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Prey choice in insectivorous steppe passerines: New insights from DNA metabarcoding

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ABSTRACT

Food availability plays a key role affecting almost every aspect of bird ecology, including bird population, distribution, reproduction, and behavior. Understanding the functional relationship between prey availability and insectivorous birds' diet is important in the current scenario of habitat loss and declining of insect and bird populations. We used a fecal metabarcoding approach coupled with prey availability sampling (arthropod abundance and biomass) to test for evidence of selective foraging in a shrub-steppe passerine assemblage over the 2017–2019 breeding seasons in semi-natural steppes of central Spain. The results showed that the six bird species selected specific arthropod taxa. Heteroptera, Orthoptera, Araneae, Lepidoptera and some Coleoptera families were the main selected prey taxa, while Formicidae appeared to be avoided, suggesting that shrub-steppe passerines tend to select prey items providing essential nutrients and energy during the breeding season, while poor quality ones are neglected. Our findings highlight the utility of metabarcoding dietary data to assess prey selection patterns in insectivorous passerines and provide valuable information for the development of conservation and management programs to ensure the long-term availability of crucial food resources for shrub-steppe birds and other insectivores.

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1. Introduction

Relationships between animal predators and their food resources are a major issue in ecological studies, as they are essential to understand population dynamics and adaptation (Berryman, 1992; Nachappa et al., 2011; Pettorelli et al., 2015). Although these interactions can be complex and context-dependent, considerable effort has been devoted to understanding species' dietary requirements (Symondson, 2002), which is essential when designing effective conservation strategies (Clare et al., 2014; Groom et al., 2017). In birds, quality as much as quantity of prey are known to play an important role in the fitness of individuals and, hence, in the viability of populations (Davis et al., 2005; Sorensen et al., 2009). Given that most bird species are insectivorous (Lopes et al., 2016) or, at least, feed arthropods to nestlings (White, 1985; Finke et al., 2020), global evidence of declines in arthropod abundance and biomass (Hallmann et al., 2017; Lister and Garcia, 2018; Cardoso et al., 2020; Wagner et al., 2021) has triggered alarm about food limitation, which may have serious consequences for insectivorous bird species (Bowler et al., 2019; Tallamy and Shriver, 2021; Grames et al., 2023).

Insectivorous birds are rarely pure opportunistic feeders, that is, they usually do not consume their prey in proportion to their availability in the environment (Kaspari and Joern, 1993). Instead, many insectivorous species forage selectively (Hódar, 1998; Naef-Daenzer et al., 2000; Hagar et al., 2007; McClenaghan et al., 2019; Evens et al., 2020), which implies that birds do not consume prey as encountered but tend to reject low profitability prey items (Backwell et al., 1998). Furthermore, the optimal foraging theory (Pyke et al., 1977) predicts that predators will be selective when prey is abundant, but less choosy when prey become scarce (Emlen, 1966; Hughes, 2009). Indeed, seasonal changes in prey selection have been reported in insectivorous birds, probably reflecting variations in arthropod availability and richness (McClenaghan et al., 2019; Davies et al., 2022; Stolz et al., 2023). In addition, the selective foraging behavior of birds may facilitate partitioning of available resources among coexisting species, limiting the effects of interspecific competition (Schoener, 1974; Correa and Winemiller, 2014).

Arthropods provide important nutrients for birds (carbohydrates, lipids, protein), but their proportion varies considerably among arthropod taxa, indicating that some prey groups have much higher relative nutritional quality than others (Robel et al., 1995; Arnold et al., 2010; Razeng and Watson, 2015; Reeves et al., 2021). Therefore, many studies have suggested that insectivorous birds may show dietary selectivity for specific arthropods to satisfy particular nutritional needs (Arnold et al., 2010; Eeva et al., 2010). However,

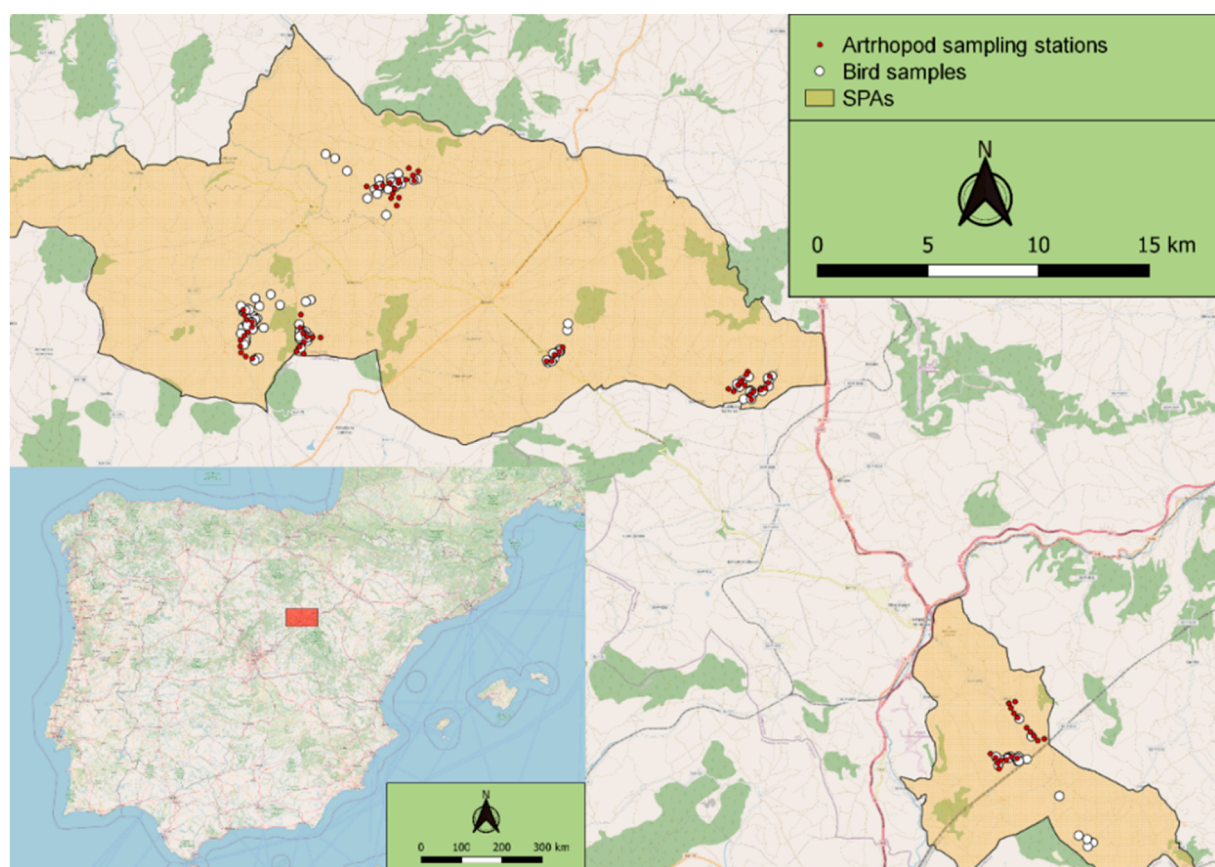


Fig. 1. Location of the study area, indicating points where fecal samples of the shrub-steppe bird species were collected (white dots) and where arthropod sampling stations were placed (red dots). Special Protection Areas (SPAs) of the European Union's Natura 2000 Network are depicted in orange (further north, 'Altos de Barahona', and further south, 'Páramo de Layna').

monitoring arthropod availability may be challenging, as arthropods are a very diverse group occupying different microhabitats and functional niches (Kremen et al., 1993), and there is a wide variety of sampling techniques for measuring arthropod availability (Santos and Fernandes, 2021). Wolda (1990) operationally defined food availability as “the abundance of potential prey items in microhabitats used by an insectivore when searching for food”, but other measures such as total arthropod biomass, relative biomass or specialized size class indices are also currently used when estimating food availability (Grames et al., 2023).

Studying the diet of birds can also be challenging, since direct observation of feeding and microscopic examination of pellets, gut contents or faeces are labor intensive and depend to a large extent on the knowledge of the person identifying the species (Pompanon et al., 2012), frequently leading to taxonomically coarse and biased results (Jedlicka et al., 2013). In addition, visual examination precludes the detection of soft-body animals that leave few or no hard remains (e.g., caterpillars; da Silva et al., 2019). DNA metabarcoding using fecal material has become increasingly popular among ecologists and ornithologists, as it provides high-resolution characterization of bird diets, allowing the identification of degraded prey even down to the species taxonomic level and the simultaneous processing of large numbers of samples with relatively low cost (Stein et al., 2014; Galan et al., 2017; Alberdi et al., 2019).

Shrub-steppe passerines, linked to flat open areas with a dominance of low ligneous vegetation (Traba et al., 2013), are small, ground-nesting birds, primarily insectivorous during the breeding season (Cramp, 1988; Hódar, 1988; de Juana and Suárez, 2020; Dunn et al., 2020; Zurdo et al., 2023). In the Iberian Peninsula, these passerines are experiencing a decline in both their population sizes and breeding ranges (López-Jiménez, 2021; Molina et al., 2022), due to the loss and fragmentation of their habitat mainly caused by agricultural intensification, abandonment of traditional sheep grazing, and changes in land use, including afforestation, new crops, and infrastructure developments (Laiolo and Tella, 2006; Traba and Morales, 2019; Traba and Pérez-Granados, 2022). In fact, the Iberian shrub-steppes are one of the most unique and threatened habitats in the European context (Sainz Ollero, 2013), where singular communities of plants (Zurdo et al., 2021), arthropods, and birds can be found (de Juana, 2005). Of particular concern is Dupont's Lark (*Chersophilus duponti*), an endangered passerine whose European distribution is restricted to the Iberian shrub-steppes (Reverter et al., 2023). Despite recent advances in determining the diet composition of shrub-steppe passerines (Zurdo et al., 2023), how their prey selection relates with the availability and quality of prey has been little explored (see Green, 1980; Hódar, 1998).

Here, we used a combination of fecal DNA metabarcoding and arthropod sampling to assess prey selection in a passerine assemblage from a shrub-steppe area in central Spain over the 2017–2019 breeding seasons. This study aimed at improving our understanding of the feeding ecology of shrub-steppes passerines. We predicted that the species studied would exhibit dietary selectivity for arthropods with higher nutritional quality. This work may provide valuable insight for the conservation and management of steppe habitats to ensure the viability of these bird species populations.

2. Methods

2.1. Study site and sampled species

Fecal and arthropod material was collected in six localities at the Special Protection Areas (SPAs) ‘Altos de Barahona’ and ‘Páramo de Layna’ (Soria province, central Spain, Fig. 1). Both are flat areas, between 1100 and 1200 m a.s.l., covered by natural steppe habitat, where cultivated fields, scattered trees and coniferous reforestation are interspersed. The landscape characteristics of the six study localities are broadly comparable, with a predominance of continental basophilic scrublands of *Genista pumila*, *G. scorpius*, *Thymus* spp., *Lavandula latifolia* and *Satureja intricata*, dry perennial grasslands, and mixed grassland-scrublands (Zurdo et al., 2021). Climate is continental Mediterranean, with a mean temperature of 10.8 °C and a mean annual rainfall of 471 mm (Aranbarri et al., 2015).

Dietary selectivity was studied for six passerine species that coexist in the study site during the breeding season: Dupont's Lark, the Eurasian Skylark *Alauda arvensis*, and the Greater Short-toed Lark *Calandrella brachydactyla* of the Alaudidae family; the Northern Wheatear *Oenanthe oenanthe* and the Western Black-eared Wheatear *Oenanthe hispanica* of the Muscicapidae family; and the Tawny Pipit *Anthus campestris* of the Motacillidae family. These steppe passerines share unique ecological and evolutionary characteristics related to their nesting habits and preference for structurally simple habitats (de Juana, 2005; Barrero et al., 2023a). They are all small, morphologically similar, ground-nesting birds subjected to a high rate of nest predation (Pérez-Granados et al., 2017). All these species are insectivorous during the breeding season, being beetles, grasshoppers and spiders the most frequently consumed prey (Zurdo et al., 2023).

2.2. Fecal sample collection

We collected feces from 259 adult individuals of the six species (81 Dupont's Lark samples; 36 Eurasian Skylark samples; 38 Tawny Pipit samples; 34 Greater Short-toed Lark samples; 30 Northern Wheatear samples; and 40 Western Black-eared Wheatear samples) captured in the six study localities across the study area during the breeding seasons (April to June) of 2017–2019. Spring-traps baited with mealworms (*Tenebrio molitor*) coupled with species-specific recordings were used to capture bird individuals. Birds were placed into individual clean cotton bags (not used more than once to prevent cross-contamination) until defecation, after which they were ringed to avoid pseudoreplication and released. Some birds defecated on the ground or stones at the spring-trap as a behavioral response to capture, being the sample collected with bleach-disinfected forceps before each use. Fecal samples were placed in individual 1.5 ml Eppendorf tubes with 98% ethanol and stored at – 20 °C until processed in the laboratory. The number of feces collected per sampling period and locality is provided in the Table S1.

2.3. Arthropod availability

We located a total of 72 field sampling stations at the six study localities to monitor arthropod abundance and biomass three times during spring (April, May, and June) in 2017, 2018 and 2019 (Fig. 1; Table S1), coinciding with the sampling period of fecal samples.

In each sampling station, terrestrial ground-dwelling arthropods were collected using three pitfall traps, placed at 5 m intervals. Pitfall traps consisted of a transparent plastic cup (230 ml, 7 cm diameter, 10 cm depth), with holes in the upper part to facilitate rain drainage. Plastic cups were buried and protected by a PVC cylinder to prevent trap collapse and filled with 175 ml of 40% ethylene glycol and a drop of soap to reduce surface tension. After seven days of activity, the three pitfall traps of each sampling station were filtered together and collected arthropods were stored in plastic tubes with 70% ethanol. Flying arthropods were also sampled at the moment of collecting pitfall traps using an entomological sweep net along the longest distance between pitfall traps (10 × 2 m band). Flying individuals were pooled with ground-dwelling arthropods and stored in the same plastic tube. This combined approach has been proven as an effective method for the capture of a broad range of terrestrial and flying arthropods (Tarjuelo et al., 2019; Gómez-Catasús et al., 2019; Reverter et al., 2021; Traba et al., 2022).

In the laboratory, we identified arthropods to the order level, except for (i) Coleoptera, for which we tried identification at least to the family level; (ii) Hemiptera, which were separated according to the former division of Heteroptera and Homoptera; and (iii) Hymenoptera, differentiated between the family Formicidae and all the other Hymenoptera. We determined abundance of each arthropod group, and measured body length (excluding legs, antennae, and other appendices) of one specimen representative of its size per taxon and pitfall using a digital caliper (± 0.01 mm) to estimate overall biomass for each arthropod group applying the specific equations from Hódar (1996), which relate weight to body length in several arthropod groups of the Mediterranean region. For a similar methodology for invertebrate biomass estimation see Traba et al. (2008); (2022), Gómez-Catasús et al. (2019) or Reverter et al. (2021). Abundance and biomass of arthropod groups per sampling station were calculated as the mean values of the pitfall traps that were active after seven days (generally the three traps, but in a few cases, some were accidentally invalidated). Arthropod availability data in terms of abundance and biomass are provided per m² and trapping day throughout the text.

2.4. Molecular lab work and bioinformatics

We extracted DNA from each fecal sample using the QIAamp® PowerFecal DNA Kit from Qiagen, following the manufacturer's instructions. Before extraction, ethanol was removed from the samples by decanting following 30 min of centrifugation and dried at 50 °C until the ethanol was vaporized. A 150-base pair (bp) target region of the 18 S rRNA gene was amplified using the eukaryotic mini-barcode miniB18S_81 described in Cabodevilla et al. (2022). Negative PCR controls were included, in which no fecal DNA was added, and processed in the next steps of the molecular analysis. A second PCR was performed to index each amplified product and attach Illumina adaptors. The amplification was performed following Cabodevilla et al. (2022). Resulting fragments were checked in Bioanalyzer before pooling in equimolar amounts. Pools were cleaned using AMPure XP beads (Beckman Coulter). The final library was sequenced in an Illumina MiSeq NGS platform using a v3 MiSeq Reagent kit, following the manufacturer's instructions. DNA extraction was performed by the Genomics and NGS Core Facility at the Centro de Biología Molecular Severo Ochoa (CBMSO, CSIC-UAM, Spain), and amplification, amplicon pooling, library preparation and sequencing by the Genomics Unit of the Fundación Parque Científico de Madrid (Spain).

To analyze sequencing results, we followed the MJOLNIR pipeline (Metabarcoding Joining Obitools and Linkage Networks In R; <https://github.com/uit-metabarcoding/MJOLNIR>; pipeline steps in Appendix S1). We quality filtered and aligned paired-end Illumina sequences using OBITools (Boyer et al., 2016) and removed chimeras using VSEARCH (Rognes et al., 2016). We clustered the sequences into molecular operational taxonomic units (MOTUs) using swarm (Mahé et al., 2015), which is based on an iterative aggregation of sequences that differ less than a given distance ($d = 1$). We assigned the resulting MOTUs to taxonomic units using ecotag (OBITools; Boyer et al., 2016) and a reference database in ecoPCR format including sequences for the metabarcoded fragment (miniB18S_81), obtained from the download of all 18S sequences from NCBI database. We finally removed pseudogenes using LULU (Frøslev et al., 2017). MOTUs were identified with the most resolved taxonomic assignment possible, and those identified to species or genus were manually confirmed using the BLAST algorithm (NCBI), as well as their presence in Spain using the databases Iberfauna (<http://iberfauna.mncn.csic.es/>) and Fauna Europaea (de Jong et al., 2014).

To clean data prior to statistical analysis, we first subtracted the maximum read count of each MOTU found in the negative PCR controls (Table S2) from all other samples in order to reduce the impact of false positives. We then excluded MOTUs not considered as potential prey items: protists, fungus, algae, vascular and nonvascular plants, mammals (mostly humans), birds, and internal parasites (phyla Nematoda and Platyhelminthes), as well as mites (orders Trombidiformes and Sarcoptiformes) and ticks (order Ixodida) because they were most likely parasites of other arthropods or of the birds themselves, and product of secondary detection (da Silva et al., 2019). We also removed MOTUs assigned to the class Collembola, due to the low probability that birds would directly prey them, given their minuscule size in Spain (generally less than 2 mm in length; Arbea and Almeida, 2022). Plant MOTUs were discarded because the study species are known to feed mainly on invertebrates during the breeding season (Billerman et al., 2020). Number of reads per sample obtained for each of the excluded non-dietary taxa is provided in Table S3 in Supplementary Material. After removing non-target taxa, we retained 1065,222 dietary reads, corresponding to 28% of the initial number of reads.

We finally removed samples with less than 100 dietary reads as they were considered to have failed (negative PCR controls and some samples of different bird species), and MOTUs representing less than 1% of the total number of dietary reads (Drake et al., 2021) to avoid incorporating false positives resulting from tag-jumping events.

2.5. Statistical analysis

Analyses were carried out using R version 4.1.2 (R Core Team, 2021). To investigate whether shrub-steppe species exhibit dietary selectivity, we used network-based null models with the R package *econullnet* (Vaughan et al., 2018), developed to test for prey selection by predators. It uses a null-modelling approach based on the hypothesis of absence of prey choice, where the frequency of consumption of prey items is merely a reflection of their availability in the field. The observed frequency of occurrence of the different prey taxa in the diet is then compared to the frequency expected based on the null model (i.e., their availability recorded in the field).

Using the function *generate_null_net*, we ran the null models based on (i) the diet data for each individual of each bird species (presence/absence data), indicating the month and the year in which the fecal sample was collected (i.e., sampling period: April, May or June 2017, 2018 or 2019), and (ii) the availability data of each arthropod taxa, that is, the abundance or biomass of each arthropod taxa identified on all sampling stations per sampling period. We performed a total of four models based on (i) abundance of all arthropod taxa, (ii) biomass of all arthropod taxa, (iii) abundance of Coleoptera families, and (iv) biomass of Coleoptera families. We ran these specific models for Coleoptera families due to their great importance in the diet of the species studied (Zurdo et al., 2023) and because they comprise a large part of the total arthropod biomass of the foraging habitat (Table 1, Fig. 2B). The models were run for 9999 iterations. Prey taxa present in the diet with a frequency significantly higher than expected by the null model indicates preferred prey, while the opposite indicates undesired prey (Vaughan et al., 2018). Results were visualized using the functions *plot_preferences* and *plot_bipartite*.

Additionally, we performed these prey selection analyses based on the relative frequencies of sequence reads of dietary taxa (i.e., relative read abundance, RRA), assuming that the number of reads generated for a particular dietary item is proportional to the relative biomass of the item consumed (Deagle et al., 2010). We run two models based on (i) biomass of all arthropod taxa and (ii) biomass of Coleoptera families. Likewise, prey taxa detected in the diet in higher quantities than expected by the null model indicates selected prey, while the opposite indicates undesired prey. To facilitate text reading, the results from RRA are provided in the [Supplementary Material](#) (Tables S11–S12 and Figs. S6–S7).

This work was approved by the Local Ethical Committee for Animal Experiments of the Universidad Autónoma de Madrid (CEI80–1468-A229).

3. Results

3.1. Diet and prey availability

After bioinformatics processing, we obtained 1017,169 diet reads from 161 fecal samples of the six shrub-steppe passerines,

Table 1

Arthropod prey availability (abundance and biomass; Mean \pm SD) estimated per each year of sampling.

	Abundance (individuals/m ² /day)			Biomass (mg/m ² /day)		
	2017	2018	2019	2017	2018	2019
Coleoptera	163.55 \pm 173.51	178.54 \pm 209.34	125.65 \pm 142.56	8196.30 \pm 9886.00	12,695.79 \pm 16,714.87	7835 \pm 10,078.30
Hymenoptera	1144.05 \pm 1660.85	215.63 \pm 443.55	328.44 \pm 794.82	2421.65 \pm 4308.86	209.71 \pm 513.80	1350.53 \pm 9628.98
Formicidae	1121.29 \pm 1655.57	209.80 \pm 441.17	323.26 \pm 794.31	2243.59 \pm 4238.71	145.22 \pm 483.61	1269.40 \pm 9631.74
Other Hymenoptera	22.76 \pm 25.47	5.83 \pm 9.91	5.19 \pm 8.82	178.06 \pm 276.76	64.50 \pm 128.38	81.12 \pm 159.68
Araneae	247.69 \pm 177.91	91.52 \pm 64.96	109.16 \pm 103.32	1473.83 \pm 1448.36	701.94 \pm 710.63	833.71 \pm 984.35
Diptera	158.54 \pm 180.43	35.18 \pm 43.92	84.36 \pm 120.59	231.85 \pm 329.14	99.45 \pm 146.96	101.74 \pm 142.05
Hemiptera	123.05 \pm 118.88	25.29 \pm 47.83	54.44 \pm 55.11	300.09 \pm 387.11	57.16 \pm 100.09	130.92 \pm 150.43
Homoptera	47.14 \pm 45.15	20.14 \pm 45.45	41.93 \pm 49.43	62.96 \pm 83.76	32.96 \pm 71.74	67.53 \pm 82.36
Heteroptera	75.91 \pm 94.89	5.14 \pm 14.47	12.52 \pm 18.74	237.14 \pm 348.98	24.20 \pm 69.82	63.39 \pm 111.70
Orthoptera	67.21 \pm 89.73	23.16 \pm 42.69	70.37 \pm 85.42	3271.54 \pm 4951.34	924.73 \pm 2105.95	1618.31 \pm 2437.72
Opiliones	35.97 \pm 62.21	17.39 \pm 25.43	17.77 \pm 31.05	243.00 \pm 433.31	87.36 \pm 141.15	87.98 \pm 147.38
Archaeognatha	14.59 \pm 25.40	29.41 \pm 46.24	15.16 \pm 23.42	88.52 \pm 258.47	318.16 \pm 493.40	144.63 \pm 233.47
Blattodea	19.57 \pm 47.51	22.37 \pm 36.96	11.78 \pm 20.44	75.58 \pm 242.94	82.20 \pm 155.62	45.53 \pm 90.42
Lepidoptera	23.25 \pm 25.42	5.93 \pm 8.17	12.21 \pm 16.03	223.83 \pm 489.04	111.68 \pm 527.22	186.23 \pm 483.47
Julida	7.19 \pm 17.68	13.99 \pm 34.26	12.52 \pm 24.49	967.86 \pm 2793.34	643.79 \pm 1933.78	510.02 \pm 1336.63
Lithobiomorpha	3.04 \pm 6.68	0.66 \pm 2.77	1.94 \pm 5.34	20.82 \pm 86.45	4.20 \pm 29.12	12.32 \pm 53.28
Mantodea	0.47 \pm 3.16	0	0.94 \pm 4.17	0.72 \pm 8.54	0	1.25 \pm 8.89
Isopoda	0.06 \pm 0.85	0.13 \pm 1.27	0.07 \pm 0.91	0.10 \pm 1.46	0.13 \pm 1.27	0.61 \pm 8.33
Pseudoscorpiones	0	0	0.13 \pm 1.28	0	0	0.61 \pm 5.84

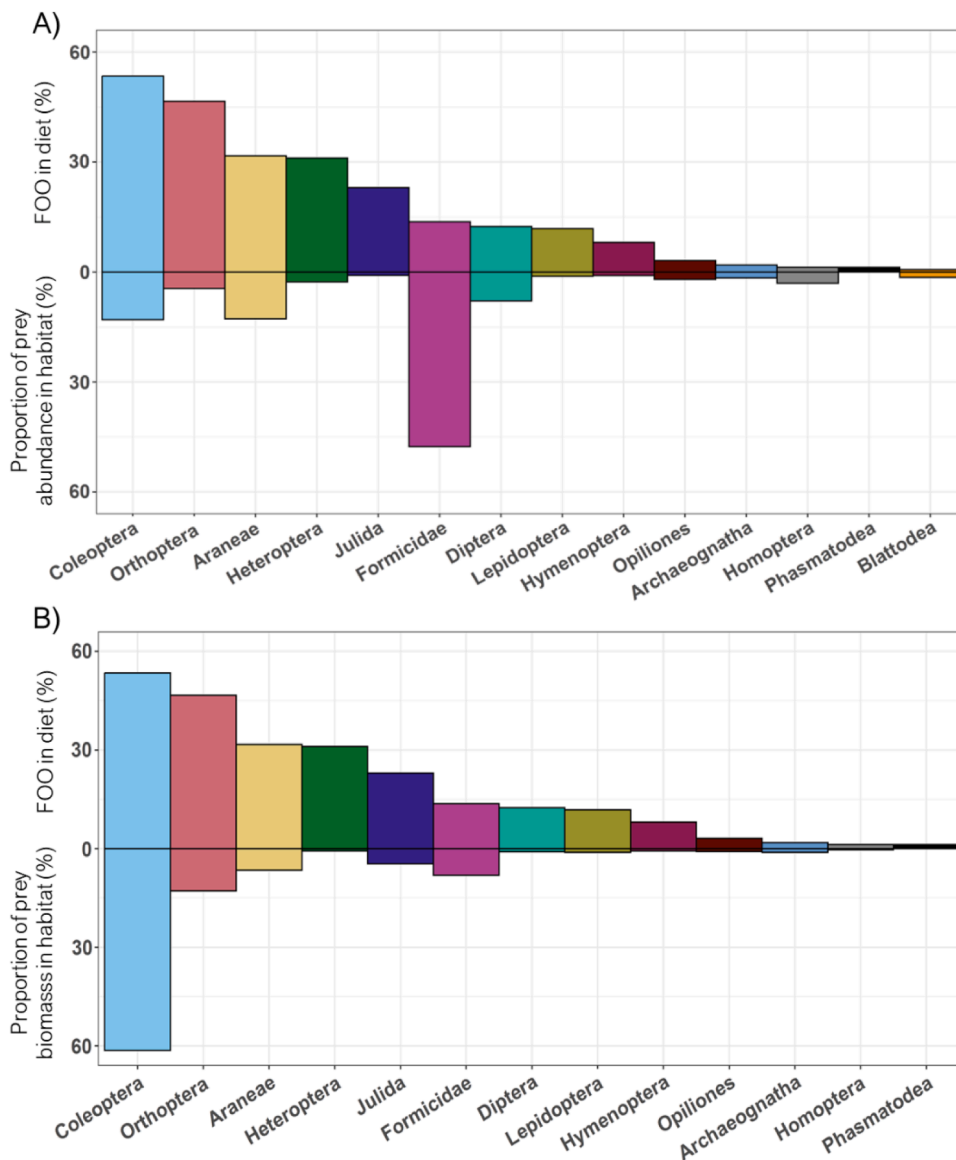


Fig. 2. Frequency of occurrence (FOO%) of the different arthropod taxa in the diet of the six shrub-steppe passerines (above the 0 line) compared to the availability of these arthropod taxa in the habitat (below the 0 line), presented as a proportion of A) the total prey abundance available in the habitat, and B) the total prey biomass available in the habitat. Each bar on the x-axis represents a different arthropod taxon. Arthropod orders with less than 1% for both FOO in diet and for the proportion of prey availability in the habitat are not depicted (i.e., Pseudoscorpiones, Isopoda, Lithobiomorpha and Mantodea for prey abundance; Pseudoscorpiones, Isopoda, Lithobiomorpha, Mantodea and Blattodea for prey biomass; Table S6).

yielding an overall success rate of 62%, although the success rate for Dupont's Lark was the lowest (44%) and that of the Western Black-eared Wheatear the highest (83%). Number of successful fecal samples after molecular analysis for each bird species is shown in [Supplementary Tables S4 and S5](#). The samples had an average of 6164 ± 3393 SD reads. We obtained 120 dietary MOTUs and identified 13 orders, 44 families, 31 genera and 17 species (Table S4). Fecal samples returned a mean of 2.99 ± 1.95 SD unique prey taxa (range = 1–11). Coleoptera was the most frequently detected order in the diet (Fig. 2A), present in 53.42% of fecal samples, followed by Orthoptera (46.58%), Araneae (31.68%), and Hemiptera (31.68%). Frequency of occurrence of each arthropod order for each bird species is provided in Table S5.

Arthropods captured in pitfall traps and sweep net were classified into 15 insect orders, arachnids, diplopods (order Julida), chilopods (order Lithobiomorpha) and terrestrial crustaceans (order Isopoda). Prey abundance per trapping day ranged from 37.17 to 14,944.41 individuals/m² (Mean \pm SD = 1203.07 ± 1394.45), while prey biomass per trapping day ranged from 0.10 to 140.53 g/m² (Mean \pm SD = 15.53 ± 16.09), with an interannual variation per arthropod group shown in Table 1. Availability data (abundance and biomass) per arthropod prey group over the three years of sampling are provided in Table S6 of the [Supplementary Material](#).

Hymenoptera, and specifically the Formicidae family (i.e., ants), contributed most to prey abundance (47.82%), but occurred infrequently in bird diets (Frequency of occurrence-FOO: 13.66%; Fig. 2A). In terms of available biomass, Coleoptera accounted for the highest proportion (61.39%) (Fig. 2B). Frequently preyed groups such as Heteroptera (FOO: 31.06%) or Julida (FOO: 22.98%) represented a much smaller proportion of available prey (abundance: 2.74% and 0.92%, respectively; biomass: 0.73% and 4.63%, respectively; Fig. 2). The order Phasmatodea was detected in the diet of birds but not collected in the arthropod samplings, while the opposite occurred for Mantodea, Lithobiomorpha and Isopoda.

3.2. Prey choice

All bird species showed dietary selectivity considering both arthropod biomass (Fig. 3 and Fig. 4) and abundance (see Fig. S1 and Fig. S2 in Supplementary Material for results using prey abundance). Indeed, we found that 33% of birds' use of prey taxa in relation to their availability was stronger or weaker than expected from the null model when prey abundance was considered (Table S7), while that proportion was 25% in the case of biomass (Table S8).

Heteropterans were consumed significantly more often than expected from their availability (abundance and biomass) in the field by all the six shrub-steppe passerines. Based on biomass, coleopterans were preyed less than expected by all species except Dupont's Lark, while based on abundance no bird species showed selection except Dupont's Lark and the Northern Wheatear, which consumed this group more than expected. Ants (Formicidae) were preyed less than expected by all bird species when considering their abundance in the habitat, but only Dupont's Lark and the Greater Short-toed Lark consumed this group less than expected from their biomass. The Greater Short-toed Lark showed significantly more consumption of orthopterans than expected from their biomass in the habitat while Dupont's Lark showed the opposite pattern. In terms of abundance, orthopterans were selected by all bird species except Dupont's Lark and the Tawny Pipit. Spiders (Araneae) were consumed more than expected from their biomass by the Tawny Pipit and the Western Black-eared Wheatear, and lepidopterans by Dupont's Lark and the Western Black-eared Wheatear based on both abundance and biomass. Millipedes (Julida) were selected by Dupont's lark and both wheatears only when their abundance was considered. Dupont's Lark, the Eurasian Skylark and the Western Black-eared Wheatear showed less consumption of homopterans than expected from their abundance in the field.

Results based on RRA of dietary taxa and arthropod biomass revealed similar prey choice patterns, but with a greater number of interactions that were weaker than expected from the null model (see Table S11 and Fig. S6 in Supplementary Material for results based on RRA).

We also found prey choice for coleopteran families: 23% and 33% of consumption by birds was stronger or weaker than expected from abundance (Table S9) and biomass (Table S10) in the habitat, respectively. Families such as Scarabaeidae, Curculionidae and Chrysomelidae were selected by most bird species, while Carabidae, Tenebrionidae, Histeridae and Meloidae were consumed less than expected from their availability in the field by some of the shrub-steppe passerines (Fig. 5 and Fig. S3 based on biomass data; Fig. S4 and Fig. S5 based on abundance data). Based on RRA, these families were also consumed in higher quantities than expected from their biomass in the field, but the number of Coleoptera families that were consumed in lower quantities than expected was greater than based on occurrence data (see Table S12 and Fig. S7 in Supplementary Material for results based on RRA).

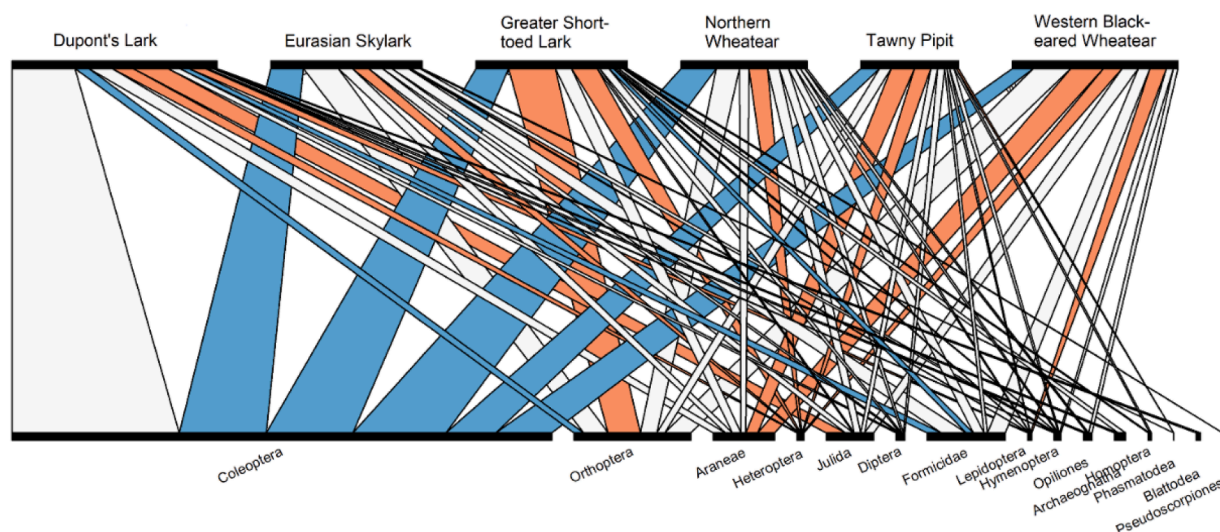


Fig. 3. Bipartite network showing the trophic interactions between shrub-steppe bird species and arthropod prey groups. White link color indicates equal dietary frequency to that expected from biomass estimated in the habitat; orange denotes higher frequency in diet than expected; and blue represents less frequent consumption than expected. Bottom box widths are proportional to the biomass of each prey group in the environment, while top box widths are proportional to the sample size for each passerine species.

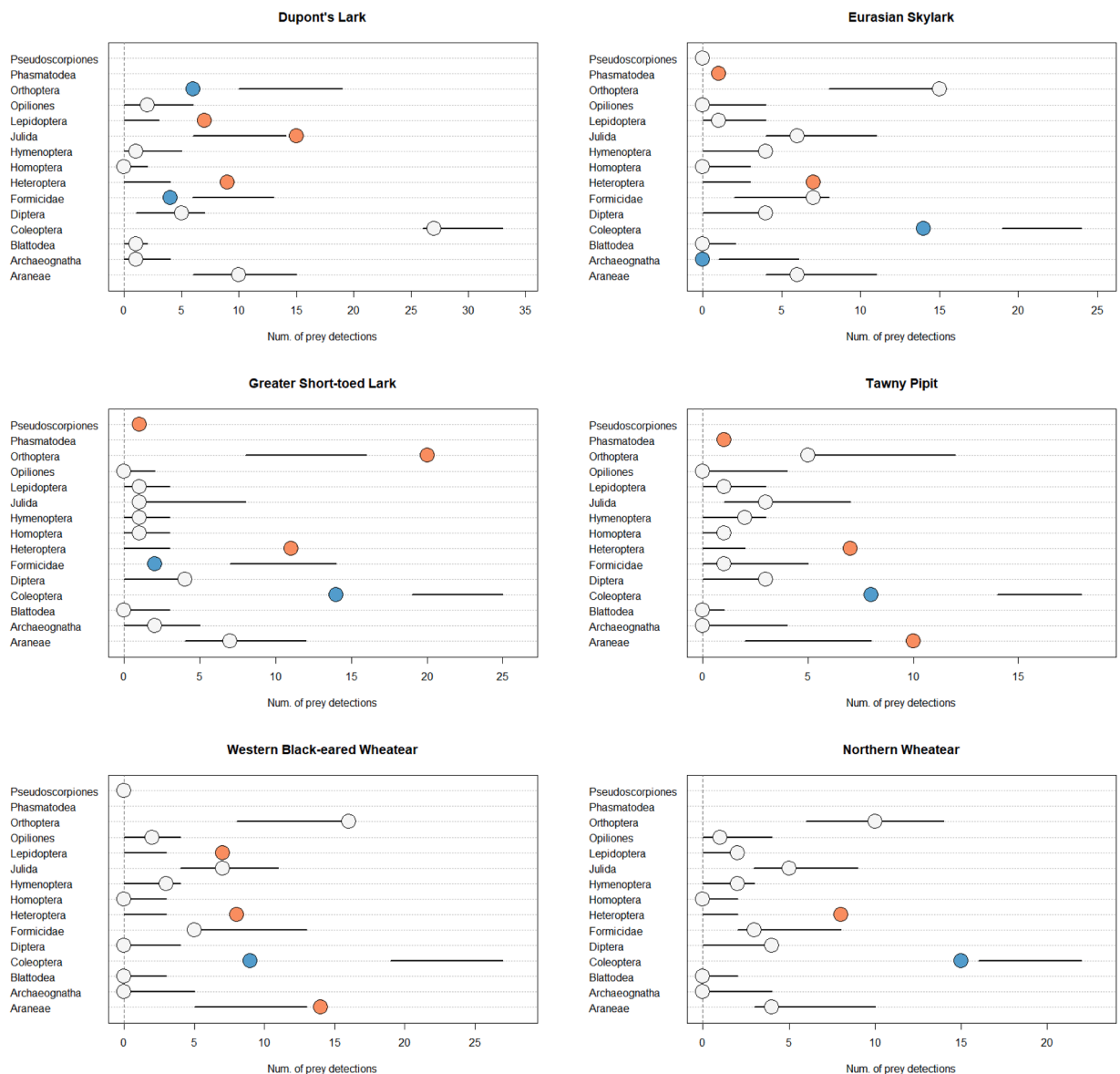


Fig. 4. Dietary selectivity plots for the six shrub-steppe passerines over the breeding season, showing the observed consumption frequencies (dots) and the predictions from the null model with 95% confidence limits (horizontal black lines). White dots denote arthropod prey groups consumed in proportion to their biomass in the field, orange dots indicate prey groups consumed more frequently than expected, and blue dots represent arthropods preyed less frequently than expected.

4. Discussion

Here we describe the relationship between food availability and diet in a shrub-steppe passerine assemblage, using fecal DNA metabarcoding and estimates of environmental arthropod availability. A previous study of these species' dietary niche (Zurdo et al., 2023) provided information about their diet composition and assessed interspecific trophic niche overlap but did not comprehensively address their prey selection patterns. This study filled a gap, showing that all shrub-steppe passerines select some food resources more often than others compared to their availability in the foraging habitat. This information can be a useful tool to guide conservation planning and habitat management (Nell et al., 2023), especially when endangered species, such as the Dupont's lark, are involved (Margalida et al., 2009).

4.1. Dietary selectivity in shrub-steppe passerines

According to our prediction, these results show a significant prey selection for some arthropod groups by all shrub-steppe bird

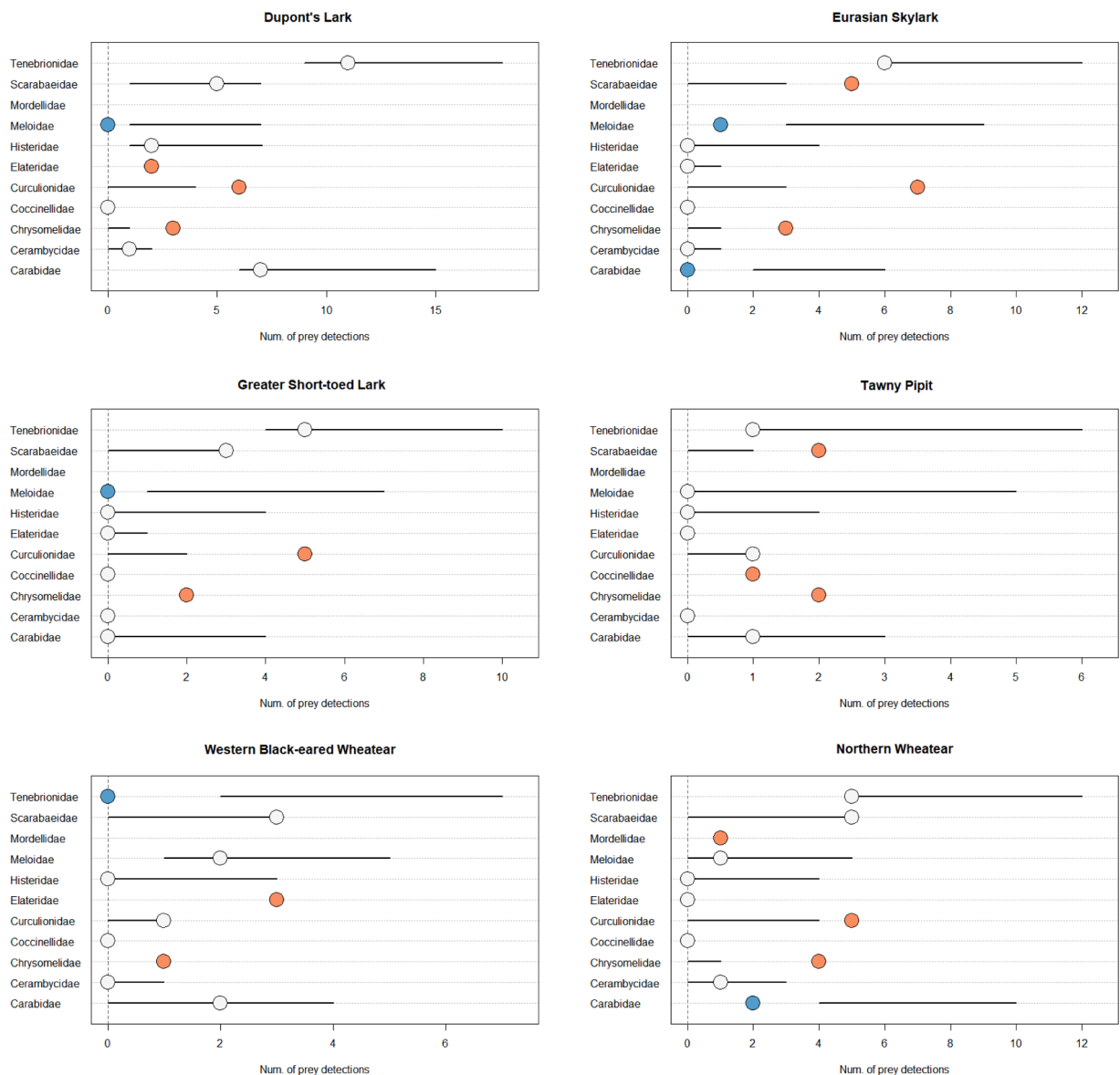


Fig. 5. Dietary selectivity plots for the six shrub-steppe passerines over the breeding season, showing the observed consumption frequencies (dots) and the predictions from the null model with 95% confidence limits (horizontal black lines). White dots denote Coleoptera families consumed in proportion to their biomass in the field, orange dots indicate prey groups consumed more frequently than expected, and blue dots represent arthropods preyed less frequently than expected.

species. However, we found differences between species in their prey selection patterns, which might facilitate trophic niche segregation and thus the coexistence of these species that are known to have a high dietary overlap (Zurdo et al., 2023). Such segregation is in agreement with previous studies that have also found differences on dominance and nesting niche within the same species (Barrero et al., 2023a, 2023b). In addition, the selectivity shown suggests that selected food resources are widely available in the environment, since as the optimal foraging theory predicts (Pyke et al., 1977), when food is abundant, individuals are likely to be more selective, choosing preys of higher quality (Emlen, 1966; Hughes, 2009).

Almost all species showed selectivity for Orthoptera based on prey abundance, but only the Greater Short-toed Lark exhibited this selectivity when prey biomass was considered. In the foraging habitat, these insects had the second highest proportion of biomass (12.82%), although their abundance was proportionally low (4.51% of total abundance). Despite this, Orthoptera was the most frequently consumed group by three of the six species (highly prominent in the Greater Short-toed Lark). Several studies concur that Orthoptera is one of the arthropod groups with the highest content of protein (Table 2), a macronutrient that plays an important role as resource of energy for many animals (Robbins, 1983), and fundamental for lean growth, reproduction and molt in birds (Machovsky-Capuska et al., 2016). Most of the samples from the Greater Short-toed Lark were collected in June, coinciding with the beginning

Table 2

Nutritional composition of the arthropod orders most frequently consumed by the six shrub-steppe birds, based on results compiled from different studies. Bird species that showed some type of selection for each prey group are also indicated. Numbers indicate the source from which nutritional information was obtained: 1. [Robel et al. \(1995\)](#); 2. [Brodmann and Reyer \(1999\)](#); 3. [Razeng and Watson \(2015\)](#); 4. [López et al. \(2016\)](#); 5. [Reeves et al. \(2021\)](#). Except for moisture (percentage of wet mass), the values of each macronutrient correspond to the percentage of dry mass. An average value was obtained when the same study provided several data for the same arthropod group. Species names are abbreviated: Dupont's Lark = DL, Eurasian Skylark = ES, Greater Short-toed Lark = GSL, Tawny Pipit = TP, Northern Wheatear = NW, Western Black-eared Wheatear = WBW. Symbols (+) and (-) indicate positive and negative selection, respectively.

		Araneae	Coleoptera	Diptera	Hemiptera	Hymenoptera	Julida	Lepidoptera	Orthoptera	Reference
Macronutrients	Protein	73.65	62.40	65.80	56.18	-	-	66.10	70.98	1
		63.79	61.41	63.27	49.95	66.53	38.20	57.31	-	2
		76.25	73.75	61.81	63.75	67.50	-	64.38	68.75	3
		-	64.44	-	-	-	37.45	-	73.96	4
		57.15	26.45	-	36.30	22.05	-	26.55	35.00	5
	Fat	17.65	24.50	11.40	24.80	-	-	17.45	11.58	1
		6.56	6.22	6.22	10.24	5.77	5.58	4.95	-	2
		9.55	12.00	9.55	16.50	-	-	12.00	7.36	3
		-	12.09	-	-	-	5.70	-	8.68	4
		10.00	14.50	-	19.50	20.01	-	9.30	7.10	5
	Carbohydrates	1.04	1.92	3.42	1.70	2.87	3.83	3.43	-	2
		-	5.94	-	-	-	45.54	-	3.99	4
	Moisture	69.18	63.33	60.33	51.81	66.43	65.09	74.07	-	2
		69.88	66.24	58.03	50.09	53.35	-	70.95	65.57	3
	Exoskeleton	6.20	37.50	-	21.70	37.40	-	13.10	10.60	5
Species with prey selection	Based on prey abundance	NW (-)	DL (+)	DL (-) WBW (-)	Heteroptera: all species (+)	Formicidae: all species (-)	DL (+)	DL (+)	ES (+)	
			NW (+)		Homoptera: DL (-)	Other Hymenoptera: ES (+)	ES (+)	WBW (+)	GSL (+)	
					ES (-)		WBW		WBW (+)	
					WBW (-)		(+)		NW (+)	
							NW (+)			
	Based on prey biomass	TP (+) WBW (+)	All species except DL (-)	-	Heteroptera: all species (+)	Formicidae: DL (-) GSL (-)	DL (+)	DL (+) WBW (+)	GSL (+) DL (-)	

of the peak of adult orthopteran abundance, which normally occurs in summer in temperate regions (Aguirre-Segura and Barranco, 2015). This could explain the observed high frequency of orthopterans consumption by this species. Furthermore, our dietary selection results indicated that, despite the higher availability of orthopterans in June, the Greater Short-toed Lark preyed on them more than expected, suggesting that the feeding strategy of this passerine species might involve an active search for this highly nutritional prey. Hódar (1998) also showed a positive selection for Orthoptera by the Western Black-eared Wheatear in two shrub-steppe areas of southern Spain. Conversely, Dupont's Lark in our study site showed lower Orthoptera consumption than expected from its biomass. We do not believe this implies a rejection of these prey items, since in terms of orthopteran abundance results indicate consumption as expected from availability. Most of Dupont's Lark samples were collected in April and May, when orthopteran adults were not yet developed, and the nymphs being smaller in body size and probably less profitable (Aguirre-Segura and Barranco, 2015), so an increase in the number of samples of this species in June may provide more conclusive results. Also, it is possible that Dupont's Lark exhibits a functional response to changes in prey populations in which at high orthopteran densities they eventually reach saturation or satiation (Holling, 1959; Salamolard et al., 2000; Beardsell et al., 2021), but additional research is needed to assess the functional responses of shrub-steppe species involved in predator-prey interactions to confirm this conjecture.

Although dietary results already indicated a high importance of Hemiptera for the overall shrub-steppe bird community (this study; Zurdo et al., 2023), we did not expect such a marked selectivity for true bugs (Heteroptera), revealed by both occurrence and quantitative (RRA) dietary data. In the study area, the availability of this group was low (Fig. 2, Table S6). This was likely not due to sampling bias, as most true bugs families are phytophagous and associated directly or indirectly with plants, either by feeding on plants or by foraging on the ground for fallen seeds or fruits (Weirauch et al., 2019), which makes both pitfall trapping and sweep netting highly effective capture methods for this group (Schwertner et al., 2021). Therefore, the selectivity for this relatively scarce insect group might be explained by its high nutritional quality, as true bugs and other hemipterans are among the prey that provide the highest fat supply (Table 2). The role of fat as the prime energy source for migratory birds has been widely demonstrated (Bairlein, 2002; Pierce and McWilliams, 2014), but it is also a major macronutrient for avian reproduction (Reynolds and Perrins, 2010). In addition, immature stages of this group are wingless and also phytophagous (Goula and Mata, 2015), which makes them highly exposed to predators. Previous studies have proven that true bugs represent an important food source for insectivorous birds in different habitats (Exnerová et al., 2003; Buchanan et al., 2006; Holland et al., 2006; Mansor et al., 2021).

Regarding the order Coleoptera, the results of the selection analysis are somewhat striking. Our results indicated that Dupont's Lark and the Northern Wheatear preyed this group more frequently than expected from their abundance, but results changed when beetle biomass was considered: all bird species except Dupont's Lark consumed them less frequently and in lower quantities than expected. Given that beetles are one of the main food sources for all the study species, and that their availability in the foraging habitat is very high, particularly in terms of biomass, this negative selection does not likely mean avoidance. Besides, these insects also stand out in terms of macronutrients (Table 2), with high levels of protein and lipids. As mentioned above in connection with the case of Dupont's Lark and orthopterans, it is also likely that these species exhibit saturation at high beetle densities. In addition, our findings showed that some beetle families were selected over others. For instance, weevils (Curculionidae) and leaf beetles (Chrysomelidae) were positively selected by most of the bird species, while dung beetles of the family Scarabaeidae were selected by the Eurasian skylark, the Tawny Pipit and the Northern Wheatear. These groups are mainly phytophagous (weevils and leaf beetles) and coprophagous (dung beetles), which can facilitate their detection and capture both for birds that feed mainly by walking or running and picking up prey from the ground and from small shrubs and herbs, and for those that also feed from perches (mainly both wheatears; Dunn et al., 2020; Collar, 2021). However, the most abundant beetles in the study area, darkling beetles (Tenebrionidae), were less frequently preyed than expected by the Western Black-eared Wheatear, as occurred with ground beetles (Carabidae) and both the Eurasian Skylark and the Northern Wheatear. Also, blister beetles (Meloidae), which are reported to synthesize cantharidin (compound highly toxic to vertebrates and invertebrates; Dettner, 1997; see however Bravo et al., 2017), were consumed less than expected from their biomass by the Eurasian Skylark, the Greater Short-toed Lark and Dupont's Lark.

Other arthropod groups positively selected by some of the bird species were spiders and lepidopterans. The Tawny Pipit and the Western Black-eared Wheatear selected spiders, while the latter and Dupont's Lark showed selectivity for lepidopterans. These are also groups with high nutritional value, as both (but particularly spiders) provide a high protein content, low exoskeleton content (Table 2) and moisture supply (Razeng and Watson, 2015), which might be an important factor for birds that obtain large part of their water from food (e.g., Dupont's Lark has apparently never been observed to drink water; Donald, 2004; de Juana and Suárez, 2020). Besides, lepidopteran larvae contain the largest proportion of carotenoids (Arnold et al., 2010; Eeva et al., 2010), which are very important pigments, antioxidants, and immune stimulants for birds (Surai et al., 2001). Also, Dupont's Lark and both wheatears exhibited prey selection for millipedes of the order Julida (based on abundance), which are an important source of carbohydrates (Table 2) and calcium, a limiting micronutrient for breeding success (Bures and Weidinger, 2003; Reynolds and Perrins, 2010).

Ants, on the contrary, were consumed below their availability in terms of abundance by all bird species, and by Dupont's Lark and the Greater Short-toed Lark based on biomass. Ants are small and highly sclerotized (Table 2), making them less valuable nutritionally (Redford and Dorea, 1984; Razeng and Watson, 2015). In addition, the presence of the unpalatable and potentially toxic formic acid in ants (Bennett et al., 1996) has been reported as the cause of ant avoidance by many bird species (Dean and Milton, 2018). Nevertheless, ants are consumed by many species of birds in diverse habitats (Holland et al., 2006; Razeng and Watson, 2012; Dean and Milton, 2018), and, for instance, in the Eurasian Skylark fecal samples analyzed in this study, they appeared with non-neglectable frequency, suggesting that these insects provide a reliable, though not especially attractive, food resource, particularly when more profitable prey may be difficult to find or less available (Hódar, 1998).

In prey selection models we have used both abundance and biomass as measures of prey availability, providing a broader and more accurate picture of trophic interactions and selection processes. Bird selection studies typically use abundance alone (Hunter et al.,

1988; Kaspari and Joern, 1993; McClenaghan et al., 2019; Evens et al., 2020; Davies et al., 2022), in part probably because it is easily obtained by passive sampling without additional measurements, manipulation or data transformation (Saint-Germain et al., 2007). However, when investigating questions such as prey availability for higher trophic levels and, in addition, working with species whose body masses differ by several orders of magnitude, which is the case for arthropods (e.g., beetles of the family Meloidae, with an average of 99.55 mg, compared to 0.78 mg for ants in our study area), the information obtained becomes ecologically more meaningful when biomass is used as a complement to abundance (Saint-Germain et al., 2007), since metabolism and energy transfer through trophic levels are intrinsically linked to body mass of individuals (Brown et al., 2004).

4.2. Use of metabarcoding for dietary selection by insectivorous

The use of DNA metabarcoding for identification of invertebrate prey from fecal samples provides comprehensive information on the diet of insectivorous species. Compared to traditional morphology-based methods, metabarcoding is less time-consuming and also avoids potential observer biases and the undetectability of soft-bodied species (Pompanon et al., 2012). However, metabarcoding did not provide information about the life stage of the arthropod prey consumed. Since some birds may have stronger selectivity for particular life stages (e.g., caterpillars in tits -Paridae-; Naef-Daenzer et al., 2000; García-Navas and Sanz, 2011), this information could reveal some life-stage dietary selection by shrub-steppe species that has likely been undetected in our study. Related to this, our study did not address prey size selection either. Many bird species select larger prey items (Evens et al., 2020; Mitchell et al., 2022), mainly to feed their nestlings (Quinney and Ankney, 1985; Naef-Daenzer et al., 2000; McClenaghan et al., 2019; Davies et al., 2022). Although we did have information on the size of the arthropods trapped in the field, it was not available for the species present in the fecal samples, since direct measure is not possible. Alternatively, we could have used data from entomological literature or arthropod guides to find approximate body lengths for each dietary item detected (see Mitchell et al., 2022; Davies et al., 2022), but the taxonomic resolution of our primer set did not allow that with the adequate confidence, as in most cases identification only to order or family level was achieved. In practice, however, the primer set had a very broad utility, detecting a large diversity of invertebrates, and with an optimal taxonomic resolution comparable to the identifications of pitfall and sweep-net captures.

Our metabarcoding approach was based both on occurrence data, which may have overestimated the importance of prey taxa consumed in small quantities (Deagle et al., 2019), and on RRA, a quantitative estimate of the relative biomass proportions of prey consumed (Neby et al., 2021), which provided similar results than occurrence data. When using occurrence data, the choice of the minimum sequence threshold used to define an occurrence is crucial (Drake et al., 2021), and the one we used in this study (1%; see bioinformatics section) has been proved to be suitable for many dietary analysis (Deagle et al., 2019) and to effectively eliminate a very high proportion of contamination, secondary detection, and sequencing errors (Drake et al., 2021). On the other hand, there is little consensus on the quantitative ability of DNA metabarcoding, i.e., the extent to which proportions of reads generated corresponds to the original proportions of prey items (Deagle et al., 2013; Piñol et al., 2018; Lamb et al., 2019), mostly due to differential degradation rates of tissues in the guts of consumers, differences of DNA density between consumed tissues, PCR primer bias and random sampling during sequencing (Murray et al., 2011; Leray and Knowlton, 2017). However, several studies have shown that RRA and ingested prey biomass correlate positively in some model systems (Lamb et al., 2019), including insectivorous birds (Verkuil et al., 2022). Although we did not perform validation tests, which are important for measuring DNA metabarcoding's potential for the quantitative assessment of diets (Neby et al., 2021; Verkuil et al., 2022), we consider that the additional analysis of prey selection based on RRA broaden the picture and strengthen our occurrence-based findings on prey selection patterns in insectivorous passerines.

4.3. Conservation implications

Reduction in food supply due to declining abundance and biomass of insects and other invertebrates is clearly affecting bird species (Bowler et al., 2019; Grames et al., 2023), but also mammals, reptiles and amphibians (Lister and Garcia, 2018; Goulson, 2019; Kehoe et al., 2021). Although such processes and the long-term consequences are still little studied, it has been demonstrated, for instance, that increasing crop intensification in shrub steppes has negative effects on arthropod biomass and on the use of space by Dupont's Lark (Reverter et al., 2021). Given the marked selectivity for some arthropod prey by shrub-steppe passerines, changes in their availability might affect bird population dynamics (Grames et al., 2023). Therefore, monitoring of selected arthropod groups should be a key task within conservation plans for these species, as well as the implementation of actions that ensure that a sufficient supply of arthropods is available to birds. The promotion of extensive sheep grazing, a traditional practice associated with Iberian steppes that has declined in recent decades (Traba and Pérez-Granados, 2022), is a recommended management measure to maintain the vegetation structure of shrub steppes and increase food availability (Gómez-Catasús et al., 2019, 2023), both directly through sheep depositions and indirectly by increasing habitat heterogeneity (Zhu et al., 2012; Newbold et al., 2014; Gómez-Catasús et al., 2023).

5. Conclusion

By using DNA metabarcoding and prey availability monitoring (including biomass), we provided evidence that shrub-steppe passerine species select prey that supply them with an important source of nutrients and energy, essential to meet the physiological requirements of breeding. These findings expand our knowledge of how shrub-steppe species interact with their prey and also among them, showing some differences in prey selection, which might facilitate the coexistence of these species in the breeding habitat. Our study highlights the importance of food availability, and especially that of some specific groups, for insectivorous species, suggesting that fluctuations in abundance and biomass of selected arthropod prey may have a substantial impact on bird populations. This has

important implications for understanding and conserving shrub-steppe bird species and other generalist insectivores, especially crucial in the current scenario of increasing loss of invertebrate abundance and diversity and negative bird population trends.

Ethical approval

This work was approved by the Local Ethical Committee for Animal Experiments of the Universidad Autónoma de Madrid (CEI80–1468-A229).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

R scripts for statistics are available for download on Zenodo (<https://doi.org/10.5281/zenodo.8179311>). Metabarcoding dietary data used in this study are provided as Table S2.

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Author contributions

JZ, MM and JT conceived the original idea. JT provided funding. JT and MM supervised the project. JZ curated and analyzed data. JZ and MR prepared original data bases. JZ, AB, DB-R, MR, JG-C, and CP-G carried out field work, and MR and JZ carried out arthropod identification. JZ took the lead in writing the manuscript and all authors provided critical feedback and contributed to the final manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2023.e02738](https://doi.org/10.1016/j.gecco.2023.e02738).

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