1   Title: Trait-based functional dietary analysis provides a better insight into

2   the foraging ecology of bats

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26

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

50   gain and/or psychomotor learning process during which they would acquire

51   more effective foraging skills.

52   5. Our approach revealed a degree of functional flexibility in the trophic niche

53   previously unknown for an insectivorous bat. R. euryale consumed a wide variety

54   of moth taxa differing in profitability throughout seasons and between

55   ontogenetic stages. We showed the validity of trait-based approaches to gain

56   new insights in the trophic specialization of predators consuming hundreds of

57   species of prey.

58

59   Keywords: Chiroptera, DNA metabarcoding, fourth-corner, functional traits,

60   moths, niche flexibility, RLQ

61

62   Introduction

63   Evolutionary processes have determined the current ecological specialization of

64   predators on particular prey items (Ferry-Graham, Bolnick, Wainwright, &

65   Avenue, 2002). Measuring the degree of prey specialization, trophic flexibility,

66   and evolutionary relationship with prey, is pivotal to understanding predators'

67   ability to cope with changing environments and their role in ecosystem

68   functioning (Devictor et al., 2010). However, the definition of ecological

69   specialization is highly context dependent (Devictor et al., 2010). In many

70   predator-prey systems –such as for insectivores– a large range of prey species

71   are involved and prey spectra can considerably vary temporally (e.g. due to

72   changes in prey phenology; Kartzinel & Pringle, 2014), spatially (e.g. differing

73   prey assemblages across distribution areas; Marciniak et al., 2007) and in

74   relation to intraspecific variation of predators (Bolnick et al., 2003; Nifong,

75 Layman, & Silliman, 2015; Zhao, Villéger, Lek, & Cucherousset, 2014).

76 Additionally, the degree of trophic specialization is commonly assessed based on

77 the taxonomic width of consumed prey taxa through classical diversity indices,

78 which offer little information on the foraging performance of individual

79 predators. A further step in foraging ecology is to understand the functional

80 mechanisms that determine why and how species and individuals are specialized

81 on particular prey types. These functional mechanisms include morphological,

82 physiological and behavioural traits of both predator and prey (Ferry-Graham et

83 al., 2002), which influence their foraging performance and fitness.

84 The functional relationship between predator and prey is based on assumptions

85 that not all prey are equally profitable for predators, and that not all evasive or

86 defensive adaptations are equally effective against predators (Spitz, Ridoux, &

87 Brind'Amour, 2014; Stephens & Krebs, 1986). Prey profitability influences the

88 individual fitness of predators and is determined by the cost-benefit balance of

89 prey consumption (Stephens & Krebs, 1986). The relative contribution of traits

90 such as prey size, aggressiveness and evasiveness determines the profitability of

91 each prey item, while the relative contribution of each prey item to the

92 predator's diet varies with prey ecological availability as well as with niche

93 variation among predator individuals (Araújo, Bolnick, & Layman, 2011). Indeed,

94 it is known that size, sex, ontogeny and individual specialization in predators

95 may all influence the trophic niche of individuals within species. Although this

96 can have significant ecological, evolutionary and conservation implications, it has

97 been overlooked in many ecological studies (Bolnick et al., 2003). For instance,

98 sex-specific energetic requirements and morphological dimorphism are likely to

99 be the main factors of intraspecific diet variation in grey seals (Beck, Iverson,

100   Bowen, & Blanchard, 2007). Pumas show age-specific prey selection, where less

101   experienced sub-adult hunters avoid adult ungulates (Elbroch, Feltner, &

102   Quigley, 2017). Some populations of sea otters exhibit extreme individual-level

103   diet specialization, which is likely related to several factors such as spatio-

104   temporal variation of prey, matrilineal transmission of foraging preferences and

105   frequency-dependent intraspecific competition (Estes, Riedman, Staedler,

106   Tinker, & Lyon, 2003). Hence, trait-based functional dietary approaches might

107   offer new insights to understand the foraging ecology and behaviour of complex

108   predator-prey systems, as they consider the functional relationship among

109   predators and prey. Such approaches have recently been tested for marine

110   predator-prey systems (Green & Côté, 2014; Spitz et al., 2014), but they have

111   never been applied to terrestrial systems.

112   Of the many predator-prey systems, insectivorous bats and their arthropod prey

113   provide an important contribution, both taxonomically and functionally, to most

114   terrestrial ecosystems (Kunz, Braun de Torrez, Bauer, Lobova, & Fleming, 2011).

115   Molecular dietary studies are revealing that bats who forage on particular insect

116   taxa are in fact consuming hundreds of species, with variation across time

117   (Arrizabalaga-Escudero et al., 2015; Razgour et al., 2011), across space (Clare et

118   al., 2013) and across individuals (Mata et al., 2016). These studies are providing

119   the first detailed insights into the highly diverse diets of insectivorous bats.

120   However, when it comes to such diverse diets, using a purely taxonomic

121   interpretation impedes the assessment of functional specialization and selective

122   behaviour. Indeed, not all insects are equally profitable, and not all bat

123   individuals are identical. Prey profitability might differ among individuals due to

124   sex-specific energetic requirements or individual differences in prey capturing

125 skills. For instance, male and female Tadarida teniotis bats differ in the type of

126 moths they consume (Mata et al., 2016). Similarly, Hamilton and Barclay (1998)

127 reported dietary diferences between adult and juvenile Eptesicus fuscus bats and

128 suggested that flight and echolocation experience may be causing such

129 differences.

130 Insectivorous bats typically forage on hundreds of insect species, which may

131 vary considerably in mass, flight characteristics and evasive or defensive

132 behaviour, traits that likely condition their profitability for bat individuals. Do

133 individual bats change their diet according to differences in the profitability of

134 available prey? Alternatively, are they foraging on particular traits irrespective of

135 prey species diversity? To which extent are those traits phylogenetically

136 conserved among prey? It is clear that the accurate measurement of the trophic

137 specialization of predators like insectivorous bats is unlikely to be achieved from

138 mere taxonomy-oriented dietary research.

139 Here, we explore the validity of trait-based dietary approaches as a novel

140 procedure to assess the functional relationship between predators like bats and

141 their insect prey. We choose as our model species a horseshoe bat species that

142 mainly consumes flying insects such as moths (Aitor Arrizabalaga-Escudero et

143 al., 2015; Goiti, Garin, Almenar, Salsamendi, & Aihartza, 2008). This predator-

144 prey system allows us to better understand the complex ecology of insectivorous

145 bats in relation to the functionally and taxonomically highly diverse Lepidoptera.

146 Evidence suggests that the echolocation system of horseshoe bats allows them to

147 distinguish small differences among prey, based on acoustic glints produced by

148 the echoes of beating wings (Koselj, Schnitzler, & Siemers, 2011; Schnitzler,

149 1987). Besides, European moths are well represented in reference COI barcode

150 databases (e.g. BOLD Systems), a prerequisite for successful molecular

151 identification of prey species from faeces. We assess the functional relationship

152 between individual bats and their moth prey by measuring the relationship

153 between individual traits of bats and traits related to prey profitability, using

154 DNA metabarcoding, across populations and seasons. Simultaneously, we

155 analyse the potential qualitative availability of moth species for bats, and we

156 explore the relationship between moth traits and moth phylogeny. We test the

157 general hypothesis that a bat species which mainly consumes flying insects like

158 moths will show a flexible foraging diet opportunistically following

159 spatiotemporal changes in the availability of functional traits displayed by

160 potential prey. Specifically, our aims are to (i) evaluate the foraging niche

161 flexibility of bats based on the functional traits of consumed and available moths

162 as well as on the phylogenetic structure of their traits, (ii) identify and measure

163 the traits of moths consumed by bats linked to profitability, and (iii) assess the

164 intraspecific trophic variation of a horseshoe bat species in relation to prey

165 traits.

166

167 2. Material and Methods

168 2.1. Study Area and Bat Captures

169 In May, July and September 2012, we captured 126 individuals of the

170 Mediterranean horseshoe bat Rhinolophus euryale (Rhinolophidae) with a harp

171 trap at their roost in two caves located in northern Spain, in the Karrantza (43°

172 15'N, 3° 22'W) and Lea-Artibai -hereafter Lea- (43° 18'N, 2° 33'W) valleys of the

173 Basque Country (see Appendix S1 in Supporting Information for details).

174 Captured bats were held individually in clean cloth bags until they defecated.

175 Bats were sexed and aged, their body mass and forearm length measured using a

176 digital balance (Pesola, precision 0.01 g) and metal vernier calliper (Medid,

177 precision 0.05 mm), respectively and their pellets collected and frozen within 6

178 h. Bats were immediately released at the roost after handling. Capture and

179 handling protocols were approved by the Ethics Committee at the University of

180 the Basque Country (Ref. CEBA/219/2012/GARIN ATORRASAGASTI) and

181 performed under license from the Regional Council of Biscay.

182

183 2.2. DNA extraction, PCR amplification and sequencing

184 DNA extraction from bat faecal samples, PCR amplification and high throughput

185 sequencing were performed following the same procedure as described in

186 Arrizabalaga-Escudero et al. (2018). The only difference was that we performed

187 two PCR replicates for each DNA extract sample in order to discriminate

188 between PCR and/or sequencing artefacts and true biological sequences (Hope

189 et al., 2014). In short, DNA was extracted from faeces using the QIAamp DNA

190 Stool Mini kit (Qiagen, UK) and a 157 bp-long fragment of the COI barcode region

191 was subsequently PCR amplified using modified ZBJ-ArtF1c and ZBJ-ArtR2c

192 primers (Zeale, Butlin, Barker, Lees, & Jones, 2011). Each sample and its replicate

193 were tagged with a unique primer combination in order to identify them

194 bioinformatically. Sequencing was performed on the Ion Torrent (Life

195 Technologies) sequencing platform using a 318 chip.

196

197 2.3. Bioinformatics processing

198 Raw reads were quality-filtered, demultiplexed by tagged primers, filtered by

199 length and collapsed into unique sequences using Prinseq 0.20.4 (Schmieder &

200 Edwards, 2011), Fastx-toolkit 0.0.13 (http://hannonlab.cshl.edu/fa

201 stx_toolkit/index.html) and AdapterRemoval (Lindgreen, 2012) toolkits. Reads

202 with less than 15 copies were discarded and only those sequences that appeared

203 in both replicates at a 100% similarity were kept for further analyses (Hope et

204 al., 2014). Reads were clustered into Molecular Operational Taxonomic Units

205 (MOTUs) using pick_otu and uclust methods at a 97% similarity threshold in

206 QIIME (Caporaso et al., 2010). Taxonomic assignments of MOTUs were done in

207 the BOLD database following the identification criteria of Clare et al. (2013).

208 MOTUs were classified as 'unknown' when they did not match any reference

209 sequence or when they did so but the matching sequence did not belong to

210 Iberian or French species. 'Unknown' and family-level identified MOTUs were

211 excluded from further analysis.

212

213 2.4. Moths: captures and identifications

214 Moths were captured with two objectives: first, to characterize the traits of the

215 moth species identified in bats' diet; and second, to analyse the qualitative

216 functional moth availability within the home range of the studied bat

217 populations. We used 14 light-traps (6W actinic Heath traps) in each locality and

218 season. An additional set of 21 light-traps was also used in July in Karrantza in

219 order to complete the reference dataset of moth species' traits for functional

220 dietary analyses. This extra set —belonging to a different project— could not be

221 added in other seasons/locations due to resource limitations. Light-traps were

222 located as evenly as possible within a 5 km radius from each of the colony roosts,

223 which is the likely foraging range of most R. euryale individuals (Goiti et al.,

224 2006), covering the six main habitat types available: broadleaved woodlands,

225 holm oak forest, hedgerows, pine plantations, eucalyptus plantations and

226 grasslands. We spread the sampling effort per habitat type according to their

227 availability (Table 1). This sampling design was decided because, even though R.

228 euryale is highly selective regarding foraging habitats (Goiti et al. 2003 and

229 2008), a substantial part of the moths they consume therein come from other

230 habitats and localities (Arrizabalaga et al. 2015). Moths were captured at least

231 for two nights at each location/season, within an interval of two nights before

232 and after bat captures. Moth sampling was avoided in humid and cold nights to

233 avoid any weather-related moth capture bias. Light-traps were activated at dusk

234 for four hours coinciding with the first activity peak of both moths (Scalercio,

235 Infusino, & Woiwod, 2008) and bats (Goiti, Aihartza, Almenar, Salsamendi, &

236 Garin, 2006). Moths were captured alive using a clothing-bag located inside the

237 light-trap. They were frozen in the bags within 1-6 hours from capture.

238 The specimens captured by all the 75 light-traps (including the extra 21 traps in

239 July) were analysed for species identification and trait measurements (see

240 section 2.5 below). Moth specimens were identified to species level whenever

241 possible, either visually by using field guides for macro- and micro-moth

242 identification (see Appendix S1 in Supporting Information for references) or

243 through DNA barcode analysis using LEP-F1/LEP-R1 primers designed by

244 Hebert et al. (2004). Note that some moth species belonging to the same or

245 related genera were almost morphologically identical and difficult to identify at

246 the species level, in particular when colouration patterns had deteriorated.

247 These specimens were classified into the following groups: Eudonia-complex

248 (including Eudonia sp. and Scoparia sp.) and ScopCabe-complex (including

249 Scopula sp., Cabera sp. and Lomographa sp.). Unfortunately, for some taxa neither

250 morphological nor genetic identification was possible and they were excluded

251 from further analysis.

252

253 2.5. Functional traits of moths and bats

254 In order to characterize the traits of the moths consumed by R. euryale, we

255 assigned functional traits of moths captured in the field (1-5 specimens per

256 species) to the species that had been molecularly identified in the diet. We

257 assume that the traits of the measured specimens are representative of moth

258 individuals consumed by bats. We defined functional traits of prey as those

259 anatomical features that likely influence the profitability of prey moths for R.

260 euryale. Thus, we measured fresh body mass, forewing length, wing loading, and

261 forewing aspect-ratio as proxies of energy content, prey size, flight speed and

262 wing shape, respectively (in Supporting Information, see Appendix S1 for

263 description of the procedure to measure each of the moth traits, and Table S2 for

264 the traits values). We defined the trait 'manoeuvrability' as the ratio between

265 hindwing and forewing areas (Manoeuvrability = hindwing area/forewing area),

266 where higher values indicate higher manoeuvring capability (sensu Jantzen &

267 Eisner, 2008). We included the ultrasound-hearing capability in moths by taking

268 this information from published sources (see Appendix S1 in Supporting

269 Information for references). Regarding bat traits, we included season and

270 location as environmental traits, and size (measured as forearm length and body

271 mass), sex and age as intraspecific traits of bats.

272

273 2.6. Phylogenetic analysis of moths

274　It is well known that DNA barcode data cannot be used to infer phylogenetic

275　relationships at deeper taxonomic levels (Rubinoff & Holland, 2005). Thus, in

276　order to be able to use our barcode data to infer a phylogenetic hypothesis with

277　branch lengths needed for downstream analyses, we used a backbone constraint

278　approach. We built a constraint topology (with no branch lengths) based on a

279　range of recent phylogenetic hypotheses for Lepidoptera (Mitter, Davis, &

280　Cummings, 2016) for all moths identified in bat faeces or light-traps. All nodes

281　that could be confidently constrained were constrained, and this in most cases

282　down to the subfamily or tribe level. Within tribes and subfamilies many of the

283　taxa sequenced in this study have not been included previously in a phylogenetic

284　study. These relationships were thus not constrained within the respective clade.

285　The full DNA barcode dataset (downloaded from the BOLD database) was then

286　analysed using RAxML-HPC2 v. 8.2.10 (Stamatakis, 2014) on XSEDE off the

287　CIPRES server (Miller, Pfeiffer, & Schwartz, 2010), with the constraint topology

288　implemented. The resulting tree thus has branch lengths calculated based on 658

289　bp of the COI gene, and estimated phylogenetic relationships of species within

290　subfamilies/tribes.

291　We then applied the root-skewness test (a Monte-Carlo test; with 999

292　repetitions) proposed by Pavoine, Baguette, and Bonsall (2010) to assess

293　whether the quantitative moth traits are phylogenetically conserved and to

294　identify any consumption pattern related to traits and phylogeny of moths. For

295　this purpose, we tested the null hypothesis that the observed test-statistic does

296　not differ from chance expectation (alternative hypothesis: the observed statistic

297　is less than expected by chance). We paired the moths' trait dataset with the

298　previously built phylogenetic tree and indicated the moth species identified in

299   the diet of bats using GraPhlAn (Asnicar, Weingart, Tickle, Huttenhower, &

300   Segata, 2015). For an easier visualization of the tree we used the tree without

301   branch lengths.

302

303   2.7. Statistical analyses

304   The relationships between prey and bat traits, as well as between the traits of

305   potentially available moths and environmental variables, were tested by RLQ

306   and fourth-corner analyses (Stéphane Dray et al., 2014) with the package ade4

307   (Dray & Dufour, 2007) in R (R Core Team, 2016). Applied to diet studies, RLQ

308   analysis and fourth-corner test are complementary methods to uncover how

309   predators filter certain species traits, linking a description of the predators to

310   prey species traits by measurements of prey consumption (Doledec et al., 1996;

311   Legendre et al., 1997).

312   These RLQ analyses were performed independently in three steps: first for

313   available moths across seasons and locations, second for all adult bats across

314   seasons and locations, and third for adult and juvenile bats during the post-

315   breeding season (juveniles were only present in the post-breeding season). For

316   availability analysis we used all samples mentioned in section 2.4 except for

317   Karrantza in July, where on behalf of comparability we only used 14 light-traps,

318   chosen at random out of the 35 set, evenly representing the habitat availability

319   as mentioned in section 2.4 (Table 1).

320   The RLQ analyses require three input matrices: R, L and Q. In our study, the first

321   matrix (L: n x p) includes the presence/absence of the p moth species in the diet

322   of n Rhinolophus euryale individuals. The second matrix (Q: p x s) describes the p

323   moth species of matrix L according to a set of s functional traits of moths (Table

324 5.1). The third matrix (R: m x n) describes the n R. euryale individuals according

325 to a set of m traits of bats (Table 5.1). From these three matrixes, RLQ analysis

326 makes a new matrix linking individual R. euryale traits and moth traits, and then

327 performs a PCA-like eigen-decomposition on that. Finally, we applied a fourth-

328 corner analysis directly on the results of the RLQ analysis, to summarize and test

329 the significance of the associations between the RLQ axes and the functional

330 traits of bats and moths.

331 Separate ordinations of each matrix are required prior to RLQ analysis. Thus, a

332 Correspondence Analysis (CA) was applied on the matrix L (species occurrence

333 in diet), and R (bats traits) and Q (moth traits) matrices were ordered by a Hill-

334 Smith analysis (Hill & Smith, 1976), since they contain both qualitative and

335 quantitative variables. Lastly, the three analyses were combined by the RLQ

336 analysis, which links Q (weighted by L) to R, using the function rlq. Graphical

337 outputs of the RLQ analysis were used to summarize the main relationships

338 between functional traits of bats and moths. Finally, Monte Carlo tests, based on

339 the total co-inertia of the RLQ analysis, were applied through function

340 randtest.rlq() of package ade4 to test the association between the R and Q

341 matrices.

342 The fourth-corner test was carried out following the approach suggested by Dray

343 et al., (2014) in applying the fourth-corner analysis directly on the results of the

344 RLQ analysis to summarize and test the significance of the associations between

345 the RLQ axes and the functional traits of bats and moths. We applied the

346 sequential test proposed by Ter Braak, Cormont, & Dray (2012) for better

347 control of type I errors (Model type = 6), we adjusted P-values by the Benjamini-

348 Hochberg (1995) correction, and the number of permutations was elevated to

349   9,999 using the function fourthcorner.rlq. The significance level of the test was

350   set at P<0.05. All RLQ matrixes are available in Dryad (doi XXXXXX).

351

352   3. Results

353   3.1. Molecular analysis of diet

354   A total of 315 MOTUs were obtained from the faecal samples of 126 R. euryale

355   individuals. We identified 63.5% (i.e. 200) of the MOTUs to species or genus level

356   and 2.2% to family level. The remaining 34.3% were classified as 'unknown'.

357   Lepidopterans accounted for 91% (i.e. 182) of the MOTUs identified to species or

358   genus level. The remaining MOTUs belonged to Diptera (5.0%), Neuroptera

359   (3.0%), Hymenoptera (0.5%) and Psocoptera (0.5%). After collapsing MOTUs by

360   identified taxa, we obtained a total of 168 lepidopteran taxa belonging to 16

361   families. The functional traits of 69.6% of those taxa were measured, completely

362   or partially, from individuals captured in the study area, 19.1% from specimens

363   at the Natural Science Museum of Araba (the Basque Country), 5.3% from BOLD

364   system scaled pictures and 3.6% from Thomas Merckx's own collected

365   specimens. We could not get data of traits of the remaining 2.4%. For further

366   analysis we only considered those taxa for which all the defined functional traits

367   were measured: 137 taxa from 12 families.

368

369   3.2. Moths: species, phylogeny and functional traits

370   We characterized moth availability in Karrantza valley from May to September,

371   and in Lea valley only in July, collecting a total of 2,873 moth specimens

372   belonging to at least 308 taxa of 18 families. We completely characterized the

373   traits of 290 species.

374 With respect to moth functional traits (Fig. 1), we found that mass, forewing

375 length, manoeuvrability, aspect-ratio and wing loading are phylogenetically

376 conserved (observed statistic-value = 0.207; expected statistic-value = 0.501; p-

377 value < 0.001), i.e. related species were more similar in measured traits than

378 expected just by chance. For instance, noctuid moths are in general characterized

379 by being heavier, faster and more manoeuvrable than geometrids, but not

380 smaller in terms of forewing length. These combinations of traits vary among

381 families. On the other hand, bats consumed moths with different trait

382 combinations and did not appear to prey exclusively on particular phylogenetic

383 groups. Nonetheless, some families appear to be either underrepresented or

384 absent in the diet of R. euryale: the macro-moth families Lasiocampidae (P in Fig.

385 1), Notodontidae (S), Sphingidae (Q), the subfamily Arctiinae (Erebidae) (T) and

386 the micro-moth families Oecophoridae (L), Gelechiidae (H), Elachistidae (G),

387 Depresaridae (I), Yponomeutidae (D) and Tineidae (A).

388 Overall, the RLQ analysis shown in Figure 2 illustrates changes of the available

389 moth assemblage at both the taxonomical and functional level through seasons,

390 and between locations within a single season. The first two axes of the RLQ

391 ordination account for 70.12% (Axis Q1/R1, horizontal) and 27.15% (Axis

392 Q2/R2, vertical) of the total co-inertia between seasons and moths' traits,

393 respectively (Figs. 2a and 2b). The length of the arrows is proportional to the

394 variation explained by a given trait. The combination of the RLQ and the fourth-

395 corner test confirmed the above-mentioned functional spatio-temporal change of

396 the moth assemblage: location and season of light-traps were significantly

397 associated with axes Q1 and Q2 (marked with grey and black squares in Table 2,

398 column A); all moth traits except Forewing Length were associated to axis R1

399 and Wing Loading to axis R2 as well (see grey and black squares in Table 2,
400 column B).

401 Main emerging patterns:

402 • The relative positions of arrows Mass and WingLoad in the lower right
403 quadrat of Fig. 2a, and arrows representing seasonality and localities in
404 Fig. 2b, indicate a general transition from presence of larger and faster
405 flying moths in May, to slower fliers in July, and to having both large fast
406 fliers and small highly manoeuvrable moths in September. Although both
407 locations showed similar light and slow flying moths in July (see positions
408 of arrows season.july.Karrantza and season.july.Lea in Fig. 2b), moths in
409 Karrantza were more manoeuvrable and with larger aspect-ratio than
410 moths in Lea (as shown by the topologic correspondence of arrows
411 AspectRatio and Maneuv in Fig. 2b with the arrows in Fig 2b).

412 • Tympanate species with higher mass and longer forewings were mostly
413 available in May: e.g. Phalera bucephala, Pheosia tremula (colour labels in
414 Fig. 2c), arrows Mass and ForeWinLen (Fig. 2a), and season.may (Fig. 2b),
415 all co-occur in the right side of the horizontal axis.

416 • Non-tympanate species with higher aspect ratio (i.e. narrower forewings),
417 low body mass and higher manoeuvrability were found in July in
418 Karrantza and Lea, and in Karrantza in September. This is shown by the
419 location of micro-moths of the families Crambidae, Pyralidae, Tortricidae,
420 Yponomeutidae and Tineidae, (colour labels in Fig. 2c), arrows
421 AspectRatio, Mass and Maneuv (Fig. 2a), and corresponding arrows
422 (season.July.Lea, season.July.Karrantza and season.september) in Fig. 2b.

423 • Species with high values of both wing loading and manoeuvrability
424   (arrows WingLoad and Manouv in Fig. 2a), such as Mythimna spp., Xestia
425   spp., Noctua spp., Phragmatobia fuliginosa and Malacosoma (colour labels
426   in Fig. 2c) were mainly found in September (Fig. 2b).

427 • Medium-sized, light and slow-flying moths, with low values of both mass
428   and wing loading (Fig. 2a) —mainly Geometridae such as Eupithecia and
429   Xanthorhoe species, Gymnoscelis rufifasciata, Lobophora halterata, (colour
430   labels in Fig. 2c)— were mainly found in July in both valleys but especially
431   in Lea (Fig. 2b).

432 • Many species showed intermediate values for the measured traits and
433   were available across different seasons and locations.

434

435 3.3. Functional relationship between adult bats and moths

436 In the second RLQ analysis the first two axes represented 83.40% (in Axis Q1/R1

437 —horizontal—) and 13.72% of the total variance (in Axis Q2/R2 —vertical—),

438 respectively. The combined RLQ and fourth-corner test confirmed that bat traits

439 breeding and post-breeding were significantly associated with axis Q1 (Table 3,

440 column A), and moth traits Mass and WingLoading with axis R1 (Table 3, column

441 B); no other bat or moth traits were significantly associated with any axis (Table

442 3).

443 In the RLQ analysis the values of the moth traits wing loading and mass increase

444 from left to right along the first axis —horizontal— (Fig. 3a). Thus, prey species

445 with higher mass and wing loading (i.e. heavier and faster fliers, located on the

446 left side of Fig. 3a) were mainly consumed in the post-breeding season (marked

447 by the arrow seaso.september in Fig. 3b), including noctuids such as Xestia,

448 Mythimna and Agrotis spp (colour labels on the left side of Fig. 3c). In contrast,

449 lighter and slower species, such as Idaea spp. and Pleuroptya ruralis, (colour

450 labels on the right side of Fig. 3c) were mainly consumed in the breeding season

451 (right side of Fig. 3b and 3d).

452 To sum up, bats consumed lighter and slower moths in the breeding season (in

453 July), and heavier and faster fliers in the post-breeding season, and seasonality

454 contributed considerably more to the variation found in the diet than the other

455 environmental and intraspecific bat variables.

456

457 3.4. Functional relationship among juvenile bats, adult bats and moths

458 In the third RLQ analysis, the ordination overall shows a functional diet

459 difference between adult and juvenile bats in relation to consumed prey-types

460 (Fig. 4). The two RLQ axes explain 94.45% (in Axis Q1/R1 —horizontal—) and

461 4.47% of the total variance (in Axis Q2/R2 —vertical—), respectively. The

462 combined RLQ and fourth-corner analysis show that the age and body mass of

463 bats (Table 4, column A) and the mass and wing loading of moths (Table 4,

464 column B) were the only traits significantly associated to the first RLQ axis —

465 horizontal, Q1/R1—, while none was significantly associated to the second axis

466 —vertical, Q2/R2—.

467 The first axis essentially shows an uphill gradient from left to right in body mass

468 and age of bats (shown by arrows age.juvenile and age.adult in Fig. 4b), in

469 relation to the mass and wing loading of consumed moths (corresponding

470 arrows in Fig. 4a). This indicates that adults (i.e. heavier bats; N = 18; marked in

471 red in Figs. 4b and 4d) tended to eat heavier and faster-flying moths (right side

472 in Fig. 4a): i.e. mainly noctuids such as Pheosia tremula, Catocala electa,

473   Scoliopteryx libatrix, Mythimna unipuncta, Xestia baja, X. xanthographa (colour

474   labels at the lower right quadrant in Fig. 4c). On the contrary, juveniles (N = 24;

475   marked in green in Figs. 4b and 4d) tended to consume very manoeuvrable

476   moths with thinner wings (i.e. higher aspect-ratio), lighter bodies and slower

477   flight capacity (i.e. lower wing loading) (Fig. 4a). These moths were mainly non-

478   tympanate micro-moth species such as Eccopisa effractella, Oncocera semirubella,

479   Pleuroptya ruralis, Udea ferrugalis, Epinotia nisella and Agriphila inquinatella

480   (colour labels at the lower left quadrant in Fig. 4c). We observed a single

481   exception: a juvenile male, heavier but no larger than other juveniles, with a diet

482   similar to that of adults (G17; Fig. 4d).

483   To sum up, adult bats were heavier and consumed heavier, larger and faster

484   moths than juveniles, whereas the latter ate lighter, smaller, slower and non-

485   tympanate moths.

486

487   4. Discussion

488   4.1. Functional relationship between bats and moths

489   To our knowledge, this is the first functional dietary approach that links

490   profitability-related traits of prey with individual traits of predators in a multi-

491   species terrestrial system. This trait-based approach reveals new insights into

492   the trophic specialization of insectivorous bats. As we predicted, our model bat

493   showed a functionally diverse diet in relation to profitability-related traits.

494   Additionally, we also identified some intraspecific functional diet variation

495   mainly related to age-differences among bats. Overall, the qualitative trait-based

496   diet results suggest that R. euryale is, indeed, a flexible, adaptive forager that

497   opportunistically preys on functionally diverse moth species. Consequently,

498   improvements to general moth habitat near horseshoe bat colonies, at the field

499   and landscape scale (e.g. reduced hedgerow trimming and increased woodland

500   cover, respectively), will benefit these colonies (Froidevaux, Broyles, & Jones,

501   2019).

502

503   4.2. Foraging niche flexibility and prey traits: evidence for opportunistic foraging

504   The trait-based dietary and phylogenetic analyses show that some moth taxa

505   were not or rarely consumed. This may be related to some specific traits which

506   are not measured here: e.g. the fast-flying sphingids (Rydell & Lancaster, 2000),

507   the well-defended erebids (Conner & Corcoran, 2012) and some tiny micro-moth

508   families that are likely too small to be profitable enough (Jones & Rydell, 2003).

509   Also, the analyses show that R. euryale hunted a wide range of species differing

510   considerably in mass, wing loading and phylogenetic relatedness. This is obvious

511   if we compare the extreme cases of the heavy and fast sphingid Mimas tiliae

512   (colour label MimaTili at the high left quadrant in Fig. 3c) versus the tiny pyralid

513   Salebriopsis albicilla (colour label SaleAlbi in Fig. 3c). Nevertheless, we believe it

514   is worth highlighting the differences in profitability detected even among those

515   'standard' medium-sized moths such as typical noctuids, geometrids and erebids,

516   which are the most frequently consumed taxa by R. euryale here, and by many

517   other bat species elsewhere (e.g. Razgour et al. 2011; Mata et al. 2016). For

518   instance, the widely consumed Xestia c-nigrum (Noctuidae, colour label XestC-Ni

519   in Fig. 3c) and Idaea biselata (Geometridae, colour label IdaeBise in Fig. 3c), both

520   common and widespread species (Redondo, Gastón, & Vicente, 2015), do not

521   differ greatly in forewing length, but differ in mass, wing loading and

522   manoeuvrability. As such, X. c-nigrum should be capable of performing faster

523 changes in flight direction than I. biselata. This indicates that, even to approach

524 its staple prey of moths, R. euryale needs to deal with a diverse variety of moth

525 types differing in profitability. Overall, our results indicate that adults shifted

526 from pursuing and capturing varying moth types in the pre-breeding season, to

527 mainly hunting slow flying moths of low energy content in the breeding season,

528 and fast and more evasive but energetically richer moths in the post-breeding

529 season. The concordance of these trends with those observed in the potentially

530 available moth assemblages suggests that bats foraged opportunistically on

531 available moth traits irrespective of phylogeny, at least in the pre-breeding and

532 breeding seasons (but see discussion for 'Individual-level trophic niche').

533 However, an important factor determining profitability, which is not considered

534 in the present study, is certainly the relative abundance of certain prey types at a

535 certain time. This limitation should be considered when interpreting our

536 qualitative results. Considering that the consumption of each prey is the result of

537 a series of actions that imply detection, decision-making, pursuing and capturing

538 (Stephens & Krebs, 1986), a slow and manoeuvrable bat like R. euryale is likely to

539 approach those diverse prey items in different ways.

540 In fact, the observed functional plasticity in diet may be related to the flexibility

541 of prey-capture strategies reported for R. euryale (Goiti, Aihartza, Garin, &

542 Zabala, 2003; Danilo Russo, Jones, & Migliozzi, 2002), as well as for many other

543 congeneric moth-eating horseshoe bats (Bontadina, Schofield, & Naef-Daenzer,

544 2002; Goiti et al., 2003; Gareth Jones & Rayner, 1989; Neuweiler et al., 1986;

545 Danilo Russo et al., 2002; Siemers & Ivanova, 2004). The energy costs of such

546 hunting strategies are very different in horseshoe bats (Voigt, Schuller, Greif, &

547 Siemers, 2010). As predicted by the foraging theory, under laboratory conditions

548   the congeneric R. ferrumequinum shifted foraging behaviour from selective to

549   opportunistic in relation to the balance between flight cost and differing

550   availabilities in prey profitability (Koselj et al., 2011). The flexibility of horseshoe

551   bats in prey-capture techniques, the influence of flight energy costs in foraging

552   energetics and the observed large variation in the profitability of consumed

553   species by R. euryale suggest that our model species may shift foraging strategies

554   with shifting prey types (as defined by traits related to profitability) and

555   abundance in their environment, whilst probably balancing the cost of the

556   foraging strategy and the energy gain. Therefore, in order to become an

557   opportunistic fluttering-insect specialist, R. euryale had to adapt its hunting style

558   to successfully consume different types of available moths. As such, although

559   taxonomically speaking R. euryale, like many other bats, might be seen as a moth

560   specialist bat, functionally speaking it could be considered a flexible adaptive

561   generalist forager. In this context, it would be interesting to functionally assess

562   the foraging flexibility of other bat guilds (e.g. bats specialized in particular prey-

563   capturing techniques like gleaning), in order to compare how different

564   evolutionary strategies have shaped the trophic niche of insectivorous bats and

565   their prey.

566

567   4.3. Individual-level trophic niche: effect of age

568   Generalist species might be classed in two groups: species whose individuals use

569   a wide range of resources and species in which individuals differ in the use of

570   resources in a shared environment (Araújo et al., 2011; Bolnick et al., 2003). The

571   latter appears to be widespread in vertebrates (Araújo et al., 2011) although

572   little evidence exists in bats (Cryan, Stricker, & Wunder, 2012; Johnston &

573 Fenton, 2001; Vesterinen et al., 2016). This is likely related to two factors: the

574 historical limitation to determine the species-level diet of insectivorous bats, and

575 the difficulties to interpret the ecological meaning of any dietary difference

576 based on the extensive lists of detected insect species. For instance, does a

577 difference in prey composition translate into a functional dietary specialization?

578 We have been able to reveal age-related functional dietary differences, a

579 phenomenon already pointed out by other authors (Hamilton & Barclay, 1998;

580 Salsamendi et al., 2008). Adult bats more frequently consumed heavier and

581 faster moth species, whereas juveniles mainly consumed lighter, slower and

582 manoeuvrable micro-moths. This result is unlikely related to any difference in

583 the availability of moth types in relation to a differential use of habitats, as

584 juveniles use the same foraging habitat types as adults, though closer to the roost

585 (Goiti et al., 2006). Hamilton and Barclay (1998) pointed out that prey

586 profitability might differ between adults and juveniles due to differing skills in

587 pursuing, capturing and handling prey. In our study, juveniles of R. euryale were

588 significantly lighter than adult bats, but not significantly smaller, suggesting that

589 their flight would be slower than that of adults (Adams, 1996). Thus, the lack of

590 hunting experience of juveniles together with their slower flight performance

591 would restrict them to forage opportunistically on the smaller and slower moths,

592 as the energetically richer but faster moths would be out of their reach. This

593 functional difference suggests that adult bats shifted from preying upon smaller

594 and slower moths in the breeding season to selectively forage upon larger and

595 faster moths in the post-breeding season, despite the fact that small and slow

596 moths were also available (i.e. consumed by juveniles). On the other hand, this

597 age-related shift in the functional diet suggests that, in order to acquire the

598   foraging skills needed to effectively adjust to environmentally changing prey

599   types, wild young bats will likely glide through some fitness gain and/or

600   psychomotor learning processes (Noble & Cratty, 2016), underlying the

601   development of motor activity and control skills involved in complex locomotion

602   tasks like aerial hawking at fast speed (Willingham, 1998). Wright, Wilkinson &

603   Moss (2011) showed that juveniles of Eptesicus fuscus learnt novel foraging skills

604   by interacting with more experienced conspecifics. Similarly, in order to improve

605   foraging efficiency some bats may adjust echolocation call characteristics by

606   learning, as suggested by captive studies (reviewed in: Russo, Ancillotto, & Jones,

607   2018).

608

609   5. Conclusions

610   Our trait-based functional approach revealed a degree of trophic niche flexibility

611   previously unknown for a specialized moth predator: R. euryale hunted a wide

612   variety of moth taxa differing in profitability across seasons and between

613   ontogenetic stages. Moreover, local moth assemblages significantly fluctuated

614   both taxonomically and functionally across seasons. These findings could only be

615   achieved due to the high resolution level of DNA metabarcoding analysis for diet

616   studies (Clare, 2014) and the development of RLQ and the fourth-corner

617   methods to analyse the functional relationship between prey traits and

618   environmental/predator characteristics (Stéphane Dray et al., 2014). The

619   identification of profitable, key prey types for predators is the first step to

620   successfully assess their trophic niche. We here show that trait-based

621   approaches provide new insights to understand the foraging ecology and

622   evolutionary relationships of complex predator-prey systems (Green & Cote

623 2014; Spitz et al. 2014), making them a powerful tool to identify and predict the

624 spatiotemporal structure of complex predator-prey systems.

625

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640

641 Data accessibility

642 Data used in the analyses will be provided in Dryad upon manuscript acceptance.

643

644 Author contributions

645 AAE, JRA, IG and UG conceived the ideas and designed methodology; AAE, JRA, IG,

646 UG and OA collected the data; AAE, GGB and NW analysed the data; AAE, TM and

647   IG led the writing of the manuscript. All authors contributed critically to the

648   drafts and gave final approval for publication.

649

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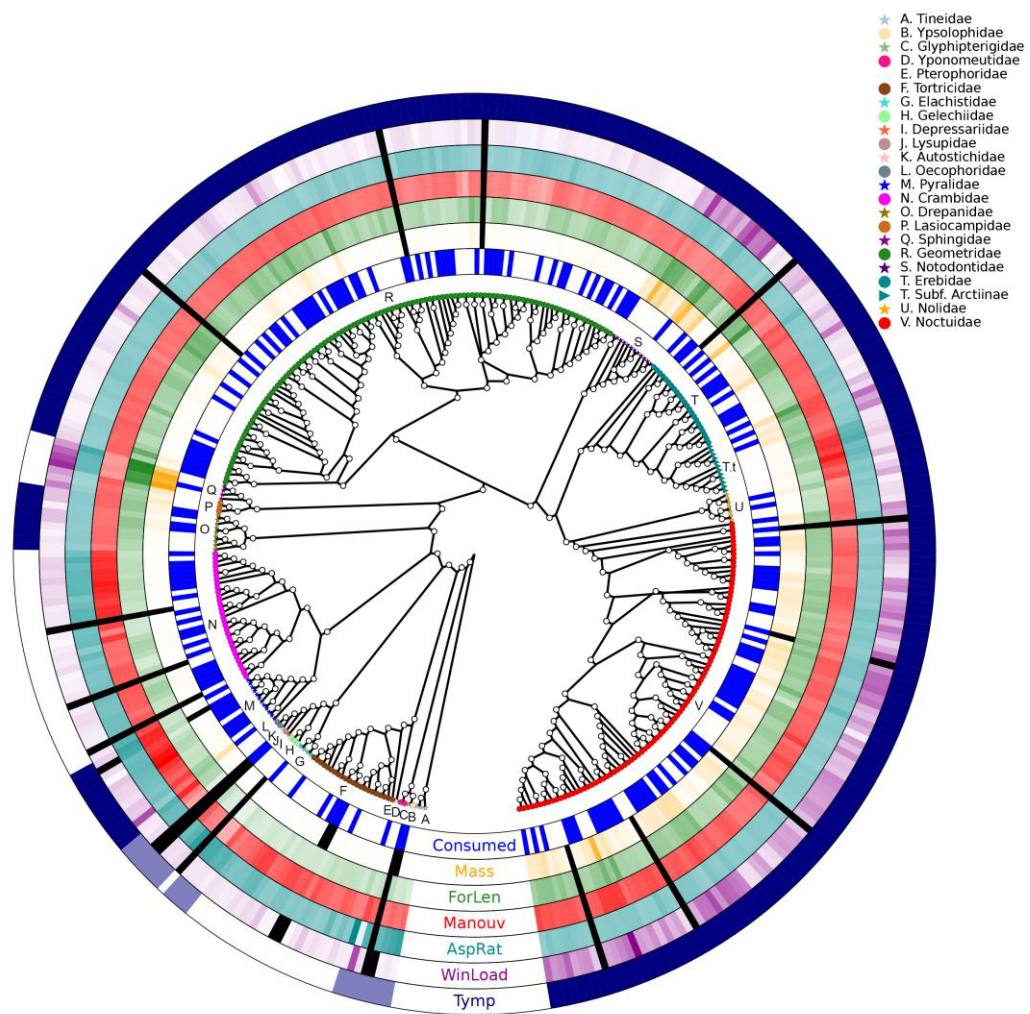
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Figure 1. Annotated phylogenetic tree of available and consumed moth taxa by R. euryale. Each coloured ring around the tree indicates a particular trait and whether it has been identified in the diet. Each ring is divided in cells, which are paired 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

892 information for this trait). The nodes of different moth families are coloured and

893 shaped by different combinations to increase the readability of the tree.

894

895

896 Figure 2. Results of RLQ analyses for the potentially available moth community:

897 a) coefficients for moth traits, b) coefficients for light-traps and c) eigenvalues

898 and scores of moth species. Panels display the first two axes only, with d-values

899 referring to grid size. Codes for prey species are available in Supporting

900 Information, Table S2. Monte-Carlo test: observed statistic = 0.156 (standardized

901 observed statistic = 4.87), with p-value < 0.01. To alleviate overplotting only

902 moth species captured in more than 2 light-traps where shown in c), where

903 coloured labels indicate species mentioned in Results.

904

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 < 0.01. 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.
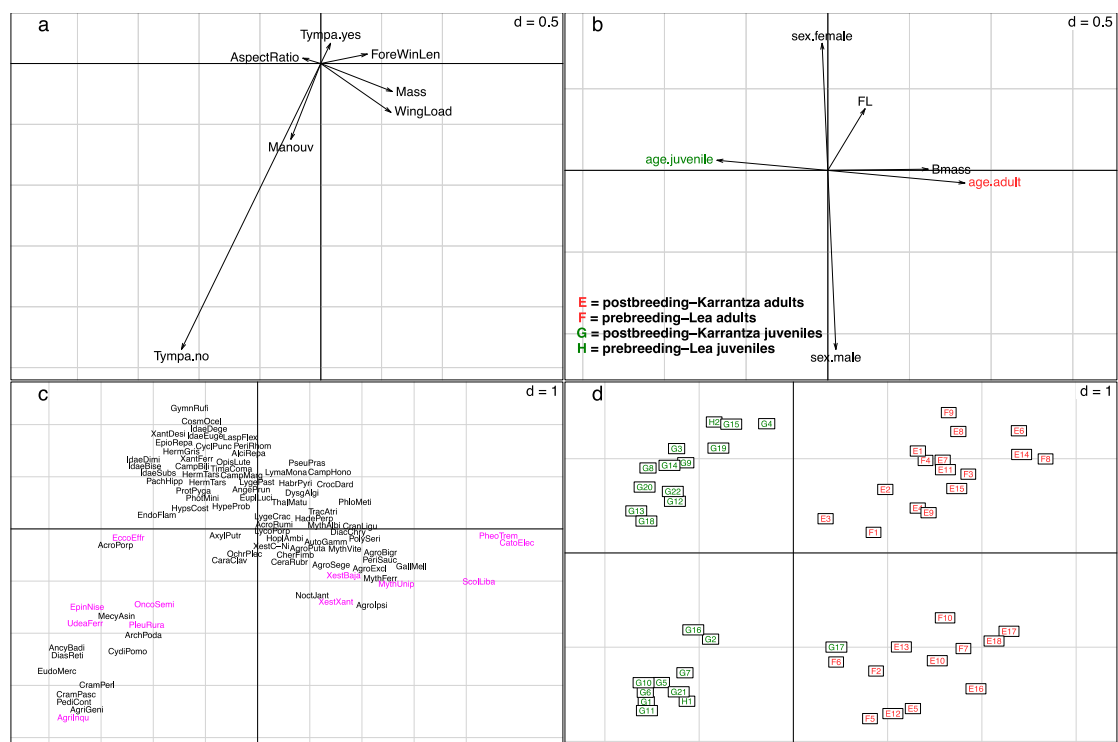
Figure 4. Results of RLQ analyses for adult and juvenile bats of the post-breeding season: a) coefficients for prey traits, b) coefficients for bat traits, c) eigenvalues and scores of prey species, and d) eigenvalues and scores of individual bats. Panels display the first two axes only, d-value = row 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 < 0.01. 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.

927 Table 1. Light-trap sampling design showing the distribution by location, season
928 and habitat type: deciduous woodland (DW), hedgerow (HG), holm woodland
929 (HOLM), coniferous plantation (CON), eucalyptus plantation (EU) and meadows
930 (MEA). *In Karrantza Valley in July, an extra set of 21 light-traps was also
931 collected (shown in brackets). **In Karrantza Valley in September one light-trap
932 failed in DW and MEA habitats —indicated by 3(-1)—.

	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

933

934

935 Table 2. Fourth-corner test results for the A) environmental traits, and B) moth
936 traits in the analysis the functional traits of moth assemblage (see Fig. 2).
937 Significant (p < 0.05) positive associations are represented by dark grey cells and
938 negative associations by light grey cells. P values are given in each cell.

(A) Environmental traits	Axis Q1	Axis Q2	(B) Moth traits	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

939

940

941 Table 3. Fourth-corner test results for the A) bat traits and B) moth traits in the

942 analysis of the functional relationship between adult bats and moths (see Fig. 3).

943 Significant (p < 0.05) positive associations are represented by dark grey cells and

944 negative associations by light grey cells. P values are given in each cell.

(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	-	-	-

945

946

947 Table 4. Fourth-corner test results for the A) bat traits and B) moth traits in the

948 analysis of functional relationship among juvenile bats, adult bats and moths (see

949 Fig. 4). Significant (p < 0.05) positive associations are represented by dark grey

950 cells and negative associations by light grey cells. P values are given in each cell.

(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

951