Trophic resource partitioning drives fine-scale coexistence in cryptic bat species

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Abstract

Understanding the processes that enable species coexistence has important implications for assessing how ecological systems will respond to global change. Morphology and functional similarity increase the potential for competition, and therefore, co-occurring morphologically similar but genetically unique species are a good model system for testing coexistence mechanisms. We used DNA metabarcoding and High Throughput Sequencing to characterise for first time the trophic ecology of two recently-described cryptic bat species with parapatric ranges, Myotis escalerai and Myotis crypticus. We collected faecal samples from allopatric and sympatric regions and locations to describe the diet both taxonomically and functionally and compare prey consumption with prey availability. The two bat species had similar diets characterised by high arthropod diversity, particularly Lepidoptera, Diptera and Araneae, and a high proportion of prey that is not volant at night, which points to extensive use of gleaning. Diet overlap at the prey-item level was lower in locally sympatric than allopatric locations, supporting trophic shift under fine-scale sympatry. Furthermore, locally sympatric samples of M. escalerai had a marginally lower proportion of not nocturnally volant prey, suggesting that the shift in diet may be driven by a change in foraging mode. Our findings suggest that fine-scale coexistence mechanisms can have implications for maintaining broad-scale diversity patterns. This study highlights the importance of including both allopatric and sympatric populations and choosing meaningful spatial scales for detecting ecological patterns. We conclude that a combination of high taxonomic resolution with a functional approach helps identify patterns of niche shift.

Introduction

Understanding the processes that enable species coexistence is a key theme of ecology with important implications for interpreting diversity patterns and predicting how systems respond to global change (Valladares, Bastias, Godoy, Granda, & Escudero, 2015). Interspecific competition is thought to have a major influence on community structure for many taxonomic groups. Niche theory (Chase & Leibold, 2003; P. Chesson, 2000; Letten, Ke, & Fukami, 2017) asserts that species coexistence is promoted through differential use of resources driven by functional differences between species, which results in communities that tend to be assembled by functionally dissimilar species (Schoener, 1974). This has been shown in numerous cases, including fish (Ross,

1986), shorebirds (Bocher et al., 2014) and rodent communities (Codron et al., 2015). Alternatively, community structure and coexistence, primarily in sessile organisms, has been often explained through neutral processes, such as dispersal or stochasticity (The neutral theory of biodiversity and biogeography; Hubbell, 2001). This framework has been often used as a null model to evaluate whether observed patterns deviate from neutral expectations (Alonso, Etienne, & McKane, 2006; McGill, Maurer, & Weiser, 2006). Yet, some studies of mobile organisms have failed to identify evidence of resource partitioning (e.g. Luiselli, 2008), suggesting that in some cases biotic interactions only play a minor role in governing community assembly, perhaps because resources are not limiting (Salinas-Ramos, Ancillotto, Bosso, Sánchez-Cordero, & Russo, 2020), and therefore neutral processes likely play a more important role.

Morphologically similar species pose a challenge for understanding mechanisms of coexistence from a niche theory perspective because they are more likely to be functionally similar, and therefore less likely to be able to use resources in a different way, a pre-requisite for resource partitioning (Weiher & Keddy, 1999). Consequently, considerable attention has been given to understanding resource partitioning among morphologically identical (cryptic) or similar co-occurring species (e.g. Gabaldon, Montero-Pau, Serra, & Carmona, 2013; Jiang, Feng, Sun, & Wang, 2008; Razgour et al., 2011). Many studies have focused on the trophic dimension, an important aspect of species' ecological niche (Schoener, 1974). DNA metabarcoding and High Throughput Sequencing (molecular diet analysis) approaches helped overcome many of the limitations of traditional morphological methods (Sousa, Silva, & Xavier, 2019), opening the door to new opportunities for studying mechanisms of species coexistence (Arrizabalaga-Escudero et al., 2018; Kruger et al., 2014; Razgour et al., 2011). However, the majority of coexistence studies focus on only sympatric populations, preventing an evaluation of how the presence of a competitor may change resource use, thus limiting the power of inferences (Salinas-Ramos et al., 2020). Moreover, most studies also focus on diet only, disregarding prey selection relative to prey availability or resource limitation (Salinas-Ramos et al. 2019). Accounting for prey selection (e.g. Rytkonen et al., 2019) can provide a more complete picture of consumer trophic preferences (Lawlor, 1980).

The processes that govern community assembly, including coexistence mechanisms, vary with spatial scale (Lewis, Bailey, Vandewoude, & Crooks, 2015; Snyder & Chesson, 2004; Viana & Chase, 2019), yet spatial scale is rarely considered in coexistence studies (Hart, Usinowicz, & Levine, 2017). A better understanding of the scale of coexistence mechanisms and how different processes interact is important for both basic and applied ecology (Peixoto, Braga, & Mendes, 2018).

This study aims to identify whether trophic ecology enables morphologically similar species to coexist across spatial scales. We focus on two recently described, morphologically nearly identical, insectivorous bat species, whose trophic ecology has not been studied to date, *Myotis crypticus* and *Myotis escalerai*. These bats are restricted to the Western Mediterranean Basin, where they overlap across the north of the Iberian Peninsula, but at the fine-scale are known to co-occur only in a few locations (Juste, Ruedi, Puechmaille, Salicini, & Ibanez, 2018). Phylogeographic analysis and species distribution modelling suggest that their ranges have been shaped by competition (Razgour, Salicini, Ibanez, Randi, & Juste, 2015). These bats therefore provide an excellent case study for understanding mechanisms of coexistence among morphologically similar species. We use DNA metabarcoding and High Throughput Sequencing to characterise the trophic ecology of *M. crypticus* and *M. escalerai* by analysing their taxonomic and functional diets and their prey selection relative to prey availability in sympatric versus allopatric populations at both fine and broad spatial scales. Given their near identical morphology and echolocation calls, the overall trophic niches of the two bats are expected to be similar and niche overlap should be high. We hypothesise that if resource partitioning is the main process facilitating coexistence, competing species will diverge in their use of resources in sympatry compared to allopatry (e.g. Klawinski, Vaughan, Saenz, & Godwin, 1994). We test the predictions that 1) trophic niche overlap and diet similarity are higher in allopatric than sympatric locations; and 2) differences in trophic niche overlap are most pronounced at the fine spatial scale where individuals of the two species share the same foraging areas.

Methods

Sampling design

Sampling took place in the Iberian Peninsula, focusing on two sympatric regions in the north where both *Myotis escalerai* and *Myotis crypticus* are found (La Rioja-Soria and southern Cantabria), and two allopatric regions: the south (Andalucia: Jaen and Granada), where only*M. escalerai* is found, and the north Atlantic coast (northern Cantabria), where only *M. crypticus* is found. Additionally, we sampled a single sympatric swarming site in Catalunya, where the two species use the same cave during the autumn mating season (Fig. 1). Within each region, 9-24 locations were sampled based on suitable habitat and accessibility, using monofilament mist nets and a harp trap placed over water sources, forest paths and cave entrances. The sampling period extended from June to September 2017, for a total of 68 sampling nights (Supplementary Table S1 for list of sampling locations). Captured bats were kept in individual cotton bags for up to 1 hour. We collected faecal samples from the cotton bags for diet analysis, and biopsy punches (3 mm) from the wing membrane of the bats to confirm species identification. Dropping samples and wing biopsies were stored in absolute and 70% ethanol, respectively. Bat sampling was carried out under local permits and ethical approval from the University of Southampton (study ID: 26627).

We sampled the arthropod community in bat sampling locations using vegetation sweeping (Barclay, 1991; Swift & Racey, 2002) to assess bat prey selectivity relative to prey availability (Jones, 1990; Kunz, 2009). We chose vegetation sweeping because of the expected low flight and gleaning behaviour of the species based on their morphology and echolocation calls, and the foraging behaviour of the morphologically similar better studied member of the cryptic species complex,*Myotis nattereri* (de Jong, 1995). During bat sampling nights, we set linear transects in each habitat type in the sampling location and swept the vegetation along each transect. After 10 sweeps, we collected the capture into a plastic bag and moved five steps further without sampling to increase spatial representativeness. Each sampling unit of 10 sweeps and five steps forward was repeated 5-10 times until the capture size was considered representative ($>$ 100 individuals). Transect length ranged between 30 and 80 m. Arthropod specimens captured were separated from vegetation remains in the field and stored in 70% ethanol.

DNA extractions and species confirmation

Bat species identity was confirmed in the Estacion Biologica de Donana Laboratory of Molecular Ecology (LEM, EBD-CSIC, Spain). DNA was extracted from wing biopsy punches through precipitation with isopropanol. Part of the hypervariable region of the mtDNA control region was amplified using the primers CSBC-F 5'-CCTCTTAAATAAGACATCTCGATGG-3' (Wilkinson & Chapman, 1992) and HV2-Mna-R 5'-ATGCGTGCGTGTGTAATGTC-3' (Garcia-Mudarra, Ibanez, & Juste, 2020). Species specific differential amplification patterns for this primer set were used to confirm species identity through gel electrophoresis (Garcia-Mudarra et al., 2020).

DNA was extracted from all bat dropping samples using the Qiagen DNA stool mini kit, following the protocol in Zeale et al. (2011). A total of 43 sweeping samples from 23 locations with at least three bat dropping samples were selected (Supplementary Table S1). From those, all arthropod individuals (N = 8366) were first identified morphologically to taxonomic order. Subsequently, whole specimens, if smaller than a drosophila, or a specimen part (leg or head) if larger, were separated out, dried and pooled together for DNA extraction. Arthropod DNA was extracted using the NucleoSpin DNA Insect kit with up to 35 mg of sample dry weight in each tube. Larger samples were split into several tubes. The following modifications were applied to the kit extraction protocol: In steep 2, vortex for 20 minutes in the MN Bead Tube Holder on a Vortex-Genie at maximum speed; after steep 3, pipette 550uL of clean supernatant in to a new 2mL Eppendorf, centrifuge again at 13,000RPM for 2 minutes and continue with steep 4; in steep 6, centrifuge for 3 minutes; in steep 7, add 50uL of ddH_20 and incubate for 3 minutes.

High Throughput Sequencing

Both dropping and sweeping samples were sequenced in the Bart's and the London Genome Centre, London, UK. DNA extracts were checked for quality and concentration on a TapeStation D1000. Two sets of primers were used together in order to reduce primer taxonomic bias (Alberdi, Aizpurua, Gilbert, & Bohmann, 2018), especially given the high diversity of prey types expected in the diet, ZBJ (Zeale, Butlin, Barker, Lees, & Jones, 2011) Forward: ZBJ-ArtF1c 5'-AGATATTGGAACWTTATATTTTATTTTTGG-3' and Reverse: ZBJ-ArtR2c 5'-WACTAATCAATTWCCAAATCCTCC-3', and ANML (Jusino et al., 2019) Forward: LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and Reverse: CO1-CFMRa 5'-GGWACTAATCAATTTCCAAATCC-3'. For the ZBJ amplicon each 15µl PCR reaction used 7.5µl of Multiplex PCR mastermix (QIAGEN, Germany), 0.25 µL of each primer (10 µM), 5 µL H2O and 2 µL template DNA. Negative and positive controls were included in PCR reactions and later sequencing. The thermal cycling protocol was as follows: 95 C for 15 min, 34 cycles of 94 C for 40s, 40 C for 1 min, 72 C for 30s, followed by a final extension of 72 C for 5 min. ANML regions were amplified in 15ul reactions following published protocols (Jusino et al., 2019). All products were visualised on a 1.5% agarose gel. Products were tagged using Fluidigm barcodes and checked on a TapeStation D1000 before pooling and sequencing on an Illumina MiSeq using paired end (2 x 250 bp) chemistry (Illumina, San Francisco, USA). We used two technical PCR replicates to reduce biases associated with PCR stochasticity. This led to each sample being sequenced four times (combination of two primer and two PCR replicates).

Bioinformatics

Sequencing runs were merged using USEARCH (Edgar, 2010) and primers and adaptors removed using cutadapt (Martin, 2011). Sequences were processed on the mBRAVE platform (http://www.boldsystems.org/bin) (Ratnasingham & Hebert, 2007) setting the following parameters: Min QV = 0 qv, Min Length = 100 bp, Max Bases with Low QV (<20) = 75%, Max Bases with Ultra Low QV (<10) = 75%, ID Distance Threshold = 1.5%, Exclude from OTU Threshold = 3%, Minimum OTU Size = 1, OTU Threshold = 2%. Sequences were compared with the BOLD reference libraries SYS-CRLINSECTA and SYS-CRLNONINSECTARTH to established Barcode Index Numbers (BINs). BINs are a type of Operational Taxonomic Unit (OTU) integrated in the BOLD system with advantages over traditional OTUs, such as being unique and stable (Ratnasingham & Hebert, 2013). We used BIN identity as a proxy of taxonomic prey item unit.

After obtaining BIN (prey item) composition per sample and run, we removed singletons, i.e. BINs that only had a single read per run and sample, because they are likely to be PCR or sequencing errors (Alberdi et al., 2018). We established the threshold for the minimum number of reads per sample to retain an identification based on comparing composition similarity between molecular and morphological ID from sweeping samples and any prey present in blanks. Additionally, we controlled for potential contamination during extractions by removing the BINs present in extraction blanks from other samples in the same extraction run if present with a high number of reads (>100 in any blank sample) or with less than 10 times more reads in samples than in blank. We controlled for contamination from the sequencing process by removing BINs present in sequencing blanks from all the samples from the same primer following the same criteria as above.

We used two alternative approaches to combine data from PCR replicates. In the first, the additive criteria, BIN composition from both PCR replicates of each sample were added together. In the second, the conservative approach, only BINs that appeared in both PCR replicates were considered (Alberdi et al., 2018). Under this second criteria only samples in which the four runs contained a minimum number of reads (>100) were considered given that a failed sequencing run in a sample would lead to a null composition for both of the PCR replicates of a primer (52 samples, Supplementary Table S3). Finally, we combined taxa recovered from both primers to obtain the prey composition per dropping sample for downstream ecological analysis. Duplicated BINs in the same sample coming from different PCR replicates or primers were removed. A flow chart describing the methods is shown in Supplementary Fig. S1. Ecological results from both approaches

were very similar, thus we present results based on the additive approach (See Supplementary Fig. S9 for diet based on the conservative approach).

Characterising the diets of the two bat species

The contribution of different elements to the diet for a set of samples was quantified using weighted Percent of Occurrence (wPOO), which measures the relative occurrence of diet elements (prey items/OTUs/BINs) in a set of samples considering first their relative proportion per sample (Deagle et al., 2019). For example, a prey item found in a sample with 9 other prey items will be interpreted to contribute in the diet 1/10 of what it would if it was the only prey item present. Contributions to diet based on the two other commonly used metrics: Percent of Occurrence (POO) and Relative Read Abundance (RRA) (Deagle et al., 2019), are shown in supplementary materials (Supplementary Fig. S2). We tested for differences in the number of BINs per sample between bat species for each of the orders that constitute at least 10% of the diet of either one of the bat species (Araneae, Diptera, Lepidoptera, and Hemiptera), using negative binomial generalised linear models (GLMs; in R) to fit data structure based on the distribution of model residuals. We measured order level and prey species level (BIN-level) diet composition overlap between bat species using Pianka's measure of niche overlap (O_{jk}) (R package: EcoSimR (Gotelli, Hart, & Ellison, 2015)). We tested with an ANOSIM test (R package vegan (Oksanen et al., 2019)) whether Jaccard distance in BIN composition was greater between than within bat species. The ANOSIM statistic R is based on the difference of mean ranks between and within groups, with a range between -1 and +1. A value of zero indicates that the group does not explain compositional differences. We visualised ordination of samples depending on their BIN composition with Non-Metric Multidimensional Scaling (NMDS, R package vegan: Oksanen et al., 2019). We calculated Levins' (1968) standardised measure of niche breadth (B_A) at the prey species (BIN) level for each bat species.

Functional diet assessment

Prey items were classified based on the literature (outlined in Supplementary Table S2) and an expert entomological taxonomist into three functional categories: non-volant, not actively-volant, nocturnally volant. Categorisation depended on their mobility and type of activity, reflecting their likelihood of being captured by gleaning or aerial hawking (Supplementary Data file S1, Supplementary Table S2). The categorisation was done at family or finer taxonomic level by checking the literature for data on daily activity patterns of each family and presence in nocturnal light traps (Supplementary Table S2 for criteria used). The non-volant category included wingless arthropod groups (Araneae, Isopoda and wingless insects such as some members of Blattodea, Orthoptera). The not actively-volant category included those able to fly but unlikely to have been captured by the bat through aerial hawking because they are not active fliers, either at night (diurnal Diptera), or not active fliers in general (e.g. Hemiptera, some Blattodea, Orthoptera and Coleoptera). The nocturnally volant category comprised arthropods with aerial and nocturnal activity, and therefore likely to be captured by aerial hawking (e.g. non-Ropalocera Lepidoptera, nocturnal Diptera, Neuroptera, Ephemeroptera, Trichoptera). This classification represents the likelihood of being captured by gleaning or aerial hawking rather than direct inference of the capture mode because nocturnally active aerial prey can also be captured by gleaning when resting on vegetation and not active nocturnal fliers could also be captured in the air (e.g. ballooning in spiders). Once all prey items were classified into functional groups, we obtained the functional diet of both bat species using weighted percent of occurrence (wPOO), and compared the percentage of not nocturnally volant (including both non-volant and not actively volant categories) per sample between bat species using a linear model.

Trophic niche overlap in allopatry vs sympatry across spatial scales

Locations from Andalucía (Mediterranean climate) and northern Cantabria (Atlantic climate) were classified as regionally allopatric. Locations from La Rioja and southern Cantabria (climatically Mediterranean to

sub-Atlantic) as regionally sympatric (based on data from Razgour et al., (2019) and EBD records). At the fine-scale within the sympatric regions, we classified locations as locally sympatric or allopatric depending on whether they were within 3 km of records of the other species based on a conservative estimation of the home-range diameter of the better studied cryptic congener *M. nattereri* (Boye & Dietz, 2005). The swarming location in Catalunya was removed from the fine-scale analysis because bats gather in swarming sites from distances of up to 60 km from their colonies for the purpose of breeding rather than foraging (Rivers, Butlin, & Altringham, 2005), and therefore it is unclear whether those individuals forage in sympatric areas (Supplementary Table S1 for sampling locations and their broad and fine-scale sympatry category).

To identify differential use of certain prey orders and functional groups, we tested separately for allopatric and sympatric locations whether 1) the number of BINs per sample for each of the main arthropod orders, and 2) the percentage of not nocturnally volant functional groups differed between bat species. We used negative binomial zero inflated GLMs and a linear model respectively. We run separate models for broad and fine spatial scales given that both fine scale sympatry and allopatry treatments are within regional sympatry. Cases where resource (prey order or functional group) use was different between bat species when sympatric but not when allopatric were regarded as evidence of resource partitioning. We measured prey species (BIN) level niche overlap (O_{jk}) between bat species in sympatry and in allopatry, and tested, using null models (R package ecosimR) whether overlap was lower or higher than random in sympatry versus allopatry. We tested whether O_{jk} differed between sympatric and allopatric locations by pooling the diet composition of each bat species per location and measuring O_{jk} between pairs of locations. At the regional scale we used a Gaussian Hurdle model due to the high density of zeroes in overlap values. At the local scale we used a linear model with log transformed values of O_{jk} to meet assumptions of normal distribution. All statistical analysis was carried out in R (R core team, 2019).

Prey consumption relative to availability

For each location, we quantified the relative availability of each arthropod order and functional group using weighted percent of occurrence (wPOO) after pooling together sweeping samples from the different habitats. Similarly, we obtained bat diet composition (wPOO) per location of each arthropod order and functional group by pooling diet composition of all individual bats. Then, we subtracted from the bat diet wPOO the prey availability wPOO to obtain prey arthropod and functional group selection per location. A higher proportion of a given arthropod order in the diet than in sweeping samples indicates the bats may be preferentially consuming this resource, based on prey availability at the sampled strata. This analysis is based on assumptions of sampling representativeness (see discussion for detailed overview of methodological limitations).

Testing primer performance and representation of the DNA metabarcoding approach

For each arthropod order we described the number and proportion of BINs identified by each primer. Morphological identification of the arthropod communities allowed us to compare the performance of the primers and metabarcoding approaches. We compared the presence of orders in each sweeping sample based on molecular and morphological identification to determine whether metabarcoding offers a good estimation of arthropod community composition.

Results

We analysed a total of 138 dropping samples for *Myotis escalerai*and 90 for *Myotis crypticus* from 49 locations, 26 of which were in the broad-scale allopatric regions and 23 in sympatric regions. Within the sympatric regions (La Rioja and southern Cantabria), 91 samples were classified as locally allopatric and 28 as locally sympatric (Fig. 1, Table 1; Supplementary Table S1). We recovered a total of 2,859,300 reads

(Supplementary Table S3 for details) from the 228 dropping samples for the four combinations of PCR replicates and primers (1,403,636 from ANML1 and 1,455,664 from ZBJ). These reads were associated into 1461 different BINs. Based on the BINs present in sequencing blanks, we removed for the ANML primer 6 BINs from the first run of 56 dropping samples, and 2 BINs for the second run of 10 samples. For the ZBJ primer, we removed 5 BINs from the first run of 10 samples and 23 BINs from the second run of 88 samples. Based on BINs present in extraction blanks, we removed a total of 39 BINs from 16 dropping samples

Characterising the diet of *M. escalerai* and *M. crypticus*

A total 19 arthropod orders were obtained based on the 1461 BINs (Supplementary Data file S1 for list of prey items obtained for each bat species). The diets of *M. escalerai* and *M. crypticus* were characterised by high arthropod diversity, and were composed mostly of the orders Lepidoptera (*M. escalerai* = 26.6 %; *M. crypticus* = 23.7%), Diptera (24.8%; 33.2%), Araneae (20.7%; 17.2%), but also included Hemiptera (11.8%; 6.2%), Coleoptera (4.8%; 5.1%), and Orthoptera (4.3%; 4.8%), among others (<5%) (Fig. 2a-b; Supplementary Fig. S2 for diet composition based on POO and RRA measures). Diet composition at the prey order level was very similar between bat species (O_{JK} = 0.98, above 1000 null models). However there were differences in the number of BINs per sample of Diptera, which was lower in *M. escalerai* (5.27 versus 6.75) (Negative binomial GLM: $z_{1,226}$=-2.03, P = 0.042), and Hemiptera, which was higher in *M. escalerai* (2.09 versus 1.68) (Negative binomial GLM: $z_{1,226}$=2.85, P = 0.004 Supplementary Fig. S3).

At the prey species (BIN) level, Levins' niche breadth was similar for both species, B_A = 0.17 for *M. escalerai* and B_A = 0.19 for *M. crypticus* . Niche overlap between species was higher than expected by chance (O_{JK} = 0.71, above 95% of 1000 null models). The samples from the two bat species showed some differences in prey item composition in NMDS ordination space (Fig. 3a, Stress: 0.25, k=3, non-metric fit R^2=0.934, Linear fit, R^2= 0.532). An analysis of similarity confirms that distance in prey item composition among samples is greater between species than within species (ANOSIM R statistic: 0.10, P= 0.001).

Trophic partitioning in sympatric versus allopatric locations

At the arthropod order level, there is no clear pattern of shift from high similarity in order composition between species to differential use in sympatry at any of both spatial scales (O_{JK} regional allopatry =0.88, O_{JK} regional sympatry = 0.96, >1000 null models, Fig 2c-d; O_{JK} local allopatry =0.95, O_{JK} local sympatry = 0.98, >1000 null models, Fig 2e-f). When examining the number of BINs of the main arthropod orders per sample, there were differences between bat species between the allopatric regions for Araneae and Hemiptera, which were both higher in *M. escalerai* (*M. escalerai* = 4.00, 1.65, *M. crypticus* = 2.16, 0.55 respectively), and for Lepidoptera, which was higher in *M. crypticus* (4.6, 11.94) (Negative binomial GLM: df=1,98, P<0.05). In the sympatric region, the higher number of Hemiptera in *M. escalerai*holds (*M. escalerai* = 2.50, *M. crypticus* 1.50), and in Lepidoptera there is a shift whereby is *M. escalerai* the one that consumes a higher number (*M. escalerai* = 6.78, *M. cryptius*= 4.09, Negative binomial GLM: P<0.05). At the fine-scale, within the sympatric region, the only difference found between the bat species was the higher number of BINs per sample of Hemiptera (2.67, 1.49) (Negative binomial GLM: $z_{1,89}$= -2.68, P=0.007) and Lepidoptera (6.70, 3.64) in *M. escalerai* in allopatric locations (Negative binomial GLM: $z_{1,89}$= -2.92, P=0.004). There were no differences in arthropod orders consumed between the bat species in locally sympatric locations (Negative binomial GLM: P>0.05; Supplementary Fig. S4).

At the prey species (BIN) level, at the broad-scale, trophic niche similarity between species was lower in allopatric than in sympatric regions (O_{JK} allopatric = 0.35, O_{JK}sympatric= 0.62). Conversely, at the fine-scale, within the sympatric region, trophic niche overlap between species was higher in locally allopatric locations (O_{JK}= 0.56) than in locally sympatric locations (O_{JK}= 0.37). Despite the low values of overlap in regionally allopatric and locally sympatric locations, in all the four cases, observed niche overlap was higher than 1000 null models. When measuring trophic niche overlap between species using pairs of locations, we observed the same pattern. At the broad scale we found higher diet overlap in sympatric than allopatric

locations (O_{JK} sympatric= 0.107±0.056, O_{JK}allopatric= 0.050±0.04; Gausian hurdle model: binomial GLM: $z_{1,316}$=4.76, P<0.05; Gaussian GLM: $t_{1,265}$=8.26, P<0.05). In contrast, at the fine-scale, niche overlap was lower among pairs of locally sympatric than among locally allopatric locations (O_{JK} sympatric= 0.099±0.065, O_{JK} allopatric= 0.126±0.057; Linear model: $F_{1,73}$=6.34, P=0.014; Fig 3b).

Functional diet analysis

Both species had a similar high percentage of non-volant (*M. escalerai* =21.4 %, *M. crypticus* =19.5%) and not actively-volant (44.6%, 45.8%) prey items in the diet. Only 34.0% and 34.7% of weighted percent of occurrence (wPOO) was composed of arthropods classified as nocturnally volant (Fig. 4a). There were no differences in the overall percentage of not nocturnally volant prey taxa (BINs) per sample between bat species (66% ±20%, 66% ±21%, Linear model: $F_{1,217}$ <0.001, P= 0.990; Fig. 4b). When analysing functional diet differences separately in allopatric versus sympatric regions, we found differences between species in allopatric regions, whereby *M. crypticus* consumed lower percentage of prey that were not nocturnally volant (allopatric regions:*M. escalerai* = 66% ±19%, *M. crypticus* =48% ±25%; $F_{1,98}$=11.72, P<0.05; Fig. 4c; sympatric regions: 65% ±22%, 71% ±17%; $F_{1,117}$=2.3, P=0.13; Fig. 4d). At the fine-scale, there were no differences among bats in locally allopatric locations (*M. escalerai* = 67% ±22%, *M. crypticus* =72% ±16%, $F_{1,89}$=1.325, P=0.250; Fig. 4e) while in locally sympatric locations the percent of prey that were not nocturnally volant was borderline lower in the diet of *M. escalerai* (52% ±17%) than *M. crypticus* (68% ±18%; $F_{1,26}$=4.03, P=0.055; Fig. 4f).

Prey consumption relative to availability

In nearly all cases, we could not detect over- or under-selection of arthropod orders by the bats relative to their availability in sweeping samples. The distribution of prey order selection values between the 1st and 3rd quartiles overlapped with zero in all cases, except in the case of *M. escalerai* and Lepidoptera, where positive selection values could indicate over-selection (1st-3rd quartile: +0.43 — +18.7; Supplementary Fig. S5).

Metabarcoding and primer performance

There were compositional differences in the prey orders that each primer recovered. A large proportion of the BINs identified in dropping samples were only recovered by one of the primers (Supplementary Fig. S6). Neuroptera, Orthoptera, Coleoptera were more frequently recovered by ZBJ, while Plecoptera and Thysanoptera, Dermaptera Mantodea where more frequently recovered by ANML (Supplementary Fig. S6). Supplementary Figure S7a-b shows composition for a subset of dropping samples comparing each primer.

In sweeping samples, 7065 insects were identified morphologically to order level, with an average of 174.3 individuals per sample (range 7-624). Using molecular tools, we recovered 899,853 reads (Supplementary Table S2), and identified 813 different BIN items. Some of the rarer orders were under-represented in the molecular analysis. Specifically, Opiliones, Dermaptera, and Archaeognatha, appeared in more than 10 sweeping samples each identified morphologically, but were rarely recovered in the molecular approach, despite being present in the reference databases (Supplementary Fig. S8).

Discussion

Morphologically almost identical species are likely to compete for resources, and therefore offer good case studies to understand processes that drive species coexistence. We find evidence of reduced trophic niche overlap in recently separated cryptic bat species in sympatric locations relative to allopatric ones based on DNA metabarcoding and high throughput sequencing. The functional analysis suggests that the subtle trophic shift seen may be driven by differential foraging mode. Our results support niche theory predictions of

the role of biotic interactions in driving species assemblages (Schoener, 1974). Trophic resource partitioning was only evident at the fine spatial scale, within areas of range overlap, suggesting that fine-scale mechanisms of coexistence could have implications for the maintenance of broad- scale diversity patterns (Godsoe, Murray, & Plank, 2015).

Trophic ecology of Myotis escalerai and Myotis crypticus

Our results reveal that the two bat species have a broad generalist diet, tend to consume prey relative to their availability and use gleaning to a high extent. We found high similarity in their trophic ecology in terms of both order and prey species composition. Both bat species' diets are mostly composed of Lepidoptera, Diptera and Araneae, but also include several other prey orders. However, *M. escalerai*consumes a higher percentage of Hemiptera, while *M. crypticu* s Diptera. Functionally, the two bat species consume an equally high proportion of prey items that are not nocturnally volant, which suggests that both bats predominantly glean prey from vegetation.

The trophic ecology of these two recently described bat species is very similar to their cryptic sister-species *M. nattereri,* which also feeds mostly on Lepidoptera, Diptera and Araneae (Hope et al., 2014; Swift, 1997; Swift & Racey, 2002; Vaughan, 1997) and is known to catch a high proportion of its prey through gleaning (Arlettaz, 1996; Hope et al., 2014; Shiel, McAney, & Fairley, 1991; Swift, 1997; Swift & Racey, 2002). Similarly to our study, Shiel et al., (1991) estimated that 68% of *M. nattereri* 's diet is made up of arthropod families that are not active at night. The row of hairs in the uropatagium border is a characteristic trait of members of the *M. nattereri* species complex that is thought to be functionally linked with gleaning (Czech, Klauer, Dehnhardt, & Siemers, 2009). Although the presence of more developed hairs in *M. escalerai* is one of the characters which separates these taxa (Juste et al., 2018), we found no difference in the extent of gleaning between the two bat species.

Trophic partitioning across spatial scales

Despite overall high trophic niche similarity between the two bat species at the prey order level, we detect a signature of trophic shift at the prey species (BIN) level, whereby diet overlap is lower in locally sympatric compared to locally allopatric locations at the fine-scale. This supports the contribution of trophic partitioning to species coexistence even when overall trophic niche overlap is high. A similar trend was seen at the functional level, whereby the proportion of prey items that are not nocturnally volant is borderline different between bat species only when locally sympatric. This suggests that the differentiation in diet composition seen at the prey species level when locally sympatric may be driven by a shift in foraging strategy (e.g. Krüger et al., 2014), through *M. escalerai* decreasing its extent of gleaning. However, our inference is limited by small sample sizes, which reduced the power of the analysis. At the arthropod order level, we find differences in the use of some arthropod orders among allopatric regions, likely due to differences in arthropod availability between the Mediterranean region, where only *M. escalerai* is found and the Atlantic region, where *M. crypticus* is present.

Several studies have identified trophic niche shifts from allopatry to sympatry, for instance between morphologically similar fish (Gkenas, Magalhães, Cucherousset, Orjuela, & Ribeiro, 2019; Schmitt & Coyer, 1983) and reptile species (Huey, Pianka, Egan, & Coons, 1974; Klawinski et al., 1994). However, in bats, previous coexistence studies looking at trophic ecology only focused on sympatric populations, and rarely found evidence of trophic resource partitioning. A few exceptions are the gleaning bats *M. nattereri* , *Plecotus auritus* and*Myotis bechsteini* in a sympatric population in central Europe (Andreas, Reiter, & Benda, 2012a), and evidence of low dietary overlap between sympatric *P. auritus* and *Plecotus macrobullaris*(Ashrafi, Beck, Rutishauser, Arlettaz, & Bontadina, 2011).

The observed trophic shift, albeit subtle, suggests that the two bat species are likely competing for food resources. It has been previously hypothesised that arthropods are abundant and do not constitute a limiting resource for bats (Arlettaz, 1999; Krüger et al., 2014). However, exclusion experiments in both tropical

(Kalka, Smith, & Kalko, 2008) and temperate forests (Böhm, Wells, & Kalko, 2011) show that bats can control the abundance of arthropods, and therefore arthropods could be a limiting resource to competitors (Salinas-Ramos et al., 2020).

Our study does not refute the possibility that other coexistence mechanisms, such as habitat or temporal partitioning (Schoener, 1974), occur among these two species, or the role of environmental variability in facilitating coexistence (Chesson & Warner, 1981). Spatial partitioning is frequently cited as a key mechanism of coexistence in other bat studies (e.g. Arlettaz, 1999; Emrich, Clare, Symondson, Koenig, & Fenton, 2014; Kunz, 1973; Russo et al., 2014). Although in many cases, contrary to our study, spatial partitioning may be driven by slight differences in bat morphology (e.g. Salsamendi et al., 2008, 2012), which would affect their performance in different habitats (Norberg, 1994). However, in our study the two species were caught in the same sampling sites, some of which were forests, where they are known to forage, suggesting they may share the same foraging sites.

A better understanding of the spatial scales of species coexistence is an important advance in our understanding of the maintenance of diversity (Hart et al., 2017). Our finding that trophic partitioning only occurs at the fine spatial scale is consistent with other bat (Peixoto et al., 2018), ant (Albrecht & Gotelli, 2001), parasitoid insects (Harvey, Snaas, Malcicka, Visser, & Bezemer, 2014) and bobcat (Lewis et al., 2015) studies, showing that interspecific interactions are more important for shaping community structure at fine rather than broad spatial scales. However, this pattern is not universal (e.g Harmačková, Remešová, & Remeš, 2019). Fine-scale coexistence mechanisms could prevent competitive effects from scaling-up (Godsoe et al., 2015), which in our study system could contribute to enabling broad-scale range overlap across the north of the Iberian Peninsula.

Prey consumption relative to availability

The diet of a species is a function of both consumer selection and trophic resource availability within the foraging habitat (Lawlor, 1980). Therefore, considering resource availability allows for a better inference of species trophic preferences. Previous studies comparing bat prey consumption with prey availability pointed to selection of certain prey orders, such as Coleoptera by *Eptesicus fuscus* (Agosta, Morton, & Kuhn, 2003), chironomid flies by *Myotis daubentoni*(Vesterinen et al., 2016) and certain prey traits like moth size by*Barbastella barbastella* (Andreas, Reiter, & Benda, 2012b). Similarly, *M. nattereri* was found to over-select arachnids, Opiliones, Coleoptera, and several Diptera families, and under-select Hemiptera (Swift & Racey, 2002). In this study we do not detect clear trends of over-selection for specific prey orders matching the generalist broad trophic niche of the studied bats. However, diet selection results should be interpreted with caution due to the difficulty of obtaining a representative estimation of arthropod availability. Any arthropod sampling technique is biased towards certain types of arthropods (Cooper & Whitmore, 1990) and the habitats sampled and their respective sampling effort may not adequately represent where bats actually forage, especially given that they can use large areas and arthropod communities change depending on habitat type (Lamarre et al., 2016) and vertical stratification (Ulyshen, 2011). Our molecular diet analysis results confirm that the two studied bat species indeed glean prey from the vegetation, and therefore the arthropod community sampled using sweep nets likely represents at least part of the prey resources available to the bats.

Methodological considerations and study limitations

Primer bias towards certain taxonomic groups is a major issue in metabarcoding studies (Elbrecht et al., 2019). In this study, prey items were frequently recovered by only one of the primers, and differences existed in the recovery of the different arthropod orders. This supports previous studies that suggest that more than one set of primers should be used when the expected diet covers a broader taxonomical spectrum (Alberdi et al., 2018). The inclusion in this study of a set of samples with known composition based on morphological analysis (albeit only at the order level) gives us some idea of potential biases in the molecular identification.

Opiliones, in particular, were morphologically identified in several sweep net samples and are known to be present in the diet of *M. nattereri* (Galan et al., 2018; Swift, 1997; Swift & Racey, 2002), but were absent from the molecularly-characterised diets of the two bats. Thus, their absence in this study is likely the result of primer amplification bias.

Parameter choice during bioinformatic analysis can modify the diet composition recovered (Alberdi et al., 2018). The strong match between the inferences drawn using the additive and the conservative approach of dealing with PCR replicates (Fig. S9), show that the results are robust to that choice, and mirror other studies showing that parameters choice does not change ecological conclusions (Clare, Chain, Littlefair, Cristescu, & Deiner, 2016). In the same manner, high similarity in diet composition based on weighted Percent of Occurrence (wPOO) and Relative Read abundance (RRA), indicates that the results are also robust to the measure used.

Because prey development stage cannot be identified using the metabarcoding approach, some of the prey species (BINs) classified as nocturnally volant may correspond to non-flying larval stages. This could be important in Lepidoptera, and could increase the inferred importance of the gleaned behaviour of both species because larval stages are known to be consumed by *M. nattereri* (Hope et al., 2014). More generally, nocturnal aerial activity is not directly quantifiable, and therefore our classification is subject to a certain degree of subjectivity. However, because arthropod orders most difficult to categorise due their diversity, like Coleoptera, are consumed in similarly low proportion by both bat species, potential classification biases are expected to be low and standardised across species. Nevertheless, due to potential classification biases and low sample sizes in sympatric locations, interpretations of functional prey shift should be considered with caution.

Conclusions

In line with niche theory predictions, we show that coexistence among morphologically identical (cryptic) species can be facilitated through fine-scale mechanisms of resource partitioning, despite high levels of trophic similarity at the broad-scale, even in sympatric regions. Hence, this study highlights the importance of using appropriated spatial scales when studying impacts of biotic interactions on community assembly (Viana & Chase, 2019). Our findings that trophic resource partitioning is only evident at the fine spatial scale, within areas of range overlap, suggest that fine-scale mechanisms of coexistence could have implications for the maintenance of broad- scale diversity patterns. This is the first study to identify a trophic shift between allopatric and sympatric populations of insectivorous bats, supporting the role of trophic resource partitioning in enabling species co-occurrence in the same foraging site. It thereby addresses some of the key limitations identified in a recent review of interspecific competition in bats (Salinas-Ramos et al., 2020). We highlight the importance of using high taxonomic resolution and allopatric populations at meaningful spatial scales for identifying patterns of niche shift, and the utility of using a functional approach that better links mechanistically with species trophic ecology. Understanding mechanisms of coexistence is essential for predicting species vulnerability under climate change because range shifts will result in new community assemblages and competitive interactions (HilleRisLambers, Harsch, Ettinger, Ford, & Theobald, 2013). This is particularly relevant in our study system as both species are restricted to the Mediterranean region, where climate change is predicted to be particularly severe (Sala et al., 2000), and both are predicted to experience range shifts and changes in range overlap under climate change (Razgour et al., 2019).

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Data Accessibility

- Data will be deposited in Dryad and made available upon acceptance.
- Sampling locations are available in Supplementary Information (Table S1).
- List of BINs (OTUs) obtained for each bat species, their identification and categorisation based on level of volancy are presented in Supplementary Data File S1.

Author contribution

OR conceptualised and designed the study. RNF, JJ and CI collected the samples. RNF, JJ, CI and ELC carried out the laboratory work. OR, RNF, ELC and CPD contributed to designing the analysis, and RNF performed it. RNF drafted the manuscript and all authors contributed to revisions.

Tables

Table 1 – Number of bat dropping samples, sweeping samples, and locations for each bat species by allopatry/sympatry classification at broad (regional) and fine (local) spatial scales.

		Total	Broad-scale allopatric	Broad-scale sympatric	Fine-scale allopatric	Fine-sca
Dropping samples	*M. escalerai*	138	82	56	46	6
	M. crypticus	90	18	72	45	22
Sweeping samples	*M. escalerai*	13	5	8	3	5
	M. crypticus	15	2	13	6	7
Locations		49	26	23	14	8

Figures

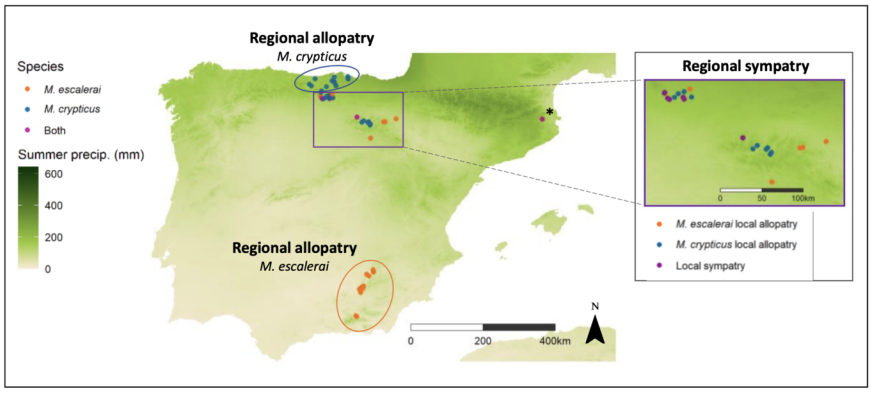

Fig. 1 – Sampling locations overlaying summer precipitation across Spain, with gradient from dry Mediterranean to wet Atlantic. Ovals encompass the two allopatric regions (Granada and Jaen for *M. escalerai* and northern Cantabria for *M. crypticus*). The rectangle encompasses the sympatric region (La Rioja and the Mediterranean climatic zone at the south of Cantabria). Insert shows the sympatric region with locally sympatric (red) versus locally allopatric (blue and orange) locations. Black star on the sympatric location at the E side of the main map in Catalunya denotes a swarming site and was excluded from the analysis.

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Fig. 2 – Overall diet composition of *M. escalerai* and *M. crypticus* using*w* eighted Percent of Occurrence (wPOO) (a, b). Dietary composition by scale of allopatry/sympatry (broad-scale: c, d, fine-scale: e, f).

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Fig. 3 – a) Non-Metric Multidimensional Scaling ordination of individual bat samples based on their BIN composition, with *M. escalerai* samples in yellow and *M. crypticus* in blue**. b)** Pianka's measure of niche overlap (O_{JK}) between the two bat species in allopatric versus sympatric locations at the regional (left) and local (right) scales. Replicates are values of overlap between pairs of locations of different bat species. Star denotes significant differences between groups.

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Fig. 4 – Functional diets of *M. escalerai* and *M. crypticus* depending on the nocturnal flight behaviour of the prey species, classified into non-volant (wingless arthropod groups), not actively-volant (able to fly but unlikely to have been captured through aerial hawking), nocturnally volant (likely to be captured by aerial hawking). Overall proportions of functional categories in the bat diets (a); proportion of not nocturnally volant prey items per dropping sample in *M. escalerai* and *M. crypticus* overall (b), in broad-scale allopatric versus sympatric regions (c) and in fine-scale locally allopatric versus locally sympatric locations (d). Star denotes significant differences between groups (Linear Model).