C, 90 s at 45 °C, 90 s at 72 °C; 35 cycles of 60 s at 94 °C, 90 s at 50 °C, 60 s at 72 °C; 72 °C for 7 min (Jusino et al., 2019). Concerning the 16s rRNA region, the amplification conditions were 2 min at 94 °C, followed by 55 cycles of 94 °C for 30 s, 55° for 30 s, 72 °C for 30 s, and a final extension of 10 min at 72 °C (Epp et al., 2012). Library preparation and sequencing were provided by an external service (San Raffaele Scientific Institute, Milan, Italy). The Illumina 16 S Metagenomic Sequencing Library Preparation protocol (Amplicon, Clean-Up and Index) was employed. This is based on a two-step PCR reaction: 1) amplification of the DNA template with overhang adapters attached, 2) attachment of indices (unique for each sample) to the adapters. Sequencing was performed using a MiSeq 600 V3 (2 × 300-bp paired-end sequencing). Two sequencing replicates per sample were conducted for each primer set, and two negative controls (one for each DNA extraction session) were produced and sequenced. The bioinformatics processing was conducted by adopting the pipeline described in Tommasi et al. (2021). Briefly, the raw sequences were paired after sequencing with QIIME2 (ver. 2019.4; https://qiime2.org/) (Bolyen et al., 2019). After primer trimming and removal of chimeric sequences, the DADA2 algorithm (Callahan et al., 2016) was used to extrapolate Exact Sequence Variants (ESVs). An expected error = 2.0 was used as an indicator of reading accuracy. Then, for each sample, we discarded those ESVs represented by a number of reads lower than 0.01% of the total reads per sample, following Andriollo et al. (2019) and Ancillotto et al. (2022). The taxonomic assignment of the obtained ESVs was done using NCBI-BLAST (Altschul et al., 1990) and the BOLD Systems IDS tool (Ratnasingham and Hebert, 2007) for 16s rDNA and COI respectively. Following Aizpurua et al. (2018), ESVs were assigned to the order level when identity values between the query and the reference sequence were ≥95%. Assignments at the family level were done with identity values > 96.5%, and species-level assignments with identity values ≥ 98%. In the case of similar identity values among multiple species, the ESVs were assigned to the genus level. All species-level assignments were manually checked and restricted only to species known to occur in the study area based on expert opinion and consulting the available distributional data of the assigned putative taxa. Non-target prey taxa (e.g., mites and other putative prey symbionts) were manually removed. To avoid the inclusion of false positives or contaminations in the network analysis, the taxonomic features detected both in negative controls and samples were discarded if represented by a total number of reads higher than the negative controls (Tommasi et al., 2021). The assigned taxa from the two sequencing replicates and markers for each sample were merged to improve the characterization of bats' diet composition (as in da Silva et al., 2019). In case of assignments to species not known or presumed to occur in the area, we only considered taxa as reliable at the genus level. Since the use of reads as a proxy of item abundance within molecular samples is still debated (Deagle et al., 2019), we scored dietary records as occurrence (presence/absence) data only. Besides being assigned to the highest possible

taxonomic level, prey items were also classified according to the following set of traits: a) favoured habitat type, dummy-coded and ordered according to the degree of naturalness (forest = 2, agricultural areas = 1, urban areas = 0), b) relevance as a pest (either forest or agricultural), ranging from 2 (major pest) to 0 (non-pest), according to the evidence provided by the European Plant Protection Organization (EPPO: https://www.eppo.int/); and c) taxon size, as estimated by wingspan (in mm), retrieved from the available literature (See Table S1 and file S2 in Supplemental files).

2.3. Statistical analyses

We investigated the functional relationships between prey and bat traits by combining RLQ and fourth-corner analyses using the *ade4* package for R (Dray and Siberchicot, 2017). Such an approach relies on the construction of three starting matrices, as described by Arrizabalaga-Escudero et al. (2019).

- Q (*p x s*): describes the *p* prey arthropod species according to one or more morphological and functional traits
- R (*m x n*): describes the "environment" of the *n* sampled bats according to a set of individual-based traits (*m*)
- L (*n x p*): combines the first two matrices, by describing the ecological interactions between bats and prey, and consists of a presence/absence matrix of *p* prey species within the diet of *n* bat individuals.

Before the RLQ analysis, multivariate ordination is needed on all three matrices, namely a Correspondence Analysis on the L matrix, since the latter includes only quantitative data, and Hill-Smith Analysis on matrices R and Q, as these include both quantitative and categorical variables. The RLQ analysis is a multivariate technique aimed at finding coefficients to obtain a linear combination of traits and environmental variables, separately, subsequently calculating the maximized covariance between these two sets of scores. In other words, the RLQ analysis maximizes the covariance between two sets of "traits", mediated by a matrix of interactions, and adopts a Monte-Carlo multivariate test (based on 9999 permutations) to assess the overall significance of the traits-environment association by calculating adjusted p-values with Bonferroni correction for multiple testing. In our dataset, traits of individual predators (bat sex, size, and scaled mass index) represent the "environmental variables" together with the sampling month (July or August), and the interaction matrix consists of the presence/absence of each prey species within each bat's diet. The fourth-corner analysis, in combination with the RLQ analysis, subsequently allows testing statistically the association between specific sets of traits (predator individuals and prey species). Before the analysis, we checked for trait collinearity by running cross-correlation analyses separately for bat and prey traits; this resulted in the selection of all traits for prey taxa (no correlation with p < 0.05 and r>|0.5|), and the exclusion of forearm length among bat traits (correlated with both sex and month of sampling). To visualize our samples' completeness in terms of ESVs prey composition, we also checked the species accumulation curve by using the *specaccum* function in the *vegan* package for R (Oksanen et al., 2013).

We then used the *bipartite* package (Dormann et al., 2014) to visualize the network of prey-predator connections, represented by trophic linkages between each bat and the pool of identified prey.

To test for differences in diet composition between male and female bats, also accounting for sampling month, we then ran a two-way PERMANOVA test on the Jaccard dissimilarity matrix calculated from the presence-absence occurrence of all prey items across all sampled bats, based on 9999 permutations. The contribution of each prey item to the observed differences was then assessed by an analysis of percentage similarity (SIMPER); results were considered as significant when p < 0.05. All tests and packages were run in R 4.2.1 (R Core Team 2013).
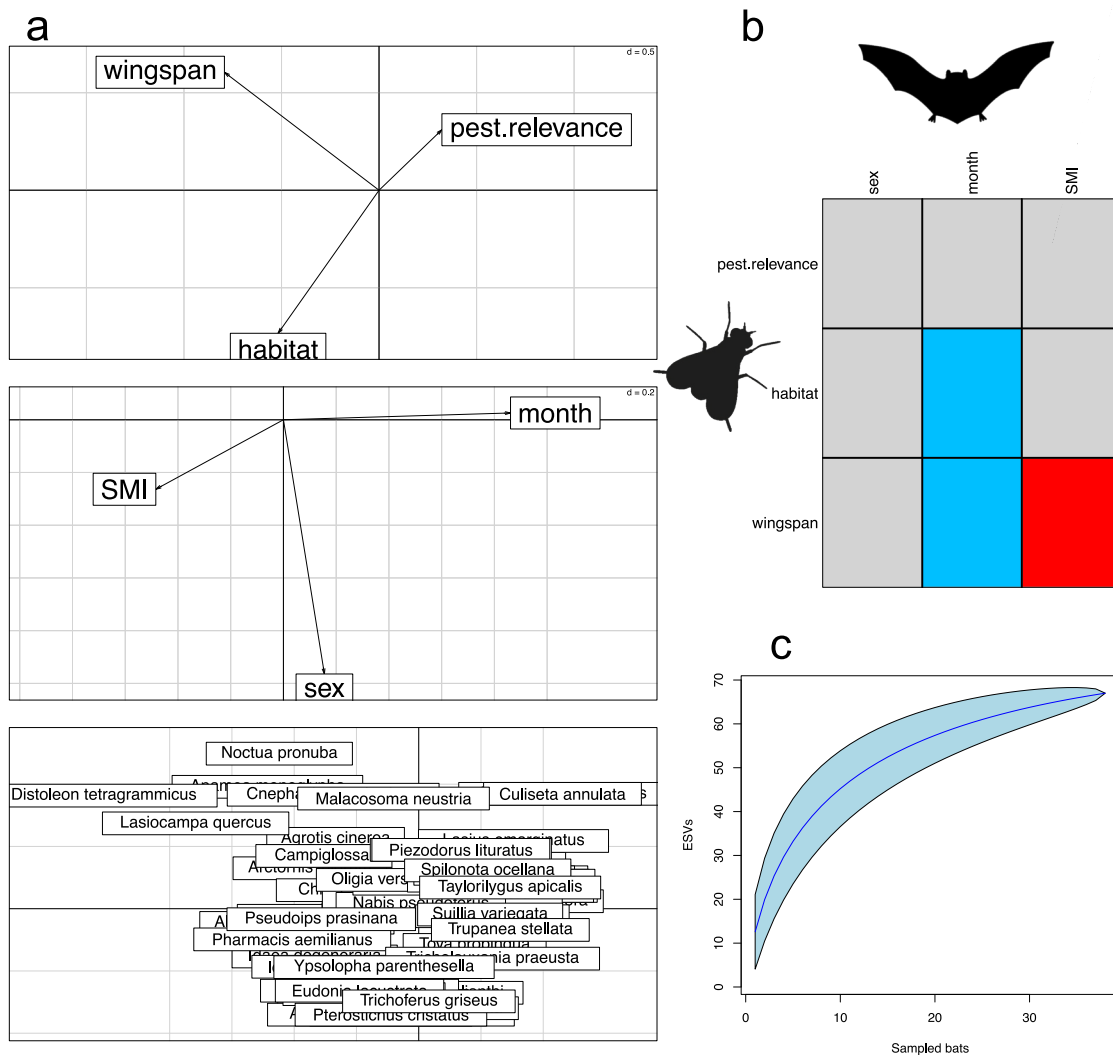
3. Results

After bioinformatic processing and quality filtering, the DNA metabarcoding of the droppings of the 38 *H. savii* (9 males, 29 females, all adults) samples yielded 6,137,543 reads in the case of the 16s rRNA marker (average per sample: 87,151) and 5,348,898 reads for the COI marker (average per sample: 74,569). These corresponded to 173 ESVs that were assigned to prey taxa, totalling 5092 bat-prey interactions. Out of these ESVs, 46.9% were identified as species, 31.7% as genus, and the remaining to order levels. The highest prey richness was that of Diptera (33.5% of assigned ESVs), followed by Lepidoptera (22.5%), Hemiptera (18.5%), Coleoptera (12.7%), Neuroptera (7.5%) and Hymenoptera (2.9%), while other orders (Blattodea, Orthoptera, Psocoptera and Strepsiptera) were only represented by one taxon per group (<1%). The obtained species accumulation curve (Fig. 1) suggests that our sample succeeded in capturing the diversity of taxa bats preyed upon in the study area, as indicated by the reached plateau. This was also evident when assessing the accumulation curves of male and female bats separately (Fig. S1 in Supplemental files), yet the larger sample of females clearly provides a more exhaustive picture of their diet. On average, each bat consumed 17 ± 6.5 ESVs (range: 9–36). Prey traits were retrieved for 67 taxa identified as species for which ecological and morphological data were available (totalling 2574 prey items, i.e., 50.5% of total recorded interactions). Overall, 57% of retrieved prey taxa (n = 46 out of 81) are known as pests or disease vectors, 42% of which (n = 19) cause serious agricultural damage – some being also important forest pests (e.g., *Actornis l-nigrum*) or disease-vectors (e.g., *Culex quinquefasciatus*). Moreover, 8 out of the 10 most frequently prey taxa are either major (*Drosophila simulans*, present in 87% of samples; *Cydia fagiglandana*, 67%; *Plutella xylostella*, 64%; *Piezodorus lituratus*, 54%; *D. suzukii*, 36%) or minor (*Nysius cymoides*, 82%; *Cosmia trapezina*, 51%; *Acanthiophilus helianthi*, 37%) pests. Of the taxa we identified as species, 58% occur in agricultural areas, the remaining in the forest (37%) or urban (5%) habitats.

The first two axes of the RLQ analysis explained 95.1 and 4.5% of the total variance, respectively. The combination of RLQ and the fourth-corner test showed that prey wingspan correlated with different sampling months (Fig. 1), i.e., prey consumed later in the season (August) were smaller (lower wingspan values) than those eaten in early summer (July). Prey's main habitat type also correlated with sampling month, with prey consumed in August being associated with less natural habitats. Finally, bats in better body conditions consumed larger prey more frequently than bats in poorer conditions (Fig. 1).

Individual bats caught their prey opportunistically, as evident by the high overall prey richness as well as by the bipartite network between individual bats and ESVs (Fig. S2 in Supplemental files). At the order level, diet composition remained comparable between male and female bats, with similar relative frequencies of occurrence of all the main prey groups (Lepidoptera: males 32.8%, females 39.5%, Hemiptera: males 11.5%, females 13.5%; Coleoptera: males 8.4%, females 8.5%; Diptera: males 37.4%, females 34.1%) except Neuroptera (males 7.6%, females 3.4%). Conversely, when assessing compositional differences at the ESV level, male and female bats showed a distinct diet composition (PERMANOVA $F_{2,90} = 82.17$, p = 0.0001), as did bats sampled early or later in the season (PERMANOVA $F_{2,90} = 83.07$, p = 0.0001), yet there was no significant interaction between these factors (PERMANOVA $F_{2,90} = -44.92$, p = n. s.). Overall, individual bats differed for 23.5% of their diet composition, with the main drivers of such dissimilarity being related to the occurrence of *Trupanea stellata*, *Limonia* sp., *P. xylostella*, *P. aemilianus*, *Piezodorus lituratus* and *D. suzukii*, cumulatively contributing to 15.5% of overall dissimilarity.

4. Discussion

The multivariate trait-based approach we adopted allowed us to disentangle the dietary habits of a bat species that is common in

Fig. 1. Results of the RLQ-fourth corner analyses on the diet of 38 adult *Hypsugo savii*, as assessed by metabarcoding techniques. a) Coefficients of individual bat traits (top panel), coefficients of prey traits (mid panel); eigenvalues scores of single prey items at the species level (lower panel). Panels display the first two multivariate axes only. Monte-Carlo test's observed statistic = 0.52, p < 0.01. b) Fourth-corner test results on the association between individual bats' (columns) and prey species' (rows) traits: significant associations (p < 0.05) between variables are shown in red (positive relationships) and blue (negative relationships). SMI = body conditions, quantified as Scaled Mass Index. C) Species accumulation curve of prey taxa (defined as Exact Sequence Variants – ESVs) detected in the diet of *Hypsugo savii*.

agroecosystems across the Mediterranean basin, showing that both individual and prey characteristics influence pest consumption by bats in human-altered (agricultural) areas. We confirmed that *H. savii* is a typical generalist forager in terms of prey diversity, showing a very high richness of preyed taxa per individual, with up to 36 ESVs, a significantly higher value than other more specialized bat species (e.g., *Plecotus* and *Barbastella*: Ancillotto et al., 2022).

Assessing whether and how frequently agricultural pests feature in the diet of a species is the first key step to understanding the ecosystem service provided by natural predators in anthropogenic environments, possibly setting the basis for future quantitative and economic assessments (Baroja Ibañez de Maeztu et al., 2021; Rodríguez-San Pedro et al., 2020). In agreement with our first hypothesis, the species' preference for foraging in non-intensive farmland habitats as assessed by previous radiotracking campaigns (Ancillotto et al., 2018) translates to the highest rates of pests (>50% of identified prey) and, more specifically, of insects associated with agroecosystems (Maslo et al., 2022). As a potential limitation of our result, we must disclose that the high rates of pests and disease vectors we found in the diet of *H. savii* may at least partly be due to a bias in the availability of barcode sequences towards economically-relevant pests. In fact, among the most frequent prey taxa we detected, some represent major pests that hamper crop yield at local and regional scales. For example, *D. suzukii* and *D. simulans* are considered major threats to the production of cherries, apricots, and other small fruits in our study area, where they have been recorded to cause losses up to 50% of the yearly yield (http://agricoltura.regione.campania.it/). *Hypsugo savii* is the commonest and most abundant bat species in the Vesuvian area (Ancillotto et al., 2021; Bosso et al., 2018); as such, the species is an excellent candidate for future quantitative assessment of insectivory's ecosystem service in agroecosystems.

In our study area, male *H. savii* have larger home ranges and cover longer distances than females (Ancillotto et al. 2018), and in support of our second hypothesis, we detected intersexual differences in diet composition, yet only at a fine-scale taxonomic resolution. In fact, male and female bats preyed, throughout the sampling season, upon similar proportions of insect orders, and even species-level differences in diet composition were not related to any specific prey trait. As such, it is likely that fine-scale differences in the prey consumed by male and female bats were mainly due to individual home-range spatial positioning and possibly to differences in their spatial behaviour (Ancillotto et al., 2018). Dietary differences among individuals were significantly driven by the bat's body condition and the month of sampling. Individual bats that fed on larger insect taxa (e.g. Lepidoptera) showed better body conditions than those preying on smaller ones (among the most frequently recorded, Dipterans), as predicted by our third hypothesis. Moths are a highly profitable prey type for bats (Kolkert et al., 2020), yet those that have evolved tympanic organs may detect bat ultrasonic calls and evade capture in most cases (see e.g., Corcoran and Conner, 2016; Ter Hofstede and Ratcliffe 2016). The bat species that frequently prey upon tympanate moths show counterstrategies to catch such otherwise inaccessible prey types (reviewed in Ter Hofstede and Ratcliffe 2016). Since *Hypsugo savii* lacks such adaptations (Kipson et al., 2022), moths (many of which tympanate) usually represent a relatively limited portion of its diet (Kipson et al., 2018). Noticeably, bats from our study featured at least 4 moth species among the taxa most frequently preyed on. As such, the mechanisms allowing *H. savii* to profitably exploit tympanate moths are yet to be defined, but the higher densities of such prey type in agricultural areas may have played a role in the high frequencies we found in our samples. Similarly, at least for females, different energy demands due to pregnancy and lactation during early summer may have driven the higher occurrence of larger prey in individual bats' diets, in comparison to late summer when juveniles become independent (McLean and Speakman, 1999). Temporal differences in prey consumption over the summer season may also have been influenced by possible phenological shifts in prey distribution or abundance.

We show that the traits of individual bats and their prey both influence prey-predator relationships and the potential ecosystem service delivered by insectivory in agroecosystems. Organic farming and other biodiversity-friendly practices (Froidevaux et al., 2017; Puig-Montserrat et al., 2021) further enhance pest suppression by bats on farmland (Russo et al., 2018), which highlights how simple practices may foster a virtuous circle between biodiversity conservation and farmland productivity (Bengtsson et al., 2005). Our study also provides support for the potential of bat diet analyses as a powerful tool for the simultaneous surveillance of arthropods from different crops and habitats, providing insights into the occurrence and distribution also of important and emerging plant disease vectors (e.g., *Philaenus spumarius* as a vector of the olive-tree pathogenic *Xylella fastidiosa*; Cornara et al., 2018) besides actual pests. Moreover, the molecular analysis of bat diet may not only promote the study and surveillance of pest arthropods in agroforestry systems (Ancillotto et al., 2022), but may be also applied to the detection of new, i.e., recently introduced (Montauban et al., 2021), or rare species. As an example, our sample featured one sequence that matched (at 98%) those of *Micromus angulatus* (Neuroptera), a species whose last record in the study region dates to the 1950s despite several more recent surveys (A. Letardi, pers. comm.) and certainly worth in-depth verification. Taken together, our results emphasize the high potential of bats as natural samplers, efficiently aiding the surveillance of a wide range of target arthropods relevant to the economy, public health and/or conservation, e.g., by pointing out whether (and where) to implement more specific field methods for insect detection and possibly management.

Finally, our study remarks on the importance of common, abundant species as potential deliverers of ecosystem services, confirming the value of studying and managing such too often neglected species appropriately.

Author contributions statement

DR and *LA* conceived the ideas and designed the methodology; *LA, AF* and *PC* identified the study site and collected the data; *GA, NT, APG, FdB, UB* and *AG* analysed the data; *LA* and *DR* led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.actao.2023.103890.

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