

Food for everyone: differential feeding habits of cryptic bat species inferred from DNA metabarcoding

Tommy Andriollo¹, Johan Michaux², and Manuel Ruedi¹

¹Museum d'histoire naturelle de la Ville de Geneve

²Université de Liège

August 16, 2021

Abstract

Ecological theory postulates that the niche of co-occurring species must differ along some ecological dimensions in order to allow their stable coexistence. Yet, many biological systems challenge this competitive exclusion principle. For instance, insectivorous bats from the Northern Hemisphere typically form local assemblages of multiple species sharing highly similar functional traits and pertaining to identical feeding guilds. Although their trophic niche can be accessed with unprecedented details using genetic identification of prey, the underlying mechanisms of resource partitioning remain vastly unexplored. Here, we studied the differential diet of three phenotypically and phylogenetically closely-related bat species of the genus *Plecotus* in an area of sympatry and throughout their entire breeding season (April-October) using DNA metabarcoding. Even at such a small geographic scale, we identified strong seasonal and spatial variation of their trophic niche at both intra- and inter-specific levels. Indeed, while the different bats fed on a distinct array of prey during spring, they showed higher niche overlap during summer and fall, when all three bat species switched their hunting behavior to feed on few temporarily abundant moths. Furthermore, by considering the ecological traits of prey species, we inferred from the menu of each bat species that feeding grounds and hunting techniques differed suggesting that niche partitioning was primarily habitat-driven. As predicted by their phylogenetic relationships, the two most-closely related bat species exhibited the most distinct foraging habitat preferences, while the third, more distantly-related species was more generalist. These results highlight the need of extensive samples to fully understand species coexistence.

Introduction

The competitive exclusion principle, or Gause's law, is a central principle in ecology positing that limited resources prevent the stable coexistence of two species relying on their similar use (Hardin, 1960). As a corollary, species living in sympatry are likely to have a differentiated use of limiting available resources –known as resource partitioning– that reduces niche overlap and competition. Resource partitioning in co-occurring species can be achieved along several dimensions of their ecological niche (Hutchinson, 1957). The most important ones are linked to habitat and food use (Schoener, 1974), but many additional processes have been described to complete this Hutchinsonian niche concept (Holt, 2009). Studying ecological niche dimensions can be challenging, especially when working with rare, elusive and morphologically cryptic species or when many closely related forms coexist in the same place. Echolocating bats represent such a group, as they can form assemblages of numerous species pertaining to the same feeding guilds, and sharing remarkably similar functional traits such as morphological and echolocation characteristics (Aldridge & Rautenbach, 1987; Mancina *et al.*, 2012; Roswag *et al.*, 2018a; Schnitzler & Kalko, 2001; Vesterinen *et al.*, 2018). A growing number of studies also revealed that bat communities are formed by species more closely-related

than expected by chance, a mechanism called phylogenetic underdispersion (Patrick & Stevens, 2014; Presley *et al.*, 2018; Riedinger *et al.*, 2013). The most common approach to characterize interspecific competition within such species assemblages relied on diet analyses (Salinas-Ramos *et al.*, 2020). Such studies benefitted from the advent of metabarcoding techniques, which provide unprecedented resolution in taxonomic identifications (Alberdi *et al.*, 2019; Clare, 2014). Despite these technical advances, mechanisms mediating species coexistence in bats still remain unclear.

Two main measurements of the trophic niche are classically used to disentangle feeding ecology of interacting species: the trophic niche breadth allows to distinguish generalist from specialist feeding strategies (e.g., Lopes *et al.*, 2015; Salinas-Ramos *et al.*, 2015), while dietary niche overlap measures the level of competition for food between species (e.g., Aldasoro *et al.*, 2019; Chang *et al.*, 2019; Kartzinel *et al.*, 2015; Leray *et al.*, 2015; Sato *et al.*, 2018). However, such ecological indices consider each prey species as a simple resource type, and little or no information is usually derived from the ecology of the prey itself (Spitz *et al.*, 2014). Some authors relied on informal considerations about major prey groups or singular prey species (e.g., Chang *et al.*, 2019; Salinas-Ramos *et al.*, 2015; Vesterinen *et al.*, 2018), while others used simple tests to assess habitat preferences of pairs of predators once prey species were assigned to different ecological categories (Razgour *et al.*, 2011; Roswag *et al.*, 2018b). Yet, more sophisticated approaches relying on multivariate statistical analyses exist to relate characteristics of multiple consumers to habitat or functional traits of their prey (Quéméré *et al.*, 2013; Spitz *et al.*, 2014). Though promising, their use is still limited and for instance did not help disentangling the ecology of two sibling species in a case study applied to bats (Arrizabalaga-Escudero *et al.*, 2018).

We explored here a biological system involving three long-eared bat species (genus *Plecotus*) occurring in Western Europe: two are closely-related sister species, *P. auritus* and *P. macrobullaris*, while the third, *P. austriacus*, is more distantly related (Juste *et al.*, 2004; Spitzenberger *et al.*, 2006). Pairs of these three species can coexist in close vicinity, including in the same building, but do not hybridize (Andriollo *et al.*, 2018) and are much more rarely found in trios. Morphologically, all three species are so similar that they may be challenging to identify in the field (Andriollo & Ruedi, 2018; Ashrafi *et al.*, 2010). They also have nearly identical echolocation call characteristics (Barataud, 2015; Dietrich *et al.*, 2006) and wing shape (Entwistle *et al.*, 1996) suggesting that all three species share similar foraging behavior as well (Schnitzler & Kalko, 2001). Indeed, long-eared bats are known to feed extensively on tympanate moths (Alberdi *et al.*, 2012; Vaughan, 1997). Whereas the diet of *P. auritus* can be more diverse as it includes also many prey from other insect orders (Ashrafi *et al.*, 2011; Motte, 2011; Razgour *et al.*, 2011), micro-histological identification of prey remains indicated that the diet of *P. austriacus* and *P. macrobullaris* cannot be differentiated, suggesting that the latter taxa compete for the same trophic niche (Ashrafi *et al.*, 2011). As these two species exhibit essentially parapatric distributions at the regional scale (Mattei-Roesli, 2010; Rutishauser *et al.*, 2012), several authors suggested that they occupy the same ecological niche, preventing their stable coexistence in sympatry (Alberdi & Aizpurua, 2018; Ashrafi *et al.*, 2011; Dietrich *et al.*, 2006; Rutishauser *et al.*, 2012). However, this observation is contradicted by the co-occurrence of both species across wide areas in the Dinaric Alps (Tvrtković *et al.*, 2005), in Corsica (Courtois *et al.*, 2011), in the Pyrenees (Alberdi *et al.*, 2014), in the French Prealps (Arthur & Lemaire, 2015), or in the Geneva region (Gilliéron *et al.*, 2015; Rutishauser *et al.*, 2012). This hypothesis of competitive exclusion is also challenged by the fact that the distribution of *P. macrobullaris* is still confined to the higher elevations in the eastern part of its range, where *P. austriacus* does not occur (Alberdi *et al.*, 2014).

To understand how interspecific competition is mediated among the highly similar long-eared bats, we studied their diet in a unique area of sympatry where multiple colonies of all three species are established in close proximity and potentially exploit overlapping feeding grounds (Gilliéron *et al.*, 2015). Following the optimal foraging theory, we hypothesized that the trophic niche of bats would be wider when availability in preferred resources is low (for moths typically in spring), while specialization on particular prey groups would take place during other periods of the year. Since insect abundance and diversity are known to vary seasonally, we designed this study based on a sampling regime covering the entire period of activity of the bats in maternity roosts (April-October). Our first goal was to document the spatial and seasonal variation of the

diet of all three species simultaneously and with a high-resolution metabarcoding technique to identify the crucial periods of interspecific competition. The second goal was to infer indirectly the feeding strategies of these predators using the ecological traits of hundreds of prey species identified in their diet. Our final aim was to understand species coexistence by revealing the mechanisms of trophic niche partitioning occurring among these three sympatric and elusive bat species.

Material and Methods

Feces sampling

We collected guano samples from nine breeding colonies of long-eared bats established within an 18 km-radius area in the Geneva region (Figure 1). Initial genetic identifications of the bats suggested that all colonies were monospecific (Andriollo & Ruedi, 2018) and occupied by brown long-eared bats *Plecotus auritus* (five colonies), by grey long-eared bats *P. austriacus* (two colonies) or by alpine long-eared bats *P. macrobullaris* (two colonies). The closest colonies of the three species were found within 2 km from each other, indicating that bats from all species could potentially feed in the same territory (Ashrafi *et al.*, 2013; Gilliéron *et al.*, 2015; Preatoni *et al.*, 2011). During the year 2015, all fresh guano was collected from clean paper sheet placed under each roost and sampled every other week from mid-April to mid-October. This timespan covers notably the three major seasons of activity typical of temperate bats, i.e. spring (from mid-April to mid-June, before pups are born), summer (from mid-June to mid-August, when pups are reared) and autumn (from mid-August to mid-October, when juveniles are weaned and adults disperse to hibernacula; Figure 1).

Extraction, sequencing and prey identification

For each of the 11 dates and the 9 locations, we extracted “community samples” consisting in an aggregate of about 60 mg of guano pellets taken from the bulk of guano collected. Such community samples therefore represent a random collection of pellets produced by several members from a colony during two weeks. Such community samples are therefore more likely to represent the overall prey spectrum consumed by animals from a given colony, rather than reflecting the hunting preference of a particular individual. This totally unobtrusive design of guano collection allowed repetitive sampling without disturbing maternity colonies and without the need to capture the animals. Besides the community samples, we also extracted six “individual samples” consisting in about 8 mg of guano (1-3 pellets) for each date. These individual samples were issued from three neighboring colonies occupied each by a different bat species (numbered 1, 6 and 8 in Figure 1). These individual samples were used to calculate percentage of prey occurrence (wPOO; Deagle *et al.*, 2018) and are equivalent to the fecal production of a single individual. The DNA extraction protocol followed Zeale *et al.* (2011) with modifications to improve yield as described in Andriollo *et al.* (2019a). PCR blanks were used to ensure that no major cross-contamination occurred during laboratory procedures and that tag-jumping was limited (Taberlet *et al.*, 2018). Additionally, all DNA extracts were randomly placed into sequencing plates to prevent any systematical bias such as contamination among adjacent wells. A 157 bp-long fragment of the COI barcode gene was amplified using ZBJ primers and a PCR setup detailed in Zeale *et al.* (2011). Although these primers were specifically designed to amplify a large range of arthropods from the diet of insectivorous bats, they may exhibit some amplification biases (Clarke *et al.*, 2014; Elbrecht *et al.*, 2016; Elbrecht *et al.*, 2019; Tournayret *et al.*, 2019). However, as they work particularly well with the prey consumed by long-eared bats (Zeale *et al.*, 2011), it seems unlikely that such potential bias would severely affect measurement of the diet from those three species. As alternative primer combinations rely on different markers (typically 16S) for which extensive reference sequence databases are lacking, this COI barcode is currently also the most efficient marker to get species-level identifications of arthropods. Library construction, sequencing of PCR products, demultiplexing of raw data, clustering of sequences in MOTUs and their taxonomic assignment were all carried out as detailed in Andriollo *et al.* (2019a).

Measurement of trophic niche

Prey species were treated as percentage of occurrence in the total dataset, weighted for each extracted sample by the number of prey species identified in the given sample (wPOO; Deagle *et al.*, 2018). This method for describing the diet was preferred as it performs better than other indices when dealing with extremely diversified diets such as that of long-eared bats (Andriollo *et al.*, 2019a). To estimate the completeness of the prey spectrum evaluated for each species and season, we used the Chao2 minimum estimator of asymptotic species richness (Chao, 1987; Colwell *et al.*, 2012) computed with the software EstimateS 9.1.0 (Colwell, 2013).

Niche breadth of each predator was measured using the Levins' index (Levins, 1968), whereas niche overlap between pairs of species was calculated using the Morisita-Horn's index (Horn, 1966; Morisita, 1959). Statistical significance of niche overlap was tested using the RA 3 randomization algorithm proposed by Lawlor (1980) and implemented in the package EcoSimR (Gotelli *et al.*, 2013). To visualize niche overlaps among the three bat species, matrices of pairwise niche overlap were projected through multidimensional scaling (MDS) using the principal coordinates analysis (PCoA) function implemented in the R package ade4 (Dray & Dufour, 2007). We also carried out a simpler principal component analysis (PCA) based on the presence absence matrix of all prey species in an attempt to identify the ones explaining similarity among samples (i.e., directly linked to niche overlap).

Ecological traits of the prey

In order to infer indirectly the hunting habitats of the three species of long-eared bats from their diet, we first gathered the main ecological preferences of all identified prey species (Appendix 1) from the entomological literature (Appendix 2). This information was sorted into 18 binary ecological traits (Table 1) mostly related to habitat, but also included whether the prey species was flying at night or not. This latter category characterizes prey such as syrphid flies or woodlice that must be gleaned from solid surfaces when hunted by night, as they are either strictly diurnal or flightless.

In order to relate the bat species, the consumed prey and their ecological traits, we used an RLQ analysis (Dolédéc *et al.*, 1996; Legendre *et al.*, 1997) as implemented in the R package ade4. This ordination method summarizes the joint structure of the following three tables (or matrices): R, containing the bat species to which a sample is issued, Q, containing the ecological traits of the prey species, and L, the linking table containing the prey spectrum of each bat species. The fourth-corner statistics was used to test the significance of relationships between bat species and ecological traits (Dray *et al.*, 2014). Both permutation of entire rows (model 2) and columns (model 4) of the linking table were carried out, and outputs of these models were combined (model 6) in order to avoid inflated type I errors (Dray & Legendre, 2008; ter Braak *et al.*, 2012). Significance of these fourth-corner statistics was assessed by performing 9'999 permutations, and p-values were adjusted by the false discovery rate method (FDR; Benjamini & Hochberg, 1995; Dray *et al.*, 2014).

Results

Curated dataset of consumed prey

The sequenced library of both community and individual guano samples (284 samples in total) produced 5'016'988 Illumina reads (raw data deposited on Zenodo doi:10/XXX), representing 1'349 distinct sequences that were clustered into 883 MOTUs. Of these MOTUs, 125 (14%) did not match to any existing reference DNA sequence and were removed from the dataset. A further 46 MOTUs were excluded as they represented obvious environmental contaminants (fungi, algae, bacteria and rotifers), or were arthropod species (21 of them) known to feed on guano but not likely to be preyed upon by bats (e.g., mites and dermestid beetles). Two species of slugs, *Deroceras reticulatum* and *Arion vulgaris*, present in three distinct samples from roosts of *P. auritus*, were discarded as well as they were certainly secondary prey of the carabid beetles eaten by the

bats, not their actual prey species (see also Galan *et al.*, 2018). Unexpectedly, the DNA of two bat species, *Pipistrellus pipistrellus* and *Eptesicus serotinus*, was detected in five of the 284 fecal samples analyzed suggesting that these species may have roosted occasionally with long-eared bats. A visual inspection of the prey composition in these five potentially contaminated fecal samples, however, indicated that these non-targeted bats contributed minimally to exogenous prey. Indeed, most prey species identified in these samples were moth or large flies (typical of long-eared bats), whereas pipistrelles would mostly feed on small dipterans (Swift *et al.*, 1985) and serotines on large beetles (Kervyn & Libois, 2008; Robinson & Stebbings, 1993), that were absent from these particular guano samples.

Molecular diet of long-eared bats

In the curated dataset of consumed prey, including the community samples for nine colonies and the individual samples for three colonies, a total of 687 distinct MOTUs were retained, 319 (46%) of which occurred in a single guano sample. A total of 602 (88%) of these distinct MOTUs were identified taxonomically to the species level, while the remaining ones were assigned to the genus, family or order level (2%, 7% and 3%, respectively; Appendix 1). Hunted prey were mostly insects (668 MOTUs), but also included 14 spiders, four woodlice and one large springtail species. Woodlice were retained in this dataset since they do occur, albeit rarely, in bat guano analyses (e.g., Kaňuch *et al.*, 2005; Leelapaibul *et al.*, 2009; Rydell *et al.*, 2016). The springtail was also kept as it is possibly large enough (3 mm) to be targeted by bats or accidentally consumed during grooming (Vesterinen *et al.*, 2013).

In the complete prey spectrum (both community and individual samples) and as expected, the most represented insect orders were Lepidoptera and Diptera (392 and 193 MOTUs, respectively). Hemiptera, Hymenoptera, Coleoptera and Neuroptera included 14 to 17 species, while other detected orders (Blattodea, Dermaptera, Mecoptera, Orthoptera, Psocodea, Raphidioptera, and Trichoptera) were only represented by a handful of species. Most species identified in these fecal samples were already recorded in local (Merz, 2012) or national faunal inventories (de Jonget *et al.*, 2014), or were likely recent colonizers for the country (Andriollo *et al.*, 2019b). Interestingly, a number of diurnal or non-flying arthropods (earwigs, orthopterans, scorpionflies, woodlice and most spiders) were only detected in fecal samples of *P. auritus*, while other arthropod orders were represented in comparable proportions in the diet of the three long-eared bat species.

When focusing on the individual samples gathered in the three neighboring colonies, the diet of *P. auritus*, *P. austriacus* and *P. macrobullaris* included 300, 171 and 157 prey species, respectively (Figure 2a). Chao2 extrapolations from accumulation curves suggest that these numbers only represent 52 to 66% of the potential prey richness, the diet of *P. auritus* being the most underestimated (Figure 2b). These extrapolations also indicated that 98 to 220 individual samples per species (instead of 66) would have been necessary to detect 95% of the total species richness, which stresses the extreme diversity of the diet of all these bats. Only 54 (12%) prey MOTUs were shared by the three bat species (Figure 2a). These shared prey species included nine very common moths (*Agrotis exclamationis*, *Agrotis ipsilon*, *Autographa gamma*, *Hoplodrina ambigua*, *Mythimna albipuncta*, *Mythimna pallens*, *Noctua pronuba*, *Nomophila noctuella* and *Xestia c-nigrum*) that were detected in more than 20% of samples of each bat species. According to wPOO estimates, lepidopterans represented by far the most preferred prey in all three long-eared bat species (73, 80 and 91% for *P. auritus*, *P. austriacus* and *P. macrobullaris*, respectively), followed by dipterans (4, 13 and 15%; Figure 2c). Guano samples gathered in summer and autumn invariably exhibited more Lepidoptera than the ones from spring, while the proportion of consumed dipterans varied considerably from one season or from one predator species to another. The same seasonal pattern and very similar proportions in arthropod orders were retrieved in the diet of the three bat species recovered in the community samples analyzed (Appendix 3). In absolute numbers, however, less prey species were identified in the 96 community samples (Appendix 3), compared to the 186 individual samples analyzed (Figure 2a), as is expected for such unequal sampling effort.

Variation of the trophic niche breadth

The dietary diversity in terms of prey species exhibited the same pattern of seasonal changes for all three bat species. The trophic niche breadth of each long-eared bat was indeed low during spring and autumn and higher during summer (Figure 3a). This pattern, however, was mostly due to an increase of consumed noctuid and geometrid moths, whose species diversity and abundance peak in summer (Altermatt, 2010). Indeed, when considering prey identified at the family level (Figure 3b), niche breadth was actually decreasing throughout the year for the three bat species. This implies that the predators consumed a restricted taxonomic range of arthropods during the summer and fall seasons. Regarding specific amplitude of the diet, *P. auritus* consistently exhibited a broader trophic niche than *P. austriacus* and *P. macrobullaris* irrespective of the season (Figure 3a), or whether we focused on individual or community samples (results not shown). This result is not only due to a higher number of colonies of *P. auritus* evaluated (Figure 1), as the Levins' index measured for individual samples (i.e. with a same effort of one analyzed colony per species) throughout all the sampling period was also higher in *P. auritus* (34.4 ± 9.2) compared to *P. austriacus* (22.7 ± 8.4) or *P. macrobullaris* (22.0 ± 9.5).

Variation in trophic niche overlap

According to the measure of niche overlap among the different bat roosts (Figure 4a), it appears that the three species of long-eared bats were more similar within a given season than same-species comparisons made across seasons. This was, however, less obvious in spring when the diet from each colony was more dispersed in the MDS representation, particularly those of *P. auritus*. This seasonal variation of diet similarity was also recovered in the PCA conducted on the prey composition of samples (Appendix 4). These similarities were driven by insect species with a marked seasonal phenology that were frequently eaten by the three bat species. For instance, the June beetle *Rhizotrogus aestivus* and the spring moth *Korscheltellus lupulinus* were characteristic of the spring samples, whereas summer noctuids such as *Cosmia trapezina* and *Hoplodrina blandawere* were most abundant in samples collected during summer. The late-summer crambid *Nomophila noctuella* and the hepialid *Triodia sylvina* (Poltavsky, 2014; Robineau *et al.*, 2007) were accordingly mostly retrieved in guano samples collected in autumn (Appendix 4).

Dietary similarities were unequal among species and season (Figure 4b). Measures of trophic niche overlap among bat species during spring were indeed lower than those during summer and autumn. The pair *P. austriacus* and *P. macrobullaris* systematically exhibited greater niche overlap than they did with *P. auritus*. The largest values of trophic niche overlap were observed between *P. austriacus* and *P. macrobullaris* (mean value across the three seasons: 0.62). *P. auritus* exhibited much lower niche overlap with *P. austriacus* (0.28) and *P. macrobullaris* (0.30). All values of overlap were statistically higher than expected by chance ($P < 0.01$).

Relationship between predator species and the ecological traits of prey

Habitat preferences of the consumed arthropods could be estimated for 605 (88%) MOTUs identified to the species level. The non-evaluated items were either MOTUs identified only to the order or family or for few species for which no specific ecological traits could be found in the literature (Appendix 2).

Fourth-corner statistics indicated that ecological traits of prey significantly ($p < 0.05$) differed from one bat species to another. *P. auritus* tended to consume prey issued from lowland cluttered habitats (woodlands, hedgerows) rather than from open or mountainous areas (such as meadows and lawns, slopes, screes and rocky areas) and was also positively and significantly associated to diurnality or flightlessness of prey (Table 1). *P. macrobullaris* exhibited opposite preferences in terms of feeding habitats, as it preyed preferentially on arthropods living in open habitats and in dry and mountainous areas (e.g., screes, slopes). Finally, *P. austriacus* showed no marked preference for specialized prey, and fourth-corner statistics indicated that it was only positively associated to ubiquitous prey species, and negatively so to prey typical of closed habitats (Table 1).

Typical prey species

A more detailed examination of the The RLQ analysis (Appendix 5) indicated that the consumed arthropods most associated with *P. auritus* faecal samples included many non-flying (spiders, woodlice, cockroaches, ground beetles, earwigs, psocopterans) or diurnal arthropods such as syrphid flies. Its diet was also associated to neuropterans and several tipulids living in woodlands. Forest tachinids were also detected (e.g., *Cyzenis albicans* and *Eloceria delecta*), although these parasitic flies could be secondary prey contained in moth larvae eaten by bats. Moth species associated to *P. auritus* included geometrids (e.g., *Camptogramma bilineata* and *Operophtera brumata*) commonly found in woodlands or urban areas, noctuids (e.g., *Anorthoa munda*, *Dichonia aprilina*, *Tiliacea citrigo* and *Amphipyra* spp.) typical of deciduous woodlands, and pest tortricid species (e.g., *Archips xylosteana* and *Cydia pomonella*) thriving in orchards or woodlands.

The ubiquitous prey identified in the diet of *P. austriacus* included flies from cultivated lands, such as the chloropid *Thaumatomyia notata* that lives in agricultural fields, meadows and grasslands, the muscid *Musca autumnalis*, a pullulating pest species for cattle and horses, and the calliphorid *Pollenia pediculata* notably found in urban areas. Moth species typical for *P. austriacus* included the noctuids *Caradrina clavipalpis* and *Mamestra brassicae* that can be found in a variety of open habitats.

Finally, the diet of *P. macrobullaris* was characterized by many prey species typically associated to mountainous areas such as dry meadows and lawns or sunny slopes and screes, including the noctuids *Euxoa aquilina* and *Bryophila domestica*, and the geometrids *Gnophos furvata*, *Hemistola chrysoprasaria*, *Horisme radicularia* and *Nychiodes obscuraria*.

Discussion

Co-occurring cryptic species constitute ideal models to investigate mechanisms of trophic niche partitioning with fine-grained resolution. Previous attempts to unravel the diet of sympatric species have been carried using DNA metabarcoding, but all either focused on distantly-related species (e.g., Emrich *et al.*, 2013; Vesterinen *et al.*, 2018), on geographically-distant or time-limited samplings (e.g., Arrizabalaga-Escudero *et al.*, 2018; Razgouret *et al.*, 2011). The present study is the first of its kind to characterize the diet of three cryptic long-eared bats living in narrow geographic vicinity, and to access with high resolution their menu of arthropods throughout an entire season of activity. Experimental results obtained from this experimental design indicate that these closely-related bats share most of their diet, but also partition their trophic niche at critical periods of the year. Furthermore, differences in habitat use and hunting strategies of these bats are highlighted by analyses of prey ecological traits.

Sympatric bats share major components of their diet

High-resolution techniques have been proven to be essential for studying the diet composition of insectivorous bats, since many species exhibit very similar diets when prey resources are identified to the ordinal level (Vesterinen *et al.*, 2018). As expected, this was particularly true in the case of long-eared bats, which are specialized hunters of tympanate moths. We confirmed here that lepidopterans represent the bulk (73-91%) of their diet, followed by dipterans (4-15%), while all other arthropod groups represented less than four percent of the prey species detected (Figure 2c). These proportions were consistent with the ones recovered in previous metabarcoding studies (Alberdi *et al.*, 2012; Razgour *et al.*, 2011; Vesterinen *et al.*, 2018) and micro-histological analyses of feces (Andreas, 2002; Ashrafi *et al.*, 2011; Motte, 2011; Robinson, 1990), suggesting that recovery biases of the selected primers (Zeale *et al.*, 2011) had a limited impact on the diet assessment. As all long-eared bats share this highly specialized menu, this observation also corroborates the high overall similarities of hunting strategies predicted from their high phenotypic resemblance. Despite their high specialization on moths, species-level identification of prey indicated that the diet of these bats was much diversified, with over 680 distinct arthropod MOTUs identified in their feces (Appendix 1). Only a fifth of moth species appeared in the menu of all three long-eared bat species (Figure 2a) but included several

common noctuid species (*Agrotis* spp., *Mythimna* spp., *Noctua* spp.) that were frequently eaten by all three long-eared bat species. These abundant and widespread taxa seem to be opportunistically hunted by other insectivorous bats from different feeding guild and could represent an important component of their diet (Arrizabalaga-Escudero *et al.*, 2018). These shared prey species were particularly common in the summer diet, indicating that the three bat species had an opportunistic feeding behavior to exploit this temporally abundant trophic resource to meet their energetic demands (Arlettaz, 1996; Clare *et al.*, 2011; Cohen *et al.*, 2020). Consistently with a plastic and opportunistic hunting behavior of all three species of long-eared bats, the prey content of the guano sampled at various times of the year was more similar within any given season regardless the identity of the predator, rather than within bat species (Figure 4a).

Niche partitioning is highly seasonal

Despite parallel shifts in the menu of these three species of bats, levels of trophic niche overlap among bat species exhibited strong variations across seasons and provided crucial information to understand niche partitioning in long-eared bats. Trophic niche overlap was lower during spring and then increased notably in summer and early autumn (Figure 4b). This relationship is somewhat counterintuitive as the moth diversity (the main prey of these bats) is also at its highest during the latter seasons (Altermatt, 2010) giving more opportunities to these bats to exploit different resources. Instead, during these summer months, few abundant moth species appeared in the menu of all three species resulting in a temporary increase in niche overlap. This pattern suggests that few, very common insects may provide nearly unlimited food resources during short periods of time, temporarily alleviating the need for niche partitioning. Conversely, the trophic niche partitioning mostly occurred during spring, when prey availability was lower, both in terms of diversity and abundance. At this time of the year, all three bat species appear to feed on a more diverse range of invertebrates (Figure 2 and Figure 3), including more beetles which were virtually absent from their autumn diet.

Consistent with the predictions of niche partitioning hypothesis, the lowest niche overlap observed in spring likely evolved as a result of stronger selective pressure over food exploitation, i.e. when availability of insect prey is more limiting. Other highly similar, sibling species showed the same opportunistic behavior to feed on locally and temporally abundant prey, whereas they relied on partitioned resources at other times of the year (Arlettaz, 1996). We suggest this seasonal constraint probably applies more generally to all insectivorous bats from temperate regions with strongly marked seasonal insect phenologies. Such seasonal niche partitioning can only be detected if the sampling regime covers most of the life cycle of these bats, whereas it could be overlooked with designs focusing on narrower time scales, as has been done in many comparative studies (e.g., Arrizabalaga-Escudero *et al.*, 2018; Roswag *et al.*, 2018b).

Niche partitioning is related to differential foraging habitats

Integrating the ecological traits of prey through RLQ analyses of the diet suggested the existence of marked differences in the preferred hunting grounds of the three bat species. In particular, we found that prey from closed habitats such as woodlands and hedgerows were overrepresented in the diet of *P. auritus*, whereas that of *P. macrobullaris* included more prey species living in open and rocky or mountainous areas. Habitat preferences of the prey consumed by *P. austriacus* were lying between those two extreme patterns, for which the only significant relationship was a positive link to ubiquitous insects and a negative one to closed habitats (Table 1). The two sister species *P. auritus* and *P. macrobullaris* appeared to partition their trophic niche through the exploration of distinct hunting grounds, while *P. austriacus* was more generalist with no marked habitat preferences.

These results also shed lights on the different trophic niche breadths observed in the three long-eared bat species, which was higher in *P. auritus* when compared to the other two bat species (Figure 3a). Indeed, while this result was consistent with the wide geographic distribution of *P. auritus* across diverse habitats in Europe, and with the strong environmental and trophic specialization of *P. macrobullaris* (Alberdi *et al.*,

2012; Alberdi *et al.*, 2014; Ashrafi *et al.*, 2011; Benda *et al.*, 2006), the widespread *P. austriacus* exhibited a surprisingly narrow trophic niche. While no firm conclusion can be made to explain this observation, we suggest it could be due to exploitation of comparatively species-poor habitats (typically, agricultural lands) by this last bat species. These results based on indirect inferences from their menu also corroborate previous observations of foraging habitats conducted with more extensive and time-consuming radiotracking data (Alberdi *et al.*, 2012; Preatoni *et al.*, 2011; Razgour *et al.*, 2011). Our indirect, RLQ approach has thus a strong potential to be applied for reconstructing the foraging ecology of elusive species without the need to capture or actually track them in their natural habitat. Finally, the inferred habitat-driven niche partitioning also corroborates a growing number of studies demonstrating that habitat selection could be a major mechanism for resource partitioning in European insectivorous bats, even when those bats are morphologically highly similar and broadly sympatric (Arlettaz, 1999; Arrizabalaga-Escudero *et al.*, 2018; Nicholls & Racey, 2006).

Niche partitioning could be mediated through differential feeding strategies

The analysis of prey traits does not only provide information on hunting habitats, but also on the different hunting techniques used by bats. Here in particular, we investigated the importance of the prey locomotion type to explain differences in diet composition of bats (Table 1). A number of typically diurnal taxa (e.g., syrphid flies) or non-flying groups (e.g., carabid beetles, spiders and woodlice) were recovered in the diet of all three species of bats, but they were significantly more prominent in the diet of *P. auritus*. These prey species are expected to be gleaned by bats from solid surfaces, not in flight. Captivity experiments demonstrated the importance of the gleaning behavior in long-eared bats (Anderson & Racey, 1991). This hunting technique is supposedly more common in *P. auritus* than in *P. austriacus* (Andreas, 2002; Bauerová, 1982; Beck, 1995), whereas non-flying prey was rarely reported in the diet of *P. macrobullaris* (Ashrafi *et al.*, 2011).

The gleaning behavior has been classically invoked to account for the presence of diurnal lepidopteran wings in bat roosts (e.g., Barataud, 1990; Meineke, 1991; Motte, 2011). Yet, during the eleven visits of nine different long-eared bat roosts throughout the year, we only recorded decayed wings of butterflies (*Aglais urticae* and *Inachis io*) in two occasions under colonies occupied by *P. auritus*. This paucity of butterfly remains in the diet of *Plecotus* was confirmed by the metabarcoding approach since no butterfly sequence was identified despite the high number of other lepidopterans recovered in each guano sample (Appendix 1). Previous molecular analyses of *Plecotus* feces recorded a single butterfly (*Argynnis paphia*) out of 160 lepidopteran species (Vesterinen *et al.*, 2018), or none at all (Alberdi *et al.*, 2012; Razgour *et al.*, 2011). Potential amplification bias can be excluded as diurnal lepidopterans were recorded in the diet of other insectivorous bats using the same primer pair (e.g., Bohmann *et al.*, 2011; Vesterinen *et al.*, 2018). Conversely, we suggest that wing remains of butterfly retrieved in attics might therefore not reflect insects preyed by bats, but could have been eaten by other animals such as spiders or rodents (Olofsson *et al.*, 2011; Wiklund *et al.*, 2008) living in the same attics.

Bats for diversity assessment and ecosystem monitoring

In the current study, long-eared bats appeared to be prodigiously good samplers of the local nocturnal biodiversity. Although we only uncovered a small portion of the total dietary diversity of long-eared bats in our experimental design (Figure 2b), we retrieved more than a fifth of all moth species known from the area sampled, including 17 that were new occurrences for this well-studied region (Andriollo *et al.*, 2019; Merz, 2012). Additionally, six neuropteran species found in the guano of long-eared bats were not listed among the 26 known to occur in the Geneva province (Andriollo *et al.*, 2016; Hollier, 2012), suggesting again that metabarcoding of bat guano is a highly efficient tool for biodiversity assessment, provided that good reference databases exist. It also illustrates that bats can be used to monitor the presence of economically-relevant species such as *Calliphora* and *Lucilia* flies, vectors of diseases for human and cattle, and the alien fruit fly *Drosophila suzukii*, a major pest and an economic concern for fruit crops in many countries (Calabria *et al.*, 2012; Mazzi *et al.*, 2017).

Besides diversity assessment and biomonitoring, bats are known to provide important ecosystem services. Insectivorous species in particular selectively shift their diet to feed on spawning pest species (Baroja *et al.*, 2019; Cohen *et al.*, 2020; Kolkert *et al.*, 2020) and are supposed to prevent major agricultural losses through insect control (Boyles *et al.*, 2011; Brown *et al.*, 2015; Kunz *et al.*, 2011). Although the contribution of long-eared bats to pest suppression cannot be quantified from our data, it is noteworthy that a fifth of their diet was composed of insects typically found in agricultural landscapes, and that a total of 57 species considered as agricultural pests were identified in their feces. In particular, the noctuids *Agrotis exclamationis*, *Agrotis ipsilon*, and *Helicoverpa armigera* that are known to cause major damages to crops were detected in 61, 43, and 22% of samples, respectively, suggesting a very common consumption of these pests. At the very least, and considering that long-eared bats intensively use man-made buildings (typically attics or barns) to establish their maternity roosts in agricultural landscapes, we hope that their contribution to pest suppression can be used as an effective argument to promote conservation efforts and peaceful coexistence of human with bats in rural areas.

Acknowledgments

We gratefully acknowledge the people who helped us with guano sampling (alphabetically): Lucie Cauwet, Janik Pralong, Carlos Rouco, Cyril Schönbächler, Emmanuel Tardy, Laurent Vallotton and Eric Verelst. We also thank the owners of buildings who rendered this study possible. Lise-Marie Pigneur (Université de Liège) helped during laboratory work, and Adrien André (Université de Liège) helped with Illumina raw data extraction. Bernard Landry, Charles Lienhard and John Hollier (MHNG) provided valuable entomological expertise. Raphaël Covain (MHNG) and Stéphane Dray (CNRS, Lyon) provided useful expertise on statistical analyses. Antton Alberdi (University of Copenhagen), Jan Pawlowski (University of Geneva), Jean Mariaux and Lionel Monod (MHNG) provided useful comments on an earlier version of the manuscript. TA also thanks Frédéric Boyer, Eric Coissac, Eric Marcon, Pierre Taberlet and Lucie Zinger for fruitful discussions during the eighth DNA metabarcoding school held in French Guiana. This study benefitted from the financial support from the Direction Générale de l'Agriculture et de la Nature de l'État de Genève and the Fondation Ernst & Lucie Schmidheiny.

References

- Alberdi, A. & Aizpurua, O. (2018). *Plecotus macrobullaris* (Chiroptera: Vespertilionidae). *Mammalian Species*, 50(958), 26–33. doi: 10.1093/mspecies/sey003
- Alberdi, A., Garin, I., Aizpurua, O. & Aihartza, J. (2012). The foraging ecology of the mountain long-eared bat *Plecotus macrobullaris* revealed with DNA mini-barcodes. *PLoS ONE*, 7(4), e35692. doi: 10.1371/journal.pone.0035692
- Alberdi, A., Aizpurua, O., Aihartza, J. & Garin, I. (2014). Unveiling the factors shaping the distribution of widely distributed alpine vertebrates, using multi-scale ecological niche modelling of the bat *Plecotus macrobullaris*. *Frontiers in Zoology*, 11(1), 77. doi: 10.1186/s12983-014-0077-6
- Alberdi, A., Aizpurua, O., Bohmann, K., Gopalakrishnan, S., Lynggaard, C., Nielsen, M. & Gilbert, M. T. P. (2019). Promises and pitfalls of using high-throughput sequencing for diet analysis. *Molecular Ecology Resources*, 19(2), 327–348. doi: 10.1111/1755-0998.12960
- Aldasoro, M., Garin, I., Vallejo, N., Baroja, U., Arrizabalaga-Escudero, A., Goiti, U. & Aihartza, J. (2019). Gaining ecological insight on dietary allocation among horseshoe bats through molecular primer combination. *PLoS ONE*, 14(7), e0220081. doi: 10.1371/journal.pone.0220081
- Aldridge, H. & Rautenbach, I. (1987). Morphology, echolocation and resource partitioning in insectivorous bats. *Journal of Animal Ecology*, 56(3), 763–778.

- Altermatt, F. (2010). Climatic warming increases voltinism in European butterflies and moths. *Proceedings of the Royal Society B: Biological Sciences*, 277(1685), 1281–1287. doi: 10.1098/rspb.2009.1910
- Anderson, M. E. & Racey, P. A. (1991). Feeding behaviour of captive brown long-eared bats, *Plecotus auritus*. *Animal Behaviour*, 42(3), 489–493. doi: 10.1016/S0003-3472(05)80048-X
- Andreas, M. (2002). Potravní ekologie netopýrů Středomoří [Feeding ecology of bats in the Mediterranean]. PhD Thesis. *Institute of Applied Ecology, Czech Agriculture University, Prague*, 163 pp.
- Andriollo, T., Blanc, M., Schönbächler, C. & Hollier, J. (2016). Données nouvelles de fourmilions (Neuroptera, Myrmeleontidae) pour le bassin genevois [New antlion (Neuroptera, Myrmeleontidae) records for the Geneva Basin]. *Entomo Helvetica*, 9, 13–18.
- Andriollo, T. & Ruedi, M. (2018). Novel molecular tools to identify *Plecotus* bats in sympatry and a review of their distribution in Switzerland. *Revue suisse de Zoologie*, 125(1), 61–72. doi: 10.5281/zenodo.1196013
- Andriollo, T., Ashrafi, S., Arlettaz, R. & Ruedi, M. (2018). Porous barriers? Assessment of gene flow within and among sympatric long-eared bat species. *Ecology and Evolution*, 8(24), 12841–12854. doi: 10.1002/ece3.4714
- Andriollo, T., Gillet, F., Michaux, J. R. & Ruedi, M. (2019a). The menu varies with metabarcoding practices: A case study with the bat *Plecotus auritus*. *PLoS ONE*, 14(7), e0219135. doi: 10.1371/journal.pone.0219135
- Andriollo, T., Landry, B., Guibert, B., Pastore, M. & Baumgart, P. (2019b). Nouveaux ajouts à la liste des Lépidoptères du canton de Genève [New additions to the list of Lepidoptera for the canton of Geneva]. *Entomo Helvetica*, 12, 9–28.
- Arlettaz, R. (1996). Feeding behaviour and foraging strategy of free-living mouse-eared bats, *Myotis myotis* and *Myotis blythii*. *Animal Behaviour*, 51 (1), 1–11.
- Arlettaz, R. (1999). Habitat selection as a major resource partitioning mechanism between the two sympatric sibling bat species *Myotis myotis* and *Myotis blythii*. *Journal of Animal Ecology*, 68(3), 460–471.
- Arrizabalaga-Escudero, A., Clare, E. L., Salsamendi, E., Alberdi, A., Garin, I., Aihartza, J. & Goiti, U. (2018). Assessing niche partitioning of co-occurring sibling bat species by DNA metabarcoding. *Molecular Ecology*, 27(5), 1273–1283. doi: 10.1111/mec.14508.
- Arthur, L. & Lemaire, M. (2015). Les chauves-souris de France, Belgique, Luxembourg et Suisse (2nd ed.). *Biotope Éditions, Mèze; Muséum national d'histoire naturelle, Paris*, 544 pp.
- Ashrafi, S., Bontadina, F., Kiefer, A., Pavlinić, I. & Arlettaz, R. (2010). Multiple morphological characters needed for field identification of cryptic long-eared bat species around the Swiss Alps. *Journal of Zoology*, 281(4), 241–248. doi: 10.1111/j.1469-7998.2010.00697.x
- Ashrafi, S., Beck, A., Rutishauser, M., Arlettaz, R. & Bontadina, F. (2011). Trophic niche partitioning of cryptic species of long-eared bats in Switzerland: implications for conservation. *European Journal of Wildlife Research*, 57(4), 843–849. doi: 10.1007/s10344-011-0496-z
- Ashrafi, S., Rutishauser, M., Ecker, K., Obrist, M. K., Arlettaz, R. & Bontadina, F. (2013). Habitat selection of three cryptic *Plecotus* bat species in the European Alps reveals contrasting implications for conservation. *Biodiversity and Conservation*, 22(12), 2751–2766. doi: 10.1007/s10531-013-0551-z
- Barataud, M. (1990). Éléments sur le comportement alimentaire des oreillards brun et gris *Plecotus auritus* (Linnaeus, 1758) et *Plecotus austriacus* (Fischer, 1829). *Le Rhinolophe*, 7, 3–10.
- Barataud, M. (2015). Acoustic ecology of European bats: species, identification, study of their habitats and foraging behaviour. Collection Inventaires & Biodiversité, vol. 8, *Biotope Éditions, Mèze; Muséum national d'histoire naturelle, Paris*, 352 pp.

- Baroja, U., Garin, I., Aihartza, J., Arrizabalaga-Escudero, A., Vallejo, N., Aldasoro, M & Goiti, U. (2019). Pest consumption in a vineyard system by the lesser horseshoe bat (*Rhinolophus hipposideros*). *PLoS ONE*, 14(7), e0219265. doi: 10.1371/journal.pone.0219265
- Bauerová, Z. (1982). Contribution to the trophic ecology of the grey long-eared bat, *Plecotus austriacus*. *Folia Zoologica*, 31, 113–122.
- Beck, A. (1995). Fecal analyses of European bat species. *Myotis*, 32(33), 109–119.
- Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 57(1), 289–300.
- Bohmann, K., Monadjem, A., Lehmkuhl Noer, C., Rasmussen, M., Zeale, M. R. K., Clare, E., ... Gilbert, M. T. P. (2011). Molecular diet analysis of two african free-tailed bats (Molossidae) using high throughput sequencing. *PLoS ONE*, 6(6), e21441. doi: 10.1371/journal.pone.0021441
- Boyles, J. G., Cryan, P. M., McCracken, G. F. & Kunz, T. H. (2011). Economic importance of bats in agriculture. *Science*, 332(6025), 41–42. doi: 10.1126/science.1201366
- Brown, V. A., Braun de Torrez, E. & McCracken, G. F. (2015). Crop pests eaten by bats in organic pecan orchards. *Crop Protection*, 67, 66–71. doi: 10.1016/j.cropro.2014.09.011
- Calabria, G., Máca, J., Bächli, G., Serra, L. & Pascual, M. (2012). First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. *Journal of Applied Entomology*, 136(1–2), 139–147. doi: 10.1111/j.1439-0418.2010.01583.x
- Chang, Y., Song, S., Li, A., Zhang, Y., Li, Z., Xiao, Y., Jiang, T., Feng, J. & Lin, A. (2019). The roles of morphological traits, resource variation and resource partitioning associated with the dietary niche expansion in the fish-eating bat *Myotis pilosus*. *Molecular Ecology*, 28(11), 2944–2954. doi: 10.1111/mec.15127
- Chao, A. (1987). Estimating the population size for capture-recapture data with unequal catchability. *Biometrics*, 43(4), 783–791. doi: 10.2307/2531532
- Clare, E. L. (2014). Molecular detection of trophic interactions: emerging trends, distinct advantages, significant considerations and conservation applications. *Evolutionary Applications*, 7(9), 1144–1157. doi: 10.1111/eva.12225
- Clare, E. L., Barber, B. R., Sweeney, B. W., Hebert, P. D. N. & Fenton, M. B. (2011). Eating local: influences of habitat on the diet of little brown bats (*Myotis lucifugus*). *Molecular Ecology*, 20(8), 1772–1780. doi: 10.1111/j.1365-294X.2011.05040.x
- Clarke, L. J., Soubrier, J., Weyrich, L. S. & Cooper, A. (2014). Environmental metabarcodes for insects: in silico PCR reveals potential for taxonomic bias. *Molecular Ecology Resources*, 14(6), 1160–1170. doi: 10.1111/1755-0998.12265
- Cohen, Y., Bar-David, S., Nielsen, M., Bohmann, K., Korine, C. (2020). An appetite for pests: Synanthropic insectivorous bats exploit cotton pest irruptions and consume various deleterious arthropods. *Molecular Ecology*, 29(6), 1185–1198. doi: 10.1111/mec.15393.
- Colwell, R. K. (2013). EstimateS: Statistical estimation of species richness and shared species from samples. Version 9 and earlier. User's Guide and application. University of Connecticut.
- Colwell, R. K., Chao, A., Gotelli, N. J., Lin, S.-Y., Mao, C. X., Chazdon, R. L. & Longino, J. T. (2012). Models and estimators linking individual-based and sample-based rarefaction, extrapolation and comparison of assemblages. *Journal of Plant Ecology*, 5 (1), 3–21. doi: 10.1093/jpe/rtr044
- Courtois, J.-Y., Rist, D. & Beuneux, G. (2011). Les chauves-souris de Corse. *Albiana, Ajaccio, France*, 166 pp.

- de Jong, Y., Verbeek, M., Michelsen, V., Bjorn, P. d. P., Los, W., Steeman, F., ... Penev, L. (2014). Fauna Europaea – all European animal species on the web. *Biodiversity Data Journal*, 2, e4034. doi: 10.3897/BDJ.2.e4034
- Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., ... Eveson, J. P. (2018). Counting with DNA in metabarcoding studies: how should we convert sequence reads to dietary data? *Molecular Ecology*, 28(2), 391–406. doi: 10.1111/mec.14734
- Dietrich, S., Szameitat, D. P., Kiefer, A., Schnitzler, H.-U. & Denzinger, A. (2006). Echolocation signals of the plecotine bat, *Plecotus macrobullaris* Kuzyakin, 1965. *Acta Chiropterologica*, 8(2), 465–475. doi: 10.3161/1733-5329(2006)8[465:esotpb]2.0.co;2
- Doledec, S., Chessel, D., ter Braak, C. J. F. & Champely, S. (1996). Matching species traits to environmental variables: a new three-table ordination method. *Environmental and Ecological Statistics*, 3(2), 143–166. doi: 10.1007/bf02427859
- Dray, S. & Dufour, A.-B. (2007). The ade4 package: implementing the duality diagram for ecologists. *Journal of Statistical Software*, 22(4), 1–20. doi: 10.18637/jss.v022.i04
- Dray, S. & Legendre, P. (2008). Testing the species traits-environment relationships: the fourth-corner problem revisited. *Ecology*, 89(12), 3400–3412. doi: 10.1890/08-0349.1
- Dray, S., Choler, P., Doledec, S., Peres-Neto, P. R., Thuiller, W., Pavoine, S. & ter Braak, C. J. F. (2014). Combining the fourth-corner and the RLQ methods for assessing trait responses to environmental variation. *Ecology*, 95(1), 14–21. doi: 10.1890/13-0196.1
- Elbrecht, V., Taberlet, P., Dejean, T., Valentini, A., Usseglio-Polatera, P., Beisel, J.-N., ... Leese, F. (2016). Testing the potential of a ribosomal 16S marker for DNA metabarcoding of insects. *PeerJ*, 4, e1966. doi: 10.7717/peerj.1966
- Elbrecht, V., Braukmann, T. W. A., Ivanova, N. V., Prosser, S. W. J., Hajibabaei, M., Wright, M., ... Steinke, D. (2019). Validation of COI metabarcoding primers for terrestrial arthropods. *PeerJ*, 7, e27801v27801. doi: 10.7717/peerj.7745
- Emrich, M.A., Clare, E.L., Symondson, W.O.C., Koenig, S.E. & Fenton, M.B. (2014). Resource partitioning by insectivorous bats in Jamaica. *Molecular Ecology*, 23 (15), 3648–3656. doi:10.1111/mec.12504
- Entwistle, A. C., Racey, P. A. & Speakman, J. R. (1996). Habitat exploitation by a gleaning bat, *Plecotus auritus*. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 351(1342), 921–931. doi: 10.1098/rstb.1996.0085
- Galan, M., Pons, J.-B., Tournayre, O., Pierre, E., Leuchtman, M., Pontier, D. & Charbonnel, N. (2018). Metabarcoding for the parallel identification of several hundred predators and their prey: application to bat species diet analysis. *Molecular Ecology Resources*, 18(3), 474–489. doi: 10.1111/1755-0998.12749
- Gillieron, J., Schonbachler, C., Rochet, C. & Ruedi, M. (2015). Atlas des chauves-souris du bassin genevois. *Faune Geneve - Volume 1, CCO-Geneve*, 262 pp.
- Gotelli, N. J., Hart, E. & Ellison, A. M. (2013). EcoSimR: A software package to fit ecological null models. Retrieved from <http://www.uvm.edu/~ngotelli/EcoSim/EcoSim.html>
- Hardin, G. (1960). The competitive exclusion principle. *Science*, 131(3409), 1292–1297. doi: 10.1126/science.131.3409.1292
- Holt, R. D. (2009). Bringing the Hutchinsonian niche into the 21st century: Ecological and evolutionary perspectives. *Proceedings of the National Academy of Sciences of the United States of America*, 106 (Supplement 2), 19659–19665. doi: 10.1073/pnas.0905137106

- Horn, H. S. (1966). Measurement of "overlap" in comparative ecological studies. *The American Naturalist*, 100 (914), 419–424.
- Hutchinson, G. E. (1957). Concluding remarks. *Cold Spring Harbor Symposia on Quantitative Biology*, 22, 415–427. doi: 10.1101/sqb.1957.022.01.039
- Juste, J., Ibanez, C., Munoz, J., Trujillo, D., Benda, P., Karataş, A. & Ruedi, M. 2004. Mitochondrial phylogeography of the long-eared bats (*Plecotus*) in the Mediterranean Palaearctic and Atlantic islands. *Molecular Phylogenetics and Evolution*, 31(3), 1114–1126. doi: 10.1016/j.ympev.2003.10.005
- Kaňuch, P., Janečková, K. & Krištín, A. (2005). Winter diet of the noctule bat *Nyctalus noctula*. *Folia Zoologica*, 54(1–2), 53–60.
- Kartzinel, T. R., Chen, P. A., Coverdale, T. C., Erickson, D. L., Kress, W. J., Kuzmina, M. L., ... Pringle, R. M. (2015). DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. *Proceedings of the National Academy of Sciences*, 112(26), 8019–8024. doi: 10.1073/pnas.1503283112
- Kervyn, T. & Libois, R. (2008). The diet of the serotine bat: A comparison between rural and urban environments. *Belgian Journal of Zoology*, 138(1), 41–49.
- Kolkert, H., Andrew, R., Smith, R., Rader, R. & Reid, N. (2020). Insectivorous bats selectively source moths and eat mostly pest insects on dryland and irrigated cotton farms. *Ecology and Evolution*, 10(1), 371–388. doi: 10.1002/ece3.5901
- Kunz, T. H., Braun de Torrez, E., Bauer, D., Lobova, T. & Fleming, T. H. (2011). Ecosystem services provided by bats. *Annals of the New York Academy of Sciences*, 1223, 1–38. doi: 10.1111/j.1749-6632.2011.06004.x
- Lawlor, L. R. (1980). Structure and stability in natural and randomly constructed competitive communities. *American naturalist*, 116(3), 394–408. doi: 10.1086/283634
- Leelapaibul, W., Bumrungsri, S. & Pattanavibool, A. (2009). Diet of wrinkle-lipped free-tailed bat (*Tadarida plicata* Buchanan, 1800) in central Thailand: Insectivorous bats potentially act as biological pest control agents. *Acta Chiropterologica*, 7(1), 111–119. doi: 10.3161/1733-5329(2005)7[111:DOWFBT]2.0.CO;2
- Legendre, P., Galzin, R. & Harmelin-Vivien, M. L. (1997). Relating behavior to habitat: solutions to the fourth-corner problem. *Ecology*, 78(2), 547–562. doi: 10.2307/2266029
- Leray, M., Meyer, C. P. & Mills, S. C. (2015). Metabarcoding dietary analysis of coral dwelling predatory fish demonstrates the minor contribution of coral mutualists to their highly partitioned, generalist diet. *PeerJ*, 3, e1047. doi: 10.7717/peerj.1047
- Levins, R. (1968). Evolution in changing environments: some theoretical explorations. vol. 2, *Princeton University Press*, 120 pp.
- Lopes, C. M., De Barba, M., Boyer, F., Mercier, C., da Silva Filho, P. J. S., Heidtmann, L. ... Taberlet, P. (2015). DNA metabarcoding diet analysis for species with parapatric vs sympatric distribution: a case study on subterranean rodents. *Heredity*, 114(5), 525–536. doi: 10.1038/hdy.2014.109
- Mancina, C. A., García-Rivera, L. & Miller, B. W. (2012). Wing morphology, echolocation, and resource partitioning in syntopic Cuban mormoopid bats. *Journal of Mammalogy*, 93(5), 1308–1317. doi: 10.1644/11-mamm-a-331.1
- Mattei-Roesli, M. (2010). Situazione del genere *Plecotus* (Chiroptera) nel Cantone Ticino (Svizzera). *Bollettino della Società ticinese di Scienze naturali*, 98, 31–34.
- Mazzi, D., Bravin, E., Meraner, M., Finger, R. & Kuske, S. (2017). Economic impact of the introduction and establishment of *Drosophila suzukii* on sweet cherry production in Switzerland. *Insects*, 8(1), 18. doi: 10.3390/insects8010018

- Meineke, T. (1991). Auswertung von Fraßresten der beiden Langohrarten *Plecotus auritus* L. und *Plecotus austriacus* Fischer. *Naturschutz und Landschaftspflege in Niedersachsen*, 26, 37–45.
- Merz, B. (2012). Liste annotée des insectes (Insecta) du canton de Genève. *Instrumenta Biodiversitatis* 8, *Muséum d'histoire naturelle, Genève*, 532 pp.
- Morisita, M. (1959). Measuring of the dispersion and analysis of distribution patterns. *Memories of the Faculty of Science, Kyushu University. Series E Biology*, 2(21), 215–235.
- Motte, G. (2011). Étude comparée de l'écologie de deux espèces jumelles de Chiroptères (Mammalia : Chiroptera) en Belgique: l'oreillard roux (*Plecotus auritus*) (Linn., 1758) et l'oreillard gris (*Plecotus austriacus*) (Fischer, 1829). PhD Thesis. *Université de Liège*, 123 pp.
- Nicholls, B. & Racey, P. A. (2006). Habitat selection as a mechanism of resource partitioning in two cryptic bat species *Pipistrellus pipistrellus* and *Pipistrellus pygmaeus*. *Ecography*, 29(5), 697–708. doi: 10.1111/j.2006.0906-7590.04575.x
- Olofsson, M., Vallin, A., Jakobsson, S. & Wiklund, C. (2011). Winter predation on two species of hibernating butterflies: monitoring rodent attacks with infrared cameras. *Animal Behaviour*, 81(3), 529–534. doi: 10.1016/j.anbehav.2010.12.012
- Patrick, L. E. & Stevens, R. D. (2014). Investigating sensitivity of phylogenetic community structure metrics using North American desert bats. *Journal of Mammalogy*, 95(6), 1240–1253. doi: 10.1644/14-mamm-a-007
- Poltavsky, A. N. (2014). Phenological groups of snout moths (Lepidoptera: Pyralidae, Crambidae) of Rostov-on-Don area (Russia). *Phegea*, 42(1), 22–24.
- Preatoni, D. G., Spada, M., Wauters, L. A., Tosi, G. & Martinoli, A. (2011). Habitat use in the female Alpine long-eared bat (*Plecotus macrobullaris*): does breeding make the difference? *Acta Chiropterologica*, 13(2), 355–364. doi: 10.3161/150811011x624820
- Presley, S. J., Cisneros, L. M., Higgins, C. L., Klingbeil, B. T., Scheiner, S. M. & Willig, M. R. (2018). Phylogenetic and functional underdispersion in Neotropical phyllostomid bat communities. *Biotropica*, 50(1), 135–145. doi: 10.1111/btp.12501
- Quéméré, E., Hibert, F., Miquel, C., Lhuillier, E., Rasolondraibe, E., Champeau, J., ... Chikhi, L. (2013). A DNA metabarcoding study of a primate dietary diversity and plasticity across its entire fragmented range. *PLoS ONE*, 8(3), e58971. doi: 10.1371/journal.pone.0058971
- Razgour, O., Clare, E. L., Zeale, M. R. K., Hanmer, J., Schnell, I. B., Rasmussen, M., ... Jones, G. (2011). High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species. *Ecology and Evolution*, 1(4), 556–570. doi: 10.1002/ece3.49
- Riedinger, V., Muller, J., Stadler, J., Ulrich, W. & Brandl, R. (2013). Assemblages of bats are phylogenetically clustered on a regional scale. *Basic and Applied Ecology*, 14(1), 74–80. doi: 10.1016/j.baae.2012.11.006
- Robineau, R., Bachelard, P., Bérard, R., Colomb, C., Demerges, D., Doux, Y., ... Tautel, C. (2007). Guide des papillons nocturnes de France. *Delachaux et Niestlé, Paris*, 287 pp.
- Robinson, M. F. (1990). Prey selection by the brown long-eared bat, *Plecotus auritus*. *Myotis*, 28, 5–18.
- Robinson, M. F. & Stebbings, R. E. (1993). Food of the serotine bat, *Eptesicus serotinus* —is faecal analysis a valid qualitative and quantitative technique? *Journal of Zoology*, 231(2), 239–248. doi: 10.1111/j.1469-7998.1993.tb01915.x
- Roswag, A., Becker, N. I. & Encarnação, J. A. (2018a). Isotopic and dietary niches as indicators for resource partitioning in the gleaner bats *Myotis bechsteinii*, *M. nattereri*, and *Plecotus auritus*. *Mammalian Biology*, 89(1), 62–70. doi: 10.1016/j.mambio.2017.12.006

- Roswag, A., Becker, N. I., Drangusch, R., Kuhring, K., Ohlendorf, B. & Encarnação, J. A. (2018b). Teasing apart cryptic species groups: Nutritional ecology and its implications for species-specific conservation of the *Myotis mystacinus* group. *Population Ecology*, 61(1), 14–24. doi: 10.1002/1438-390X.1003
- Rutishauser, M. D., Bontadina, F., Braunisch, V., Ashrafi, S. & Arlettaz, R. (2012). The challenge posed by newly discovered cryptic species: disentangling the environmental niches of long-eared bats. *Diversity and Distributions*, 18(11), 1107–1119. doi: 10.1111/j.1472-4642.2012.00904.x
- Rydell, J., Bogdanowicz, W., Boonman, A., Pettersson, S., Suchecka, E. & Pomorski, J. J. (2016). Bats may eat diurnal flies that rest on wind turbines. *Mammalian Biology*, 81(3), 331–339. doi: 10.1016/j.mambio.2016.01.005
- Salinas-Ramos, V. B., Herrera Montalvo, L. G., León-Regagnon, V., Arrizabalaga-Escudero, A. & Clare, E. L. (2015). Dietary overlap and seasonality in three species of mormoopid bats from a tropical dry forest. *Molecular Ecology*, 24(20), 5296–5307. doi: 10.1111/mec.13386
- Salinas-Ramos, V. B., Ancillotto, L., Bosso, L., Sánchez-Cordero, V. & Russo, D. (2020). Interspecific competition in bats: state of knowledge and research challenges. *Mammal Review*, 50(1), 68–81. doi: 10.1111/mam.12180
- Sato, J. J., Shimada, T., Kyogoku, D., Komura, T., Uemura, S., Saitoh, T. & Isagi, Y. (2018). Dietary niche partitioning between sympatric wood mouse species (Muridae: *Apodemus*) revealed by DNA meta-barcoding analysis. *Journal of Mammalogy*, 99(4), 952–964. doi: 10.1093/jmammal/gyy063
- Schnitzler, H.-U. & Kalko, E. K. V. (2001). Echolocation by insect-eating bats. *Bioscience*, 51(7), 557–569. doi: 10.1641/0006-3568(2001)051[0557:ebieb]2.0.co;2
- Schoener, T. W. (1974). Resource partitioning in ecological communities. *Science*, 185(4145), 27–39. doi: 10.1126/science.185.4145.27
- Spitz, J., Ridoux, V. & Brind’Amour, A. (2014). Let’s go beyond taxonomy in diet description: testing a trait-based approach to prey–predator relationships. *Journal of Animal Ecology*, 83(5), 1137–1148. doi: 10.1111/1365-2656.12218
- Spitzenberger, F., Strelkov, P., Winkler, H. & Haring, E. (2006). A preliminary revision of the genus *Plecotus* (Chiroptera, Vespertilionidae) based on genetic and morphological results. *Zoologica Scripta*, 35(3), 187–230. doi: 10.1111/j.1463-6409.2006.00224.x
- Swift, S. M., Racey, P. A. & Avery, M. I. (1985). Feeding ecology of *Pipistrellus pipistrellus* (Chiroptera: Vespertilionidae) during pregnancy and lactation. II. Diet. *The Journal of Animal Ecology*, 54(1), 217–225. doi: 10.2307/4632
- Taberlet, P., Bonin, A., Zinger, L. & Coissac, E. (2018). Environmental DNA: for biodiversity research and monitoring. *Oxford University Press*, 253 pp. doi: 10.1093/oso/9780198767220.001.0001
- ter Braak, C. J. F., Cormont, A. & Dray, S. (2012). Improved testing of species traits–environment relationships in the fourth corner problem. *Ecology*, 93(7), 1525–1526. doi: 10.1890/12-0126.1
- Tournayre, O., Leuchtmann, M., Filippi-Codaccioni, O., Trillat, M., Piry, S., Pontier, D., ... Galan, M. (2019). In silico and empirical evaluation of twelve COI & 16S metabarcoding primer sets for insectivorous diet analyses. *Ecology and Evolution*, 10(13), 6310–6332. doi: 10.1002/ece3.6362
- Tvrtković, N., Pavlinić, I. & Haring, E. (2005). Four species of long-eared bats (*Plecotus*, Geoffroy, 1818; Mammalia, Vespertilionidae) in Croatia: field identification and distribution. *Folia Zoologica*, 54(1–2), 75–88.
- Vaughan, N. (1997). The diets of British bats (Chiroptera). *Mammal Review*, 27(2), 77–94. doi: 10.1111/j.1365-2907.1997.tb00373.x

Vesterinen, E. J., Lilley, T., Laine, V. N. & Wahlberg, N. (2013). Next generation sequencing of fecal DNA reveals the dietary diversity of the widespread insectivorous predator Daubenton's Bat (*Myotis daubentonii*) in Southwestern Finland. *PLoS ONE*, 8(11), e82168. doi: 10.1371/journal.pone.0082168

Vesterinen, E. J., Puisto, A. I. E., Blomberg, A. S. & Lilley, T. M. (2018). Table for five, please: Dietary partitioning in boreal bats. *Ecology and Evolution*, 8(22), 10914–10937. doi: 10.1002/ece3.4559

Wiklund, C., Vallin, A., Friberg, M. & Jakobsson, S. (2008). Rodent predation on hibernating peacock and small tortoiseshell butterflies. *Behavioral Ecology and Sociobiology*, 62(3), 379–389. doi: 10.1007/s00265-007-0465-4

Zeale, M. R. K., Butlin, R. K., Barker, G. L. A., Lees, D. C. & Jones, G. (2011). Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Molecular Ecology Resources*, 11(2), 236–244. doi: 10.1111/j.1755-0998.2010.02920.x

Data Accessibility

- DNA sequences of prey with complete sampling information and taxonomic assignments: Zenodo TBD
- Matrix of ecological traits for all metabarcoded prey species is provided in the Supporting Information file with bibliographic references

Author Contributions

MR TA conceived and designed the experiments and conducted the sampling. TA JRM performed the laboratory work. TA analyzed the data. TA MR JRM contributed to the final manuscript.

Tables

Table 1. Fourth-corner test between bat species and ecological traits of consumed prey. Significance of adjusted p-values are given as follows: −/+++ (P < 0.01); −/+ (P < 0.05); n.s.: not significant.

	Predator	Predator	Predator
Ecological traits of prey	P. auritus	P. austriacus	P. macrobullaris
Closed habitats	+++	—	-
Woodlands, forests	+++	n.s. (-)	-
Exclusively diurnal or flightless	+++	-	-
Hedgerows	+	n.s. (-)	n.s. (-)
Shrublands	n.s. (+)	n.s. (-)	n.s. (-)
Urban areas	n.s. (+)	n.s. (-)	n.s. (-)
Semi-open habitats	n.s. (+)	n.s. (-)	n.s. (-)
Lowlands	n.s. (-)	n.s. (+)	n.s. (-)
Cultivated lands	n.s. (-)	n.s. (+)	n.s. (+)
Ubiquitous	n.s. (-)	+	n.s. (-)
Mesophilous areas	n.s. (-)	n.s. (+)	n.s. (+)
Dry areas	n.s. (-)	n.s. (-)	+
Screes	n.s. (-)	n.s. (-)	+
Meadows	-	n.s. (-)	+++
Lawns	-	n.s. (-)	+++
Mountainous areas	-	n.s. (-)	+++

	Predator	Predator	Predator
Slopes	-	n.s. (-)	+++
Open habitats	—	n.s. (+)	+++

Figure captions

Figure 1. Sampling dates (upper panel) and geographic location of the nine colonies of long-eared bats studied in the Geneva region. Elevation on the map is shaded from pale (low) to dark grey (high). Blue circles represent colonies of *P. auritus* : (1) Satigny, (2) pont Butin, (3) Choulex, (4) Presinge and (5) Sappey; orange ones colonies of *P. austriacus* : (6) Collex and (7) Hermance; purple ones colonies of *P. macrobullaris* : (8) Cartigny and (9) Léaz. The sampling regime aimed to gather either “community samples” (symbolized by large plastic tubes) or “individual samples” (small plastic tubes). The inset (top left) provides a general view of the study area near the Lake Geneva in southwestern Switzerland.

Figure 2. Total number of prey and their frequency of occurrence (wPOO) detected in individual samples of guano gathered in one colony each of the three bat species. (a) Area-proportional Euler diagram of the prey species richness detected for the three bat species. **(b)** Extrapolated accumulation curves indicating the number of detected prey species for each bat species and season. **(c)** Proportions of arthropod orders found in the diet of bats in the different seasons.

Figure 3. Seasonal trophic niche breadth variation (Levins’ index) measured for the three long-eared bat species. (a) Complete dataset, with all prey items kept and identified to the species level and considered as weighted occurrence data (wPOO). **(b)** Prey identified to the family level only (Family level).

Figure 4. Trophic niche overlap (Morisita-Horn measure) among *Plecotus* species, colonies and seasons. (a) Multi-dimensional scaling (MDS) of trophic niche overlap calculated among fecal samples collected in different colonies and across seasons. Each bat species is represented by a different color and each season by a different symbol. Three inset barplots represent the observed phenology of prey species appearing in the diet of *Plecotus* bats and characteristic of specific sampling periods. **(b)** Trophic niche overlap measured throughout three periods of the year among the three long-eared bat species. For each pairwise comparison, the lowest values of niche overlap are observed during spring.





