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Trait-based functional dietary analysis provides a better insight into the foraging ecology of bats

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Abstract

1. The degree of trophic specialization determines the ability of predators to cope with changing foraging conditions, but in predators that prey on hundreds of species it is challenging to assess, especially when prey identity varies among predator individuals and across space and time.
2. Here, we test the hypothesis that a bat species foraging on flying insects like moths will show ample flexibility in trophic niche, and this irrespective of phylogenetic relationships among moths, so as to cope with a high diversity of prey types that vary across seasons. We predict that individual bats will show functional dietary differences consistent with energetic requirements and hunting skills.
3. We used DNA metabarcoding to determine the diet of 126 Mediterranean horseshoe bats (*Rhinolophus euryale*) from two different sites during three seasons. Simultaneously, we measured moth availability and characterized the traits of 290 moth taxa. Next, we explored the relationship between phylogeny and traits of all consumed and available moth taxa. Finally, we assessed the relationship between individual traits of bats and traits related to prey profitability, for which we used the RLQ and fourth-corner statistical techniques.
4. Seasonality was the main factor explaining the functional dietary variation in adult bats, with moths consumed irrespective of their phylogenetic relationships. While adults consumed moths with a broad range in wing loading, body mass and echolocation detection ability, juveniles consumed slower, smaller and lighter moths, which suggests that young individuals may undergo some fitness gain and/or psychomotor learning process during which they would acquire more effective foraging skills.
5. Our approach revealed a degree of functional flexibility in the trophic niche previously unknown for an insectivorous bat. *Rhinolophus euryale* consumed a wide variety of moth taxa differing in profitability throughout seasons and between ontogenetic stages. We showed the validity of trait-based approaches to gain new insights in the trophic specialization of predators consuming hundreds of species of prey.

KEYWORDS

Chiroptera, DNA metabarcoding, fourth-corner, functional traits, moths, niche flexibility, RLQ

1 | INTRODUCTION

Evolutionary processes have determined the current ecological specialization of predators on particular prey items (Ferry-Graham, Bolnick, Wainwright, & Avenue, 2002). Measuring the degree of prey specialization, trophic flexibility, and evolutionary relationship with prey, is pivotal to understanding predators' ability to cope with changing environments and their role in ecosystem functioning (Devictor et al., 2010). However, the definition of ecological specialization is highly context dependent (Devictor et al., 2010). In many predator-prey systems—such as for insectivores—a large range of prey species are involved and prey spectra can considerably vary temporally (e.g. due to changes in prey phenology; Kartzinell & Pringle, 2014), spatially (e.g. differing prey assemblages across distribution areas; Marciniak, Nadolski, Nowakowska, Loga, & Banbura, 2007) and in relation to intraspecific variation of predators (Bolnick et al., 2003; Nifong, Layman, & Silliman, 2015; Zhao, Villéger, Lek, & Cucherousset, 2014). Additionally, the degree of trophic specialization is commonly assessed based on the taxonomic width of consumed prey taxa through classical diversity indices, which offer little information on the foraging performance of individual predators. A further step in foraging ecology is to understand the functional mechanisms that determine why and how species and individuals are specialized on particular prey types. These functional mechanisms include morphological, physiological and behavioural traits of both predator and prey (Ferry-Graham et al., 2002), which influence their foraging performance and fitness.

The functional relationship between predator and prey is based on assumptions that not all prey are equally profitable for predators, and that not all evasive or defensive adaptations are equally effective against predators (Spitz, Ridoux, & Brind'Amour, 2014; Stephens & Krebs, 1986). Prey profitability influences the individual fitness of predators and is determined by the cost-benefit balance of prey consumption (Stephens & Krebs, 1986). The relative contribution of traits such as prey size, aggressiveness and evasiveness determines the profitability of each prey item, while the relative contribution of each prey item to the predator's diet varies with prey ecological availability as well as with niche variation among predator individuals (Araújo, Bolnick, & Layman, 2011). Indeed, it is known that size, sex, ontogeny and individual specialization in predators may all influence the trophic niche of individuals within species. Although this can have significant ecological, evolutionary and conservation implications, it has been overlooked in many ecological studies (Bolnick et al., 2003). For instance, sex-specific energetic requirements and morphological dimorphism are likely to be the main factors of intraspecific diet variation in grey seals (Beck, Iverson, Bowen, & Blanchard, 2007). Pumas show age-specific prey selection, where less experienced sub-adult hunters avoid adult ungulates (Elbroch,

Feltner, & Quigley, 2017). Some populations of sea otters exhibit extreme individual-level diet specialization, which is likely related to several factors such as spatio-temporal variation of prey, matrilineal transmission of foraging preferences and frequency-dependent intraspecific competition (Estes, Riedman, Staedler, Tinker, & Lyon, 2003). Hence, trait-based functional dietary approaches might offer new insights to understand the foraging ecology and behaviour of complex predator-prey systems, as they consider the functional relationship among predators and prey. Such approaches have recently been tested for marine predator-prey systems (Green & Côté, 2014; Spitz et al., 2014), but they have never been applied to terrestrial systems.

Of the many predator-prey systems, insectivorous bats and their arthropod prey provide an important contribution, both taxonomically and functionally, to most terrestrial ecosystems (Kunz, Braun de Torrez, Bauer, Lobo, & Fleming, 2011). Molecular dietary studies are revealing that bats who forage on particular insect taxa are in fact consuming hundreds of species, with variation across time (Arrizabalaga-Escudero et al., 2015; Razgour et al., 2011), across space (Clare et al., 2013) and across individuals (Mata et al., 2016). These studies are providing the first detailed insights into the highly diverse diets of insectivorous bats. However, when it comes to such diverse diets, using a purely taxonomic interpretation impedes the assessment of functional specialization and selective behaviour. Indeed, not all insects are equally profitable, and not all bat individuals are identical. Prey profitability might differ among individuals due to sex-specific energetic requirements or individual differences in prey capturing skills. For instance, male and female *Tadarida teniotis* bats differ in the type of moths they consume (Mata et al., 2016). Similarly, Hamilton and Barclay (1998) reported dietary differences between adult and juvenile *Eptesicus fuscus* bats and suggested that flight and echolocation experience may be causing such differences.

Insectivorous bats typically forage on hundreds of insect species, which may vary considerably in mass, flight characteristics and evasive or defensive behaviour, traits that likely condition their profitability for bat individuals. Do individual bats change their diet according to differences in the profitability of available prey? Alternatively, are they foraging on particular traits irrespective of prey species diversity? To which extent are those traits phylogenetically conserved among prey? It is clear that the accurate measurement of the trophic specialization of predators like insectivorous bats is unlikely to be achieved from mere taxonomy-oriented dietary research.

Here, we explore the validity of trait-based dietary approaches as a novel procedure to assess the functional relationship between predators like bats and their insect prey. We choose as our model species a horseshoe bat species that mainly consumes flying insects such as moths (Arrizabalaga-Escudero et al., 2015;

Goiti, Garin, Almenar, Salsamendi, & Aihartza, 2008). This predator-prey system allows us to better understand the complex ecology of insectivorous bats in relation to the functionally and taxonomically highly diverse Lepidoptera. Evidence suggests that the echolocation system of horseshoe bats allows them to distinguish small differences among prey, based on acoustic glints produced by the echoes of beating wings (Koselj, Schnitzler, & Siemers, 2011; Schnitzler, 1987). In addition, European moths are well represented in reference COI barcode databases (e.g. BOLD Systems), a prerequisite for successful molecular identification of prey species from faeces. We assess the functional relationship between individual bats and their moth prey by measuring the relationship between individual traits of bats and traits related to prey profitability, using DNA metabarcoding, across populations and seasons. Simultaneously, we analyse the potential qualitative availability of moth species for bats, and we explore the relationship between moth traits and moth phylogeny. We test the general hypothesis that a bat species which mainly consumes flying insects like moths will show a flexible foraging diet opportunistically following spatiotemporal changes in the availability of functional traits displayed by potential prey. Specifically, our aims are to (a) evaluate the foraging niche flexibility of bats based on the functional traits of consumed and available moths as well as on the phylogenetic structure of their traits, (b) identify and measure the traits of moths consumed by bats linked to profitability, and (c) assess the intraspecific trophic variation of a horseshoe bat species in relation to prey traits.

2 | MATERIALS AND METHODS

2.1 | Study area and bat captures

In May, July and September 2012, we captured 126 individuals of the Mediterranean horseshoe bat *Rhinolophus euryale* (Rhinolophidae) with a harp trap at their roost in two caves located in northern Spain, in the Karrantza (43°15'N, 3°22'W) and Lea-Artibai -hereafter Lea- (43°18'N, 2°33'W) valleys of the Basque Country (see Supporting Information, Appendix S1 for details). Captured bats were held individually in clean cloth bags until they defecated. Bats were sexed and aged, their body mass and forearm length measured using a digital balance (Pesola, precision 0.01 g) and metal vernier calliper (Medid, precision 0.05 mm), respectively and their pellets collected and frozen within 6 hr. Bats were immediately released at the roost after handling. Capture and handling protocols were approved by the Ethics Committee at the University of the Basque Country (Ref. CEBA/219/2012/GARIN ATORRASAGASTI) and performed under license from the Regional Council of Biscay.

2.2 | DNA extraction, PCR amplification and sequencing

DNA extraction from bat faecal samples, PCR amplification and high throughput sequencing were performed following the same

procedure as described in Arrizabalaga-Escudero et al. (2018). The only difference was that we performed two PCR replicates for each DNA extract sample in order to discriminate between PCR and/or sequencing artefacts and true biological sequences (Hope et al., 2014). In short, DNA was extracted from faeces using the QIAamp DNA Stool Mini kit (Qiagen, UK) and a 157 bp-long fragment of the COI barcode region was subsequently PCR amplified using modified ZBJ-ArtF1c and ZBJ-ArtR2c primers (Zeale, Butlin, Barker, Lees, & Jones, 2011). Each sample and its replicate were tagged with a unique primer combination in order to identify them bioinformatically. Sequencing was performed on the Ion Torrent (Life Technologies) sequencing platform using a 318 chip.

2.3 | Bioinformatics processing

Raw reads were quality-filtered, demultiplexed by tagged primers, filtered by length and collapsed into unique sequences using Prinseq 0.20.4 (Schmieder & Edwards, 2011), Fastx-toolkit 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html) and AdapterRemoval (Lindgreen, 2012) toolkits. Reads with less than 15 copies were discarded and only those sequences that appeared in both replicates at a 100% similarity were kept for further analyses (Hope et al., 2014). Reads were clustered into Molecular Operational Taxonomic Units (MOTUs) using pick_otu and uclust methods at a 97% similarity threshold in QIIME (Caporaso et al., 2010). Taxonomic assignments of MOTUs were done in the BOLD database following the identification criteria of Clare et al. (2013). Molecular Operational Taxonomic Units were classified as 'unknown' when they did not match any reference sequence or when they did so but the matching sequence did not belong to Iberian or French species. 'Unknown' and family-level identified MOTUs were excluded from further analysis.

2.4 | Moths: Captures and identifications

Moths were captured with two objectives: first, to characterize the traits of the moth species identified in bats' diet; and second, to analyse the qualitative functional moth availability within the home range of the studied bat populations. We used 14 light traps (6W actinic Heath traps) in each locality and season. An additional set of 21 light traps was also used in July in Karrantza in order to complete the reference dataset of moth species' traits for functional dietary analyses. This extra set —belonging to a different project— could not be added in other seasons/locations due to resource limitations. Light traps were located as evenly as possible within a 5 km radius from each of the colony roosts, which is the likely foraging range of most *R. euryale* individuals (Goiti, Aihartza, Almenar, Salsamendi, & Garin, 2006), covering the six main habitat types available: broad-leaved woodlands, holm oak forest, hedgerows, pine plantations, eucalyptus plantations and grasslands. We spread the sampling effort per habitat type according to their availability (Table 1). This sampling design was decided because, even though *R. euryale* is highly selective regarding foraging habitats (Goiti, Aihartza, Garin,

TABLE 1 Light trap sampling design showing the distribution by location, season and habitat type: deciduous woodland (DW), hedgerow (HG), holm woodland (HOLM), coniferous plantation (CON), eucalyptus plantation (EU) and meadows (MEA)

	Karrantza Valley			Lea Valley
	May	July*	September**	July
DW	3	3 (4)	3 (-1)	3
HG	2	2 (4)	2	2
HOLM	2	2 (3)	2	2
CON	2	2 (3)	2	3
EU	2	2 (3)	2	2
MEA	3	3 (4)	3 (-1)	2
Total	14	14 (21)	12	14

*In Karrantza Valley in July, an extra set of 21 light traps was also collected (shown in brackets).

**In Karrantza Valley in September one light trap failed in DW and MEA habitats –indicated by 3(-1)–.

& Zabala, 2003; Goiti et al., 2008), a substantial part of the moths they consume therein come from other habitats and localities (Arrizabalaga-Escudero et al., 2015). Moths were captured at least for two nights at each location/season, within an interval of two nights before and after bat captures. Moth sampling was avoided in humid and cold nights to avoid any weather-related moth capture bias. Light traps were activated at dusk for four hours coinciding with the first activity peak of both moths (Scalercio, Infusino, & Woiwod, 2008) and bats (Goiti et al., 2006). Moths were captured alive using a clothing-bag located inside the light trap. They were frozen in the bags within 1–6 hr from capture.

The specimens captured by all the 75 light traps (including the extra 21 traps in July) were analysed for species identification and trait measurements (see Section 2.5 below). Moth specimens were identified to species level whenever possible, either visually by using field guides for macro- and micro-moth identification (see Appendix S1 for references) or through DNA barcode analysis using LEP-F1/LEP-R1 primers designed by Hebert, Penton, Burns, Janzen, and Hallwachs (2004). Note that some moth species belonging to the same or related genera were almost morphologically identical and difficult to identify at the species level, in particular when colouration patterns had deteriorated. These specimens were classified into the following groups: *Eudonia*-complex (including *Eudonia* sp. and *Scoparia* sp.) and *ScopCabe*-complex (including *Scopula* sp., *Cabera* sp. and *Lomographa* sp.). Unfortunately, for some taxa neither morphological nor genetic identification was possible and they were excluded from further analysis.

2.5 | Functional traits of moths and bats

In order to characterize the traits of the moths consumed by *R. euryale*, we assigned functional traits of moths captured in the field (1–5 specimens per species) to the species that had been molecularly identified in the diet. We assume that the traits of the measured

specimens are representative of moth individuals consumed by bats. We defined functional traits of prey as those anatomical features that likely influence the profitability of prey moths for *R. euryale*. Thus, we measured fresh body mass, forewing length, wing loading, and forewing aspect-ratio as proxies of energy content, prey size, flight speed and wing shape, respectively (see Supporting Information, Appendix S1 for description of the procedure to measure each of the moth traits, and Table S2 for the traits values). We defined the trait “manoeuvrability” as the ratio between hindwing and forewing areas (Manoeuvrability = hindwing area/forewing area), where higher values indicate higher manoeuvring capability (sensu Jantzen & Eisner, 2008). We included the ultrasound-hearing capability in moths by taking this information from published sources (see Appendix S1 for references). Regarding bat traits, we included season and location as environmental traits, and size (measured as forearm length and body mass), sex and age as intraspecific traits of bats.

2.6 | Phylogenetic analysis of moths

It is well known that DNA barcode data cannot be used to infer phylogenetic relationships at deeper taxonomic levels (Rubinoff & Holland, 2005). Thus, in order to be able to use our barcode data to infer a phylogenetic hypothesis with branch lengths needed for downstream analyses, we used a backbone constraint approach. We built a constraint topology (with no branch lengths) based on a range of recent phylogenetic hypotheses for Lepidoptera (Mitter, Davis, & Cummings, 2017) for all moths identified in bat faeces or light traps. All nodes that could be confidently constrained were constrained, and this in most cases down to the subfamily or tribe level. Within tribes and subfamilies many of the taxa sequenced in this study have not been included previously in a phylogenetic study. These relationships were thus not constrained within the respective clade. The full DNA barcode dataset (downloaded from the BOLD database) was then analysed using RAXML-HPC2 v. 8.2.10 (Stamatakis, 2014) on XSEDE off the CIPRES server (Miller, Pfeiffer, & Schwartz, 2010), with the constraint topology implemented. The resulting tree thus has branch lengths calculated based on 658 bp of the COI gene, and estimated phylogenetic relationships of species within subfamilies/tribes.

We then applied the root-skewness test (a Monte-Carlo test; with 999 repetitions) proposed by Pavoine, Baguette, and Bonsall (2010) to assess whether the quantitative moth traits are phylogenetically conserved and to identify any consumption pattern related to traits and phylogeny of moths. For this purpose, we tested the null hypothesis that the observed test-statistic does not differ from chance expectation (alternative hypothesis: the observed statistic is less than expected by chance). We paired the moths' trait dataset with the previously built phylogenetic tree and indicated the moth species identified in the diet of bats using GraPhlAn (Asnicar, Weingart, Tickle, Huttenhower, & Segata, 2015). For an easier visualization of the tree we used the tree without branch lengths.

2.7 | Statistical analyses

The relationships between prey and bat traits, as well as between the traits of potentially available moths and environmental variables, were tested by **RLQ** and fourth-corner analyses (Dray et al., 2014) with the package **ADE4** (Dray & Dufour, 2007) in R (R Core Team, 2016). Applied to diet studies, **RLQ** analysis and fourth-corner test are complementary methods to uncover how predators filter certain species traits, linking a description of the predators to prey species traits by measurements of prey consumption (Dodelec, Chessel, Braak, & Champely, 1996; Legendre, Galzin, & Harmelin-Vivien, 1997).

These **RLQ** analyses were performed independently in three steps: first for available moths across seasons and locations, second for all adult bats across seasons and locations, and third for adult and juvenile bats during the post-breeding season (juveniles were only present in the post-breeding season). For availability analysis we used all samples mentioned in Section 2.4 except for Karrantza in July, where on behalf of comparability we only used 14 light traps, chosen at random out of the 35 set, evenly representing the habitat availability as mentioned in Section 2.4 (Table 1).

The **RLQ** analyses require three input matrices: **R**, **L** and **Q**. In our study, the first matrix (**L**: $n \times p$) includes the presence/absence of the p moth species in the diet of n *R. euryale* individuals. The second matrix (**Q**: $p \times s$) describes the p moth species of matrix **L** according to a set of s functional traits of moths (Table S2). The third matrix (**R**: $m \times n$) describes the n *R. euryale* individuals according to a set of m traits of bats (Table S2). From these three matrixes, **RLQ** analysis makes a new matrix linking individual *R. euryale* traits and moth traits, and then performs a PCA-like eigen-decomposition on that. Finally, we applied a fourth-corner analysis directly on the results of the **RLQ** analysis, to summarize and test the significance of the associations between the **RLQ** axes and the functional traits of bats and moths.

Separate ordinations of each matrix are required prior to **RLQ** analysis. Thus, a Correspondence Analysis (CA) was applied on the matrix **L** (species occurrence in diet), and **R** (bats traits) and **Q** (moth traits) matrixes were ordered by a Hill-Smith analysis (Hill & Smith, 1976), since they contain both qualitative and quantitative variables. Lastly, the three analyses were combined by the **RLQ** analysis, which links **Q** (weighted by **L**) to **R**, using the function `rlq`. Graphical outputs of the **RLQ** analysis were used to summarize the main relationships between functional traits of bats and moths. Finally, Monte Carlo tests, based on the total co-inertia of the **RLQ** analysis, were applied through function `randtest.rlq()` of package **ADE4** to test the association between the **R** and **Q** matrixes.

The fourth-corner test was carried out following the approach suggested by Dray et al., (2014) in applying the fourth-corner analysis directly on the results of the **RLQ** analysis to summarize and test the significance of the associations between the **RLQ** axes and the functional traits of bats and moths. We applied the sequential test proposed by Ter Braak, Cormont, and Dray (2012) for better control of type I errors (Model type = 6), we adjusted p -values by the Benjamini and Hochberg (1995) correction, and the number

of permutations was elevated to 9,999 using the function `fourth-corner.rlq`. The significance level of the test was set at $p < 0.05$. All **RLQ** matrixes are available in Dryad (Arrizabalaga-Escudero et al., 2019).

3 | RESULTS

3.1 | Molecular analysis of diet

A total of 315 MOTUs were obtained from the faecal samples of 126 *R. euryale* individuals. We identified 63.5% (i.e. 200) of the MOTUs to species or genus level and 2.2% to family level. The remaining 34.3% were classified as "unknown". Lepidopterans accounted for 91% (i.e. 182) of the MOTUs identified to species or genus level. The remaining MOTUs belonged to Diptera (5.0%), Neuroptera (3.0%), Hymenoptera (0.5%) and Psocoptera (0.5%). After collapsing MOTUs by identified taxa, we obtained a total of 168 lepidopteran taxa belonging to 16 families. The functional traits of 69.6% of those taxa were measured, completely or partially, from individuals captured in the study area, 19.1% from specimens at the Natural Science Museum of Araba (the Basque Country), 5.3% from BOLD system scaled pictures and 3.6% from Thomas Merckx's own collected specimens. We could not get data of traits of the remaining 2.4%. For further analysis we only considered those taxa for which all the defined functional traits were measured: 137 taxa from 12 families.

3.2 | Moths: Species, phylogeny and functional traits

We characterized moth availability in Karrantza valley from May to September, and in Lea valley only in July, collecting a total of 2,873 moth specimens belonging to at least 308 taxa of 18 families. We completely characterized the traits of 290 species.

With respect to moth functional traits (Figure 1), we found that mass, forewing length, manoeuvrability, aspect-ratio and wing loading are phylogenetically conserved (observed statistic-value = 0.207; expected statistic-value = 0.501; p -value < .001), i.e. related species were more similar in measured traits than expected just by chance. For instance, noctuid moths are in general characterized by being heavier, faster and more manoeuvrable than geometrids, but not smaller in terms of forewing length. These combinations of traits vary among families. On the other hand, bats consumed moths with different trait combinations and did not appear to prey exclusively on particular phylogenetic groups. Nonetheless, some families appear to be either underrepresented or absent in the diet of *R. euryale*: the macro-moth families Lasiocampidae (P in Figure 1), Notodontidae (S), Sphingidae (Q), the subfamily Arctiinae (Erebidae) (T) and the micro-moth families Oecophoridae (L), Gelechiidae (H), Elachistidae (G), Depressariidae (I), Yponomeutidae (D) and Tineidae (A).

Overall, the **RLQ** analysis shown in Figure 2 illustrates changes of the available moth assemblage at both the taxonomical and functional

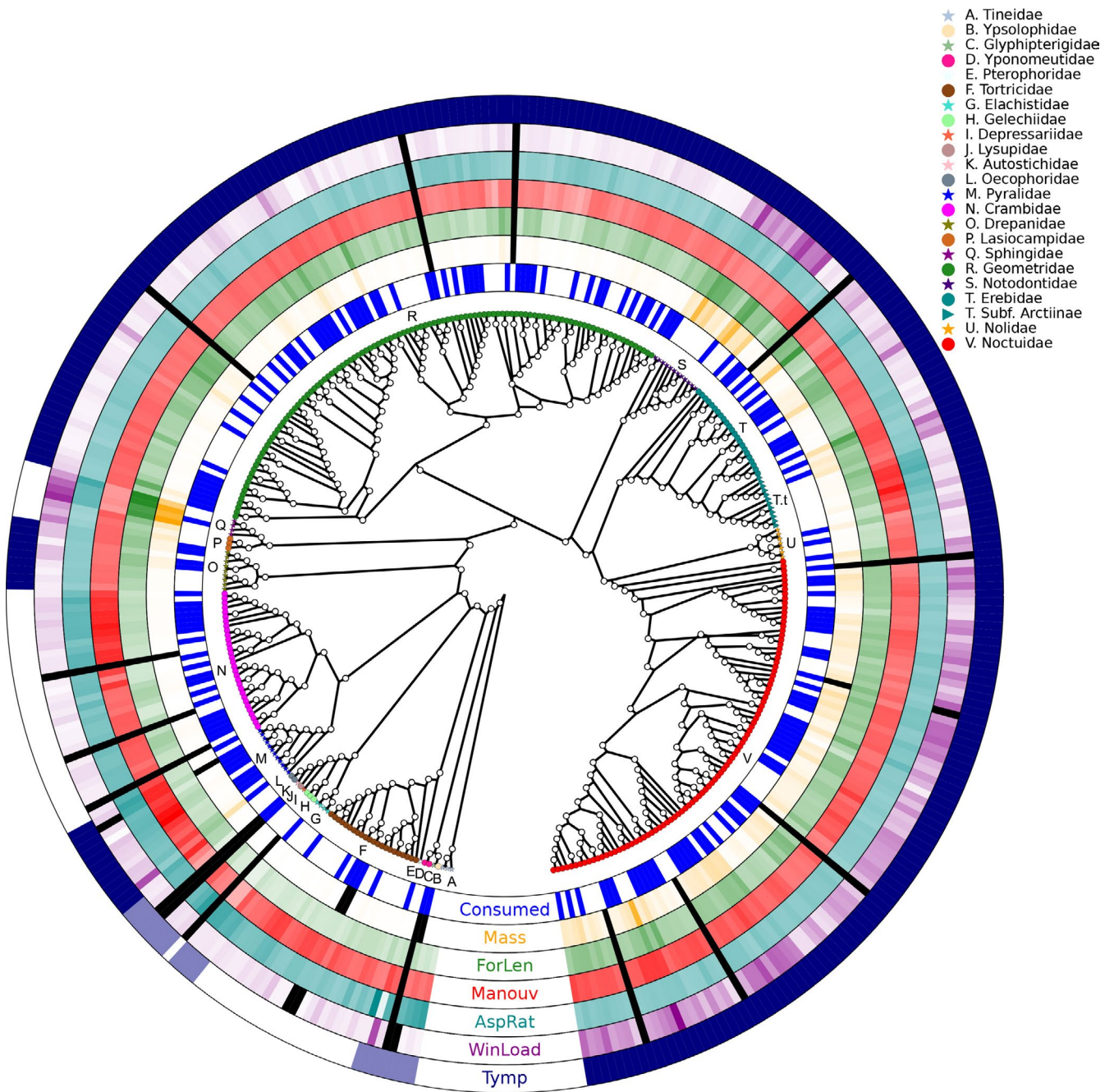


FIGURE 1 Annotated phylogenetic tree of available and consumed moth taxa by *Rhinolophus euryale*. Each coloured ring around the tree indicates a particular trait and whether it has been identified in the diet. Each ring is divided in cells, which are paired with their corresponding moth taxon and colour-toned in proportion to the value of the trait. For quantitative traits, darker colours indicate higher values. For qualitative traits, coloured cells indicate the presence of that attribute. From the inner ring outwards: (i) *Consumed*, in blue, indicates the identification of a particular taxa in the diet of the bats, (ii) *Mass* of the moths, in yellow, (iii) forewing length *ForLen*, in green, (iv) Manoeuvrability *Manouv*, in red, (v) aspect-ratio *AspRat*, in greenish blue, (vi) wing loading *WinLoad*, in purple, and (vii) tympanation *Tymp*, in dark blue (pale blue indicates no information for this trait). The nodes of different moth families are coloured and shaped by different combinations to increase the readability of the tree

level through seasons, and between locations within a single season. The first two axes of the **RLQ** ordination account for 70.12% (Axis Q1/R1, horizontal) and 27.15% (Axis Q2/R2, vertical) of the total co-inertia between seasons and moths' traits, respectively (Figure 2a,b). The length of the arrows is proportional to the variation explained by a given trait. The combination of the **RLQ** and the fourth-corner test

confirmed the above-mentioned functional spatio-temporal change of the moth assemblage: location and season of light traps were significantly associated with axes Q1 and Q2 (marked with grey and black squares in Table 2, column A); all moth traits except Forewing Length were associated to axis R1 and Wing Loading to axis R2 as well (see grey and black squares in Table 2, column B).

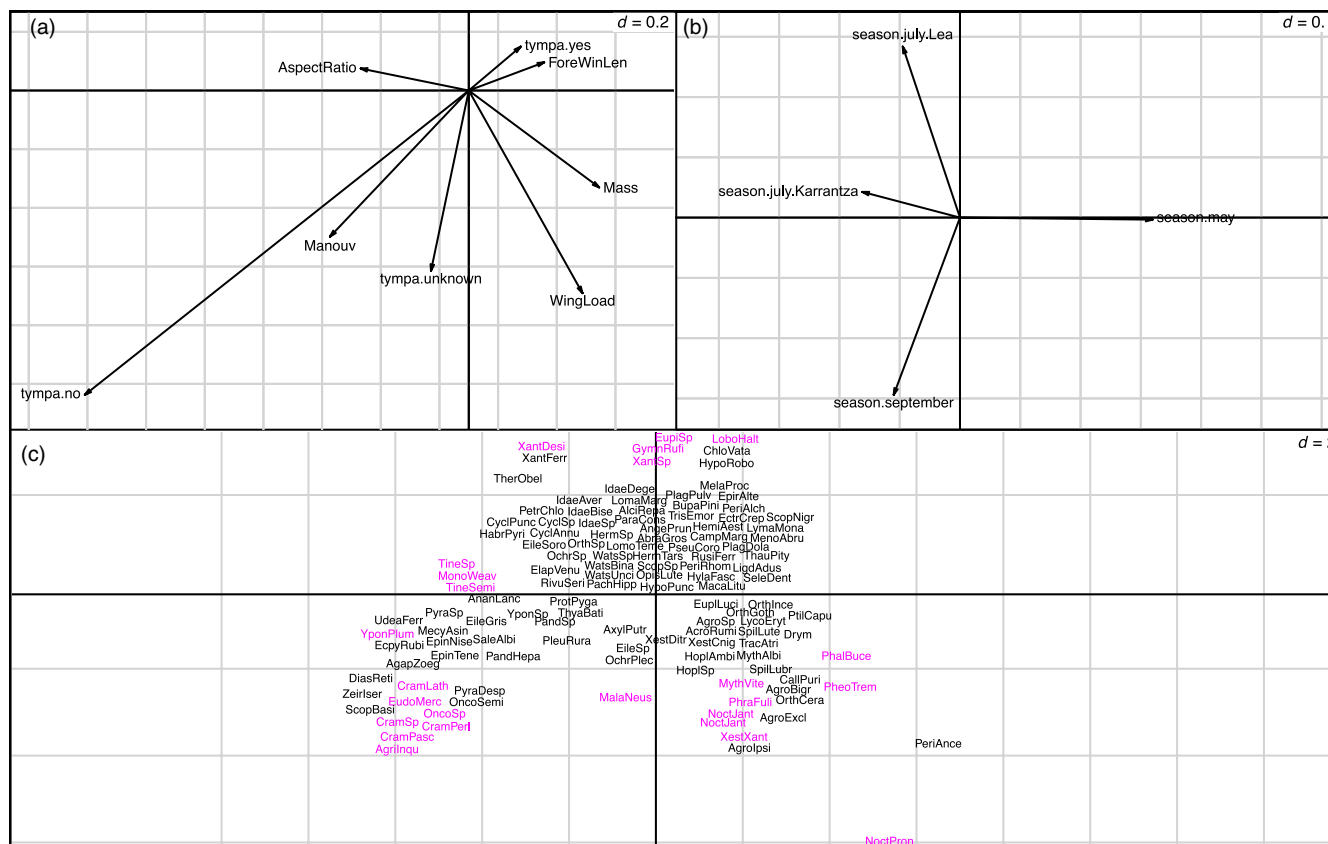


FIGURE 2 Results of RLQ analyses for the potentially available moth community: (a) coefficients for moth traits, (b) coefficients for light traps and (c) eigenvalues and scores of moth species. Panels display the first two axes only, with d-values referring to grid size. Codes for prey species are available in Supporting Information, Table S2. Monte-Carlo test: observed statistic = 0.156 (standardized observed statistic = 4.87), with p -value < 0.01. To alleviate overplotting only moth species captured in more than 2 light traps where shown in (c), where coloured labels indicate species mentioned in Results

TABLE 2 Combination of fourth-corner and RLQ results in the analysis the functional traits of moth assemblage (see Figure 2): (A) fourth-corner tests between the first two RLQ axes for moths traits (AxQ1/AxQ2) versus light trap captures; (B) fourth-corner tests between the first two RLQ axes for light trap captures (AxR1/AxR2) versus moths traits

(A) Environmental traits			(B) Moth traits		
	Axis Q1	Axis Q2		Axis R1	Axis R2
May	0.000	0.971	Tympa. No	0.021	0.199
July—Lea	0.307	0.014	Tympa. Yes	0.021	0.199
July—Karrantza	0.037	0.731	Tympa. Unknown	0.912	0.848
September	0.242	0.012	Mass	0.023	0.265
—	—	—	Forewing length	0.199	0.841
—	—	—	Manoeuvrability	0.021	0.100
—	—	—	Aspect ratio	0.049	0.848
—	—	—	Wing loading	0.045	0.021

Note: Significant ($p < 0.05$) positive associations are represented by dark grey cells and negative associations by light grey cells. p values are given in each cell.

Main emerging patterns:

- The relative positions of arrows *Mass* and *WingLoad* in the lower right quadrat of Figure 2a, and arrows representing seasonality and localities in Figure 2b, indicate a general transition from presence of larger and faster flying moths in May, to slower fliers in July, and to having both large fast fliers and small highly

manoeuvrable moths in September. Although both locations showed similar light and slow flying moths in July (see positions of arrows *season.july.Karrantza* and *season.july.Lea* in Figure 2b), moths in Karrantza were more manoeuvrable and with larger aspect-ratio than moths in Lea (as shown by the topologic correspondence of arrows *AspectRatio* and *Manouv* in Figure 2a with the arrows in Figure 2b).

- Tympanate species with higher mass and longer forewings were mostly available in May: e.g. *Phalera bucephala*, *Pheosia tremula* (colour labels in Figure 2c), arrows *Mass* and *ForeWinLen* (Figure 2a), and *season.may* (Figure 2b), all co-occur in the right side of the horizontal axis.
- Non-tympanate species with higher aspect ratio (i.e. narrower forewings), low body mass and higher manoeuvrability were found in July in Karrantza and Lea, and in Karrantza in September. This is shown by the location of micro-moths of the families Crambidae, Pyralidae, Tortricidae, Yponomeutidae and Tineidae, (colour labels in Figure 2c), arrows *AspectRatio*, *Mass* and *Manouv* (Figure 2a), and corresponding arrows (*season.July.Lea*, *season.July.Karrantza* and *season.september*) in Figure 2b.
- Species with high values of both wing loading and manoeuvrability (arrows *WingLoad* and *Manouv* in Figure 2a), such as *Mythimna* spp., *Xestia* spp., *Noctua* spp., *Phragmatobia fuliginosa* and *Malacosoma* (colour labels in Figure 2c) were mainly found in September (Figure 2b).
- Medium-sized, light and slow-flying moths, with low values of both mass and wing loading (Figure 2a) —mainly Geometridae such as *Eupithecia* and *Xanthorhoe* species, *Gymnoscelis rufifasciata*, *Lobophora halterata*, (colour labels in Figure 2c)— were mainly found in July in both valleys but especially in Lea (Figure 2b).
- Many species showed intermediate values for the measured traits and were available across different seasons and locations.

3.3 | Functional relationship between adult bats and moths

In the second **RLQ** analysis the first two axes represented 83.40% (in Axis Q1/R1 —horizontal—) and 13.72% of the total variance (in Axis Q2/R2 —vertical—), respectively. The combined **RLQ** and fourth-corner test confirmed that bat traits *breeding* and *post-breeding* were significantly associated with axis Q1 (Table 3, column A), and moth traits *Mass* and *WingLoad* with axis R1 (Table 3, column B); no other bat or moth traits were significantly associated with any axis (Table 3).

In the **RLQ** analysis the values of the moth traits *wing loading* and *mass* increase from left to right along the first axis —horizontal— (Figure 3a). Thus, prey species with higher mass and wing loading (i.e. heavier and faster fliers, located on the left side of Figure 3a) were mainly consumed in the post-breeding season (marked by the arrow *seaso.september* in Figure 3b), including noctuids such as *Xestia*, *Mythimna* and *Agrotis* spp (colour labels on the left side of Figure 3c). In contrast, lighter and slower species, such as *Idaea* spp. and *Pleuroptya ruralis*, (colour labels on the right side of Figure 3c) were mainly consumed in the breeding season (right side of Figure 3b,d).

To sum up, bats consumed lighter and slower moths in the breeding season (in July), and heavier and faster fliers in the post-breeding season, and seasonality contributed considerably more to the variation found in the diet than the other environmental and intraspecific bat variables.

3.4 | Functional relationship among juvenile bats, adult bats and moths

In the third **RLQ** analysis, the ordination overall shows a functional diet difference between adult and juvenile bats in relation to consumed prey-types (Figure 4). The two **RLQ** axes explain 94.45% (in Axis Q1/R1 —horizontal—) and 4.47% of the total variance (in Axis Q2/R2 —vertical—), respectively. The combined **RLQ** and fourth-corner analysis show that the age and body mass of bats (Table 4, column A) and the mass and wing loading of moths (Table 4, column B) were the only traits significantly associated to the first **RLQ** axis —horizontal, Q1/R1—, while none was significantly associated to the second axis —vertical, Q2/R2—.

The first axis essentially shows an uphill gradient from left to right in body mass and age of bats (shown by arrows *age.juvenile* and *age.adult* in Figure 4b), in relation to the mass and wing loading of consumed moths (corresponding arrows in Figure 4a). This indicates that adults (i.e. heavier bats; $N = 18$; marked in red in Figure 4b,d) tended to eat heavier and faster-flying moths (right side in Figure 4a): i.e. mainly noctuids such as *Pheosia tremula*, *Catocala electa*, *Scoliopteryx libatrix*, *Mythimna unipuncta*, *Xestia baja*, *X. xanthographa* (colour labels at the lower right quadrant in

(A) Bat traits	Axis Q1	Axis Q2	(B) Moth traits	Axis R1	Axis R2
Female	0.118	0.803	Tympa. No	0.139	0.248
Male	0.118	0.803	Tympa. Yes	0.139	0.248
Mass	0.978	0.835	Mass	0.000	0.725
Forewing length	0.482	0.803	Forewing length	0.139	0.139
Lea	0.482	0.553	Manoeuvrability	0.248	0.139
Karrantza	0.482	0.553	Aspect ratio	0.234	0.139
Pre-Breeding	0.553	0.094	Wing loading	0.000	0.619
Breeding	0.027	0.414	–	–	–
Post-Breeding	0.001	0.752	–	–	–

Note: Significant ($p < 0.05$) positive associations are represented by dark grey cells and negative associations by light grey cells. p values are given in each cell.

TABLE 3 Combination of fourth-corner and RLQ results in the analysis of the functional relationship between adult bats and moths (see Figure 3): (A) fourth-corner tests between the first two RLQ axes for moths traits (AxQ1/AxQ2) versus bat traits; (B) fourth-corner tests between the first two RLQ axes for bat traits (AxR1/AxR2) versus moths traits

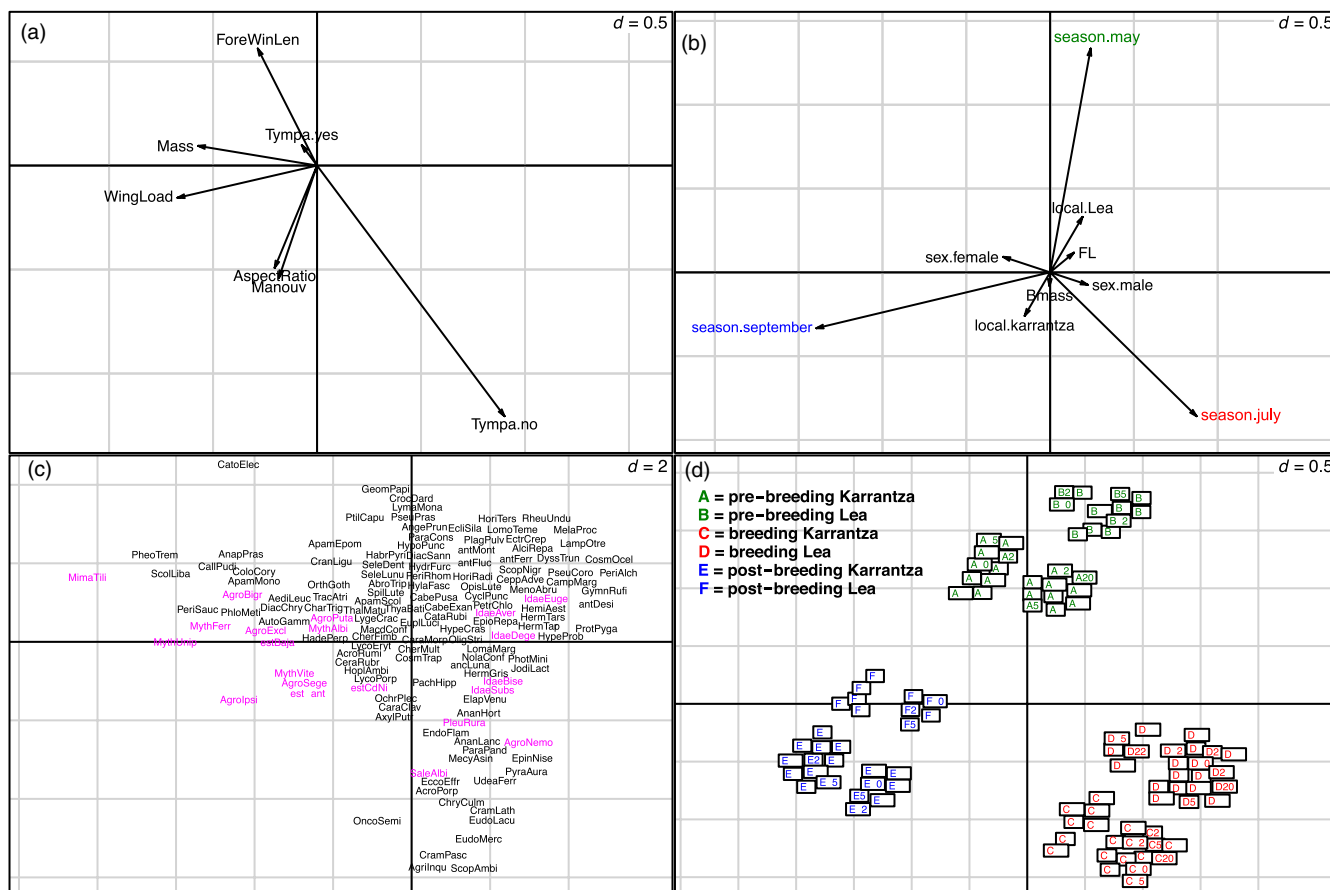


FIGURE 3 Results of RLQ analyses for all adult bats: (a) coefficients for moth traits, (b) coefficients for bat traits, (c) eigenvalues and scores of prey species, and (d) eigenvalues and scores of individual bats. Panels display the first two axes. Monte-Carlo test: observed statistic = 0.513 (standardized observed statistic = 5.62), with p -value < .01. Jittering has been applied to alleviate overplotting in panels c and d. Codes for prey species and bat individuals are available in Supporting Information, Table S2. Coloured labels in panel c indicate moth species mentioned in *Results*. Coloured labels in panel d indicate the main bat groups correlated with seasons. d -values = row scores units

Figure 4c). On the contrary, juveniles ($N = 24$; marked in green in Figure 4b,d) tended to consume very manoeuvrable moths with thinner wings (i.e. higher aspect-ratio), lighter bodies and slower flight capacity (i.e. lower wing loading; Figure 4a). These moths were mainly non-tympanate micro-moth species such as *Eccopisa effractella*, *Oncocera semirubella*, *Pleuroptya ruralis*, *Udea ferrugalis*, *Epinotia nisella* and *Agriphila inquinatella* (colour labels at the lower left quadrant in Figure 4c). We observed a single exception: a juvenile male, heavier but no larger than other juveniles, with a diet similar to that of adults (G17; Figure 4d).

To sum up, adult bats were heavier and consumed heavier, larger and faster moths than juveniles, whereas the latter ate lighter, smaller, slower and non-tympanate moths.

4 | DISCUSSION

4.1 | Functional relationship between bats and moths

To our knowledge, this is the first functional dietary approach that links profitability-related traits of prey with individual traits

of predators in a multi-species terrestrial system. This trait-based approach reveals new insights into the trophic specialization of insectivorous bats. As we predicted, our model bat showed a functionally diverse diet in relation to profitability-related traits. Additionally, we also identified some intraspecific functional diet variation mainly related to age-differences among bats. Overall, the qualitative trait-based diet results suggest that *R. euryale* is, indeed, a flexible, adaptive forager that opportunistically preys on functionally diverse moth species. Consequently, improvements to general moth habitat near horseshoe bat colonies, at the field and landscape scale (e.g. reduced hedgerow trimming and increased woodland cover, respectively), will benefit these colonies (Froidevaux, Broyles, & Jones, 2019).

4.2 | Foraging niche flexibility and prey traits: evidence for opportunistic foraging

The trait-based dietary and phylogenetic analyses show that some moth taxa were not or rarely consumed. This may be related to some specific traits which are not measured here: e.g. the fast-flying sphingids (Rydell & Lancaster, 2000), the well-defended erebids (Conner

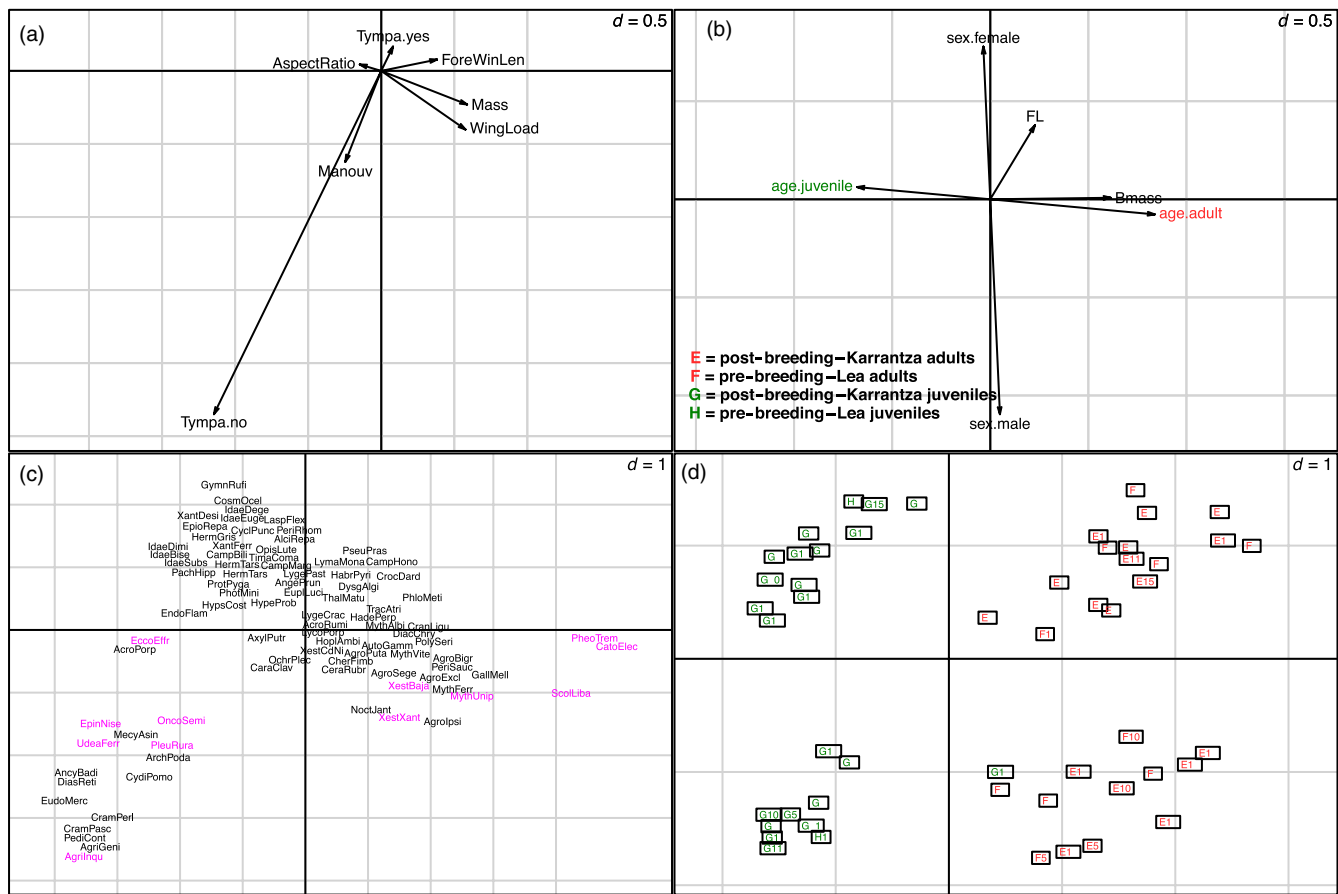


FIGURE 4 Results of RLQ analyses for adult and juvenile bats of the post-breeding season: (a) coefficients for prey traits, (b) coefficients for bat traits, (c) eigenvalues and scores of prey species, and (d) eigenvalues and scores of individual bats. Panels display the first two axes only, d -value = row scores units. Codes for prey species and bat individuals are available in Supporting Information, Table S2. Monte-Carlo test: observed statistic = 0.511 (standardized observed statistic = 6.54), with p -value < .01. Jittering has been applied to alleviate overplotting in panels c and d. Coloured labels in panel c indicate moth species mentioned in *Results*. Coloured labels in panel d indicate the main bat groups

(A) Bat traits	Axis Q1	Axis Q2	(B) Moth traits	Axis R1	Axis R2
Adult	0.000	0.983	Tympa. No	0.109	0.168
Juvenile	0.000	0.983	Tympa. Yes	0.109	0.168
Female	0.978	0.132	Mass	0.000	0.754
Male	0.978	0.132	Forewing length	0.060	0.900
Mass	0.000	0.997	Manoeuvrability	0.215	0.136
Forearm length	0.274	0.750	Aspect ratio	0.506	0.900
–	–	–	Wing loading	0.000	0.573

Note: Fourth-corner test results for the (A) bat traits and (B) moth traits in the analysis of functional relationship among juvenile bats, adult bats and moths (see Figure 4). Significant ($p < 0.05$) positive associations are represented by dark grey cells and negative associations by light grey cells. p values are given in each cell.

& Corcoran, 2012) and some tiny micro-moth families that are likely too small to be profitable enough (Jones & Rydell, 2003). Also, the analyses show that *R. euryale* hunted a wide range of species differing considerably in mass, wing loading and phylogenetic relatedness. This is obvious if we compare the extreme cases of the heavy and fast sphingid *Mimas tiliae* (colour label MimaTili at the high left quadrant

in Figure 3c) versus the tiny pyralid *Salebriopsis albicilla* (colour label SaleAlbi in Figure 3c). Nevertheless, we believe it is worth highlighting the differences in profitability detected even among those “standard” medium-sized moths such as typical noctuids, geometrids and erebids, which are the most frequently consumed taxa by *R. euryale* here, and by many other bat species elsewhere (e.g. Mata et

TABLE 4 Combination of fourth-corner and RLQ results in the analysis of the functional relationship among juvenile bats, adult bats and moths (see Figure 4): (A) fourth-corner tests between the first two RLQ axes for moths traits (AxQ1/AxQ2) versus bat traits; (B) fourth-corner tests between the first two RLQ axes for bat traits (AxR1/AxR2) versus moths traits

al., 2016; Razgour et al., 2011). For instance, the widely consumed *Xestia c-nigrum* (Noctuidae, colour label XestC-Ni in Figure 3c) and *Idaea biselata* (Geometridae, colour label IdaeBise in Figure 3c), both common and widespread species (Redondo, Gastón, & Vicente, 2015), do not differ greatly in forewing length, but differ in mass, wing loading and manoeuvrability. As such, *X. c-nigrum* should be capable of performing faster changes in flight direction than *I. biselata*. This indicates that, even to approach its staple prey of moths, *R. euryale* needs to deal with a diverse variety of moth types differing in profitability. Overall, our results indicate that adults shifted from pursuing and capturing varying moth types in the pre-breeding season, to mainly hunting slow flying moths of low energy content in the breeding season, and fast and more evasive but energetically richer moths in the post-breeding season. The concordance of these trends with those observed in the potentially available moth assemblages suggests that bats foraged opportunistically on available moth traits irrespective of phylogeny, at least in the pre-breeding and breeding seasons (but see discussion for 'Individual-level trophic niche'). However, an important factor determining profitability, which is not considered in the present study, is certainly the relative abundance of certain prey types at a certain time. This limitation should be considered when interpreting our qualitative results. Considering that the consumption of each prey is the result of a series of actions that imply detection, decision-making, pursuing and capturing (Stephens & Krebs, 1986), a slow and manoeuvrable bat like *R. euryale* is likely to approach those diverse prey items in different ways.

In fact, the observed functional plasticity in diet may be related to the flexibility of prey-capture strategies reported for *R. euryale* (Goiti et al., 2003; Russo, Jones, & Migliozi, 2002), as well as for many other congeneric moth-eating horseshoe bats (Bontadina, Schofield, & Naef-Daenzer, 2002; Goiti et al., 2003; Jones & Rayner, 1989; Neuweiler et al., 1986; Russo et al., 2002; Siemers & Ivanova, 2004). The energy costs of such hunting strategies are very different in horseshoe bats (Voigt, Schuller, Greif, & Siemers, 2010). As predicted by the foraging theory, under laboratory conditions the congeneric *R. ferrumequinum* shifted foraging behaviour from selective to opportunistic in relation to the balance between flight cost and differing availabilities in prey profitability (Koselj et al., 2011). The flexibility of horseshoe bats in prey-capture techniques, the influence of flight energy costs in foraging energetics and the observed large variation in the profitability of consumed species by *R. euryale* suggest that our model species may change foraging strategies with shifting prey types (as defined by traits related to profitability) and abundance in their environment, whilst probably balancing the cost of the foraging strategy and the energy gain. Therefore, in order to become an opportunistic fluttering-insect specialist, *R. euryale* had to adapt its hunting style to successfully consume different types of available moths. As such, although taxonomically speaking *R. euryale*, like many other bats, might be seen as a moth specialist bat, functionally speaking it could be considered a flexible adaptive generalist forager. In this context, it would be interesting to functionally assess the foraging flexibility of other bat guilds (e.g. bats specialized in particular prey-capturing techniques like gleaning), in order

to compare how different evolutionary strategies have shaped the trophic niche of insectivorous bats and their prey.

4.3 | Individual-level trophic niche: Effect of age

Generalist species might be classed in two groups: species whose individuals use a wide range of resources and species in which individuals differ in the use of resources in a shared environment (Araújo et al., 2011; Bolnick et al., 2003). The latter appears to be widespread in vertebrates (Araújo et al., 2011) although little evidence exists in bats (Cryan, Stricker, & Wunder, 2012; Johnston & Fenton, 2001; Vesterinen et al., 2016). This is likely related to two factors: the historical limitation to determine the species-level diet of insectivorous bats, and the difficulties to interpret the ecological meaning of any dietary difference based on the extensive lists of detected insect species. For instance, does a difference in prey composition translate into a functional dietary specialization? We have been able to reveal age-related functional dietary differences, a phenomenon already pointed out by other authors (Hamilton & Barclay, 1998; Salsamendi et al., 2008). Adult bats more frequently consumed heavier and faster moth species, whereas juveniles mainly consumed lighter, slower and manoeuvrable micro-moths. This result is unlikely related to any difference in the availability of moth types in relation to a differential use of habitats, as juveniles use the same foraging habitat types as adults, though closer to the roost (Goiti et al., 2006). Hamilton and Barclay (1998) pointed out that prey profitability might differ between adults and juveniles due to differing skills in pursuing, capturing and handling prey. In our study, juveniles of *R. euryale* were significantly lighter than adult bats, but not significantly smaller, suggesting that their flight would be slower than that of adults (Adams, 1996). Thus, the lack of hunting experience of juveniles together with their slower flight performance would restrict them to forage opportunistically on the smaller and slower moths, as the energetically richer but faster moths would be out of their reach. This functional difference suggests that adult bats shifted from preying upon smaller and slower moths in the breeding season to selectively forage upon larger and faster moths in the post-breeding season, despite the fact that small and slow moths were also available (i.e. consumed by juveniles). On the other hand, this age-related shift in the functional diet suggests that, in order to acquire the foraging skills needed to effectively adjust to environmentally changing prey types, young bats will likely glide through some fitness gain and/or psychomotor learning processes (Noble & Cratty, 2016), underlying the development of motor activity and control skills involved in complex locomotion tasks like aerial hawking at fast speed (Willingham, 1998). Wright, Wilkinson, and Moss (2011) showed that juveniles of *Eptesicus fuscus* learnt novel foraging skills by interacting with more experienced conspecifics. Similarly, in order to improve foraging efficiency some bats may adjust echolocation call characteristics by learning, as suggested by captive studies (reviewed in: Russo, Ancillotto, & Jones, 2018).

5 | CONCLUSIONS

Our trait-based functional approach revealed a degree of trophic niche flexibility previously unknown for a specialized moth predator: *R. euryale* hunted a wide variety of moth taxa differing in profitability across seasons and between ontogenetic stages. Moreover, local moth assemblages significantly fluctuated both taxonomically and functionally across seasons. These findings could only be achieved due to the high resolution level of DNA metabarcoding analysis for diet studies (Clare, 2014) and the development of RLQ and the fourth-corner methods to analyse the functional relationship between prey traits and environmental/predator characteristics (Dray et al., 2014). The identification of profitable, key prey types for predators is the first step to successfully assess their trophic niche. We here show that trait-based approaches provide new insights to understand the foraging ecology and evolutionary relationships of complex predator-prey systems (Green & Côté, 2014; Spitz et al., 2014), making them a powerful tool to identify and predict the spatiotemporal structure of complex predator-prey systems.

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AUTHORS' CONTRIBUTIONS

A.A.E., J.R.A., I.G. and U.G. conceived the ideas and designed methodology; A.A.E., J.R.A., I.G., U.G. and O.A. collected the data; A.A.E., G.G.B. and N.W. analysed the data; A.A.E., T.M., I.G. and J. R. A. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data used in the analyses are available in the Dryad Digital Repository: <https://doi.org/10.5061/dryad.f611bn3> (Arrizabalaga-Escudero et al., 2019).

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