











# Dietary niche overlap and resource partitioning among six steppe passerines of Central Spain using DNA metabarcoding

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Trophic niche partitioning is a mechanism that facilitates the coexistence of ecologically similar species by sharing their resource use. However, detailed information of the trophic niche in insectivorous birds is usually limited by the lack of accurate identification of consumed food resources. The use of DNA metabarcoding has proved useful for molecular identification of the taxa present in bird faecal samples. Here, we used this molecular technique to study the diets of six steppe passerine species distributed in two Special Protection Areas in central Spain, and to characterize the dietary niche overlap and the prey composition differences between bird species. In total, we distinguished 112 diet items, covering 39 arthropod families of 13 orders. Although significant dietary differences existed in prey species composition, our results indicated a 74% overlap in steppe bird dietary niche, mostly due to high consumption of abundant arthropod prey such as beetles, grasshoppers and spiders in the breeding season by all bird species. The lowest overlap was found for the dietary niches of the Greater Short-toed Lark *Calandrella brachydactyla* and Dupont's Lark *Chersophilus duponti*, a scarce and threatened species, which appeared to be the species with the most distinct dietary niche within the community. Our results make a significant contribution to the knowledge of shrub-steppe bird diets and their trophic interactions, indicating that some extent of interspecific resource partitioning occurs in the study area, notably between Dupont's Lark and the Greater Short-toed Lark. Our study demonstrates the value of DNA metabarcoding in the assessment of passerine diets and provides useful ecological results for the design of biodiversity conservation programmes in the increasingly scarce and threatened steppe habitats.

**Keywords:** diet, faecal metabarcoding, grasshoppers, Julidae, shrub-steppes, trophic niche.

Ecological niche theory is a cornerstone of ecology. Central to this theory is the role of interspecific competition in structuring ecological communities and evolutionary diversification (Chase & Leibold 2003). Niche theory predicts that species will

limit the effects of interspecific competition by partitioning space, time or resources to coexist (MacArthur & Levins 1967, Chesson 2000, Salsamendi *et al.* 2012). Species coexistence and competition for resources over evolutionary time contribute to shape the configuration of communities (Leibold *et al.* 2004). Food niche partitioning is one of the main mechanisms through which many

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species limit interspecific competition (Schoener 1974, Kent & Sherry 2020), and therefore dietary niche overlap studies are of paramount importance for understanding community structure and the coexistence of ecologically similar species (Vieira & Port 2007, Orłowski & Karg 2013, Arrizabalaga-Escudero *et al.* 2018, Bosenbecker & Bugoni 2020). An abundance of food resources may lead to high dietary niche overlap between species, but when foraging resources become less abundant, trophic niche overlap is expected to decrease as interspecific competition becomes stronger (Cody 1974, Schoener 1982). However, optimal foraging theory (MacArthur & Pianka 1966, Schoener 1971) predicts that dietary niche breadth will increase as the availability of food resources decreases, as consumption of a wide range of sub-optimal prey types may relax competition levels (Krebs & Davies 1981). Therefore, when foraging resources are scarce, although dietary niche overlap between species should decrease, the breadth of each species' trophic niche should increase. Interspecific competition may also induce character displacement (Brown & Wilson 1956, Dayan & Simberloff 2005), i.e. the evolution of phenotypic differences in size and shape that would reduce resource overlap, and hence competition, between coexisting species.

In birds, traditional approaches for diet studies have involved invasive methods including induced regurgitations (Laursen 1978, Carlisle & Holberton 2006), application of ligatures to nestlings (Herranz *et al.* 1993, Villard & Pavis 1998) or dissection of the stomachs of collected birds (Haw & Clout 1999, Kok *et al.* 2000), as well as non-invasive methods such as direct observation of feeding (Cezilly & Wallace 1988, Catry *et al.* 2012), optical examination of faecal contents (Redpath *et al.* 2001, Bravo *et al.* 2017), or biochemical methods involving isotopic and lipid analysis (Owen *et al.* 2013, Catry *et al.* 2016, Paiva *et al.* 2018). Conventional techniques provide valuable data for assessing the qualitative and quantitative composition of diets, but have important limitations, with variations in their ability to distinguish prey species, and they are labour intensive (Pompanon *et al.* 2012). In recent years, ornithologists and ecologists have taken advantage of DNA metabarcoding to identify species using faecal samples (Valentini *et al.* 2009, Pompanon *et al.* 2012, Hoenig *et al.* 2022). This approach provides higher taxonomic resolution and detectability (Shokralla *et al.*

2012, Bohmann *et al.* 2014). However, it also has certain limitations, such as errors introduced during polymerase chain reactions (PCRs), the choice of an appropriate primer set, or the need for high-quality taxonomic reference databases (Taberlet *et al.* 2012, Alberdi *et al.* 2019). So far, faecal DNA metabarcoding has been successfully applied to characterize the diets of seabirds (Deagle *et al.* 2010, Bowser *et al.* 2013, Xavier *et al.* 2018, Carreiro *et al.* 2020) and landbirds (King *et al.* 2015, Crisol-Martínez *et al.* 2016, Moran *et al.* 2019, Evens *et al.* 2020, Kusack *et al.* 2022). All types of diets have already been studied in landbirds, namely carnivorous (Hacker *et al.* 2021), insectivorous (Jedlicka *et al.* 2017, McClenaghan *et al.* 2019, Cabodevilla *et al.* 2021, Chung *et al.* 2021, Mitchell *et al.* 2022), herbivorous (Ando *et al.* 2013, Yang *et al.* 2016) and omnivorous (da Silva *et al.* 2019, Cabodevilla *et al.* 2021). However, applying faecal DNA metabarcoding techniques for evaluating trophic niche partitioning in avian communities remains limited to a few studies (Trevelline *et al.* 2018, Bumelis *et al.* 2021, Mansor *et al.* 2021, Spence *et al.* 2021, Garfinkel *et al.* 2022) and, to the best of our knowledge, with none assessing dietary niche overlap in steppe passerine communities (Morales & Traba 2016).

Steppe birds, the most threatened bird group in Europe (Sanderson *et al.* 2005, Santos & Suárez 2005), are directly dependent on open and treeless landscapes dominated by grass or small shrubs (Morales & Traba 2016). Particularly, shrub-steppe birds, linked to areas with woody vegetation (Traba *et al.* 2013), have received remarkably little scientific attention (Laiolo & Tella 2006, Gómez-Catasús 2021), and the research gap is especially important for small-sized steppe birds, such as passerines (Morales & Traba 2016).

Shrub-steppes occupy large areas of the Iberian Peninsula (Sainz Ollero & van Staaldin 2012) and present a relatively homogeneous physiognomy under very diverse climate conditions (Tellería *et al.* 1988). Since the middle of the last century, Iberian shrub-steppes have been undergoing rapid anthropogenic changes, mainly due to agricultural intensification, land-use changes (afforestation, infrastructure development), and the abandonment of traditional sheep grazing (Laiolo & Tella 2006, Traba & Morales 2019, Traba & Pérez-Granados 2022), leading to the reduction and fragmentation of these habitats and, hence, to an increase in the decline of shrub-steppe bird

populations (Santos & Suárez 2005, Laiolo & Tella 2006, Traba & Morales 2019). Some steppe bird species, such as the endangered Dupont's Lark *Chersophilus duponti*, the Greater Short-toed Lark *Calandrella brachydactyla*, Thekla's Lark *Galerida theklae* and the Western Black-eared Wheatear *Oenanthe hispanica*, among others, present their main European populations in the Iberian shrub-steppes (Santos & Suárez 2005), which makes these landscapes of great conservation value in the European context. However, knowledge about Iberian shrub-steppe bird assemblages from a community viewpoint is scarce. Therefore, understanding the interspecific interactions of species that coexist, and so may have been competing for resources in evolutionary time, is of great ecological and conservation interest, especially in the face of the high anthropogenic transformations of the shrub-steppe habitat.

In this study, we used faecal DNA metabarcoding to determine the diet composition of a passerine assemblage of the shrub-steppes of central Spain. Study species are primarily insectivorous in the breeding season, with beetles and grasshoppers being the most important prey items cited for all species in the sparse existing literature (e.g. Cramp 1988, Hódar 1998, de Juana *et al.* 2020, Dunn *et al.* 2020). We investigated differences in prey composition between species and whether shrub-steppe passerines exhibit dietary niche partitioning of food resources as expected from their coexistence in the steppe habitat. We predicted dietary similarities in the consumption of the most abundant and main prey types due to similarity between their foraging strategies, which imply prey searching mainly on the ground (Billerman *et al.* 2020), but we also expected dietary niche partitioning between species, which may favour coexistence.

## METHODS

### Study species, study site and sample collection

Our study is focused on six passerine species that coexist in the southern 'páramos' (high-altitude, steppe-dominated plateaus) of Soria (central Spain): the endangered Dupont's Lark, the Eurasian Skylark *Alauda arvensis* and the Greater Short-toed Lark of the family Alaudidae; the Tawny Pipit *Anthus campestris* of the family

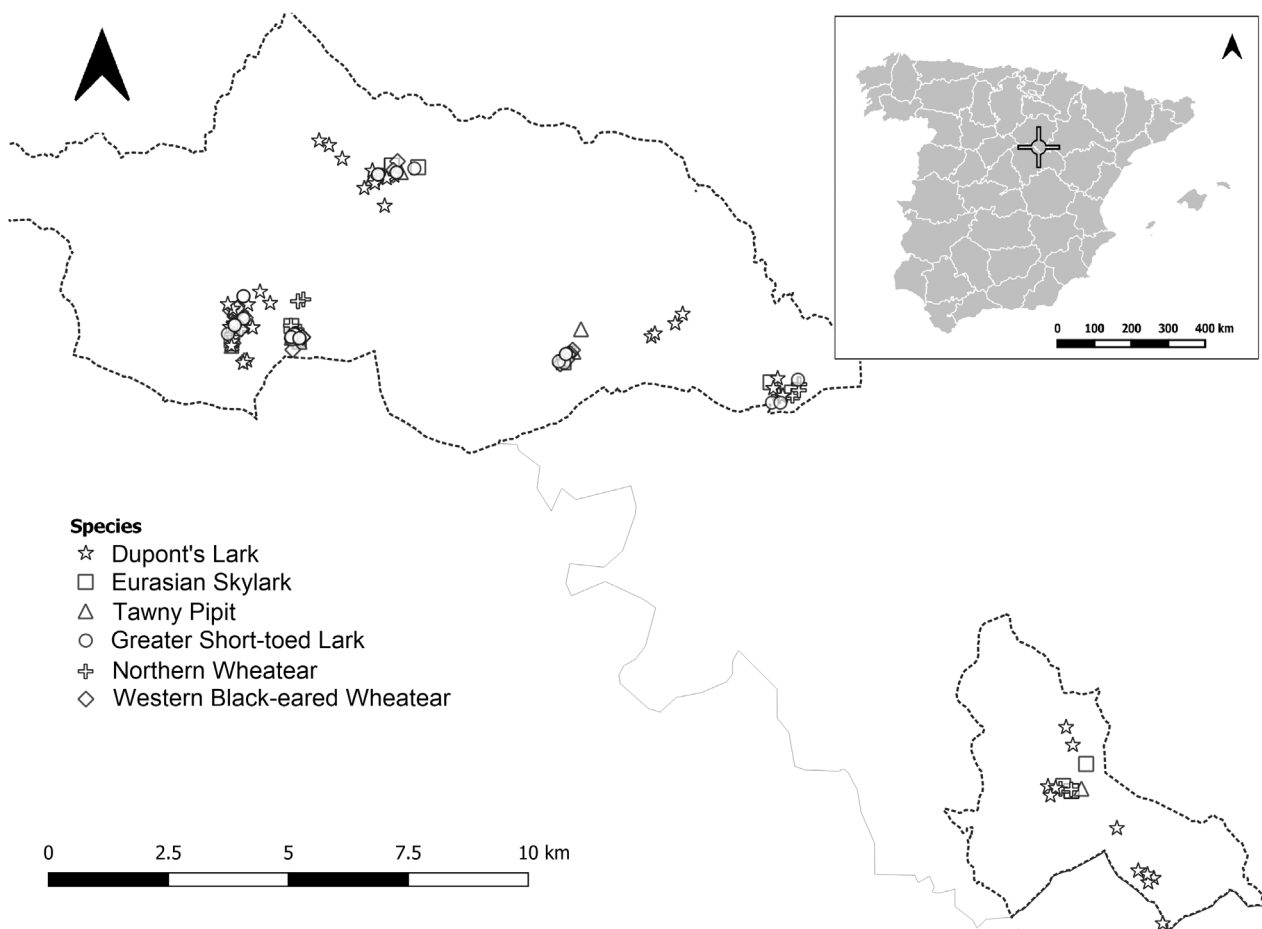
Motacillidae; and two species of the family Muscipidae, the Northern Wheatear *Oenanthe oenanthe* and the Western Black-eared Wheatear. These species of similar size inhabit open landscapes with sparse and low vegetation cover, coexisting in the breeding season in Iberian shrub-steppes, and feeding on similar invertebrates. More detailed information about the bird species is presented in Table S1.

We captured 225 adult individuals of the six species in eight localities (patches closer than 1 km are considered the same locality) across the Special Protection Areas Altos de Barahona and Páramo de Layna (Soria, Spain; Fig. 1), during the breeding seasons of 2017–2019. The study area is a flat (slope < 10%), open and treeless landscape mosaic between 1100 and 1200 m above sea level dominated by continental basophilic scrublands of *Genista pumila*, *Genista scorpius*, *Thymus* spp. and *Satureja intricata*, dry perennial grasslands, and mixed grassland–scrublands (Zurdo *et al.* 2021). Cereal fields and conifer reforestations are interspersed in the shrub-steppe matrix. Climate is continental Mediterranean, with a mean temperature of 10.8 °C and a mean annual rainfall of 471 mm (Aranbarri *et al.* 2015).

Birds were captured with spring-traps baited with mealworms (*Tenebrio molitor*) and using a species-specific recording to attract them. All birds were ringed, which allowed us to avoid duplicate samples from the same individual. Birds defecated spontaneously as a behavioural response to capture in the spring-trap or handling. We collected 89 Dupont's Lark faecal samples, 26 Eurasian Skylark samples, 28 Tawny Pipit samples, 30 Greater Short-toed Lark samples, 22 Northern Wheatear samples and 30 Western Black-eared Wheatear samples, all from different individuals. Samples were collected from clean cotton bags (washed with bleach between each use to minimize contamination) or directly from the ground or stones at the location of capture, and were stored in individual 1.5-mL plastic vial tubes with 98% ethanol and refrigerated at –20 °C until processed in the laboratory.

### Sample processing

The QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany; Ref. 12830–50) was used to extract DNA from faecal material, following the manufacturer's instructions. Before the extraction



**Figure 1.** Location of the study area in southern Soria (central Spain). Points where faecal samples of the different steppe bird species were collected are indicated in the key. Special Protection Areas (SPAs) of the European Union's Natura 2000 Network are indicated by dashed lines (further north, 'Altos de Barahona', and further south, 'Páramo de Layna').

protocol, ethanol was removed from the faecal samples by decanting following 30 min of centrifugation and dried at 50 °C until the ethanol was vaporized. DNA concentration ( $\mu\text{g}/\text{mL}$ ) and quality (A260/280 and A260/230 ratios) were quantified in a Nanodrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Each faecal sample was analysed separately. DNA extraction was performed by the Genomics and NGS Core Facility at the Centro de Biología Molecular Severo Ochoa (CBMSO, CSIC-UAM, Spain).

The eukaryotic mini-barcode miniB18S\_81 designed by Cabodevilla *et al.* (2022) was used to amplify prey DNA, targeting the 18S rRNA gene. This marker was initially selected because steppe birds can feed on a wide range of invertebrates

(insects, spiders and snails), but also on plants, so we were interested in amplifying both the invertebrate and the plant components of the diet. A negative PCR control was included, to which no faecal DNA was added, and processed with the rest of the samples in the next steps of the molecular analysis. A second PCR was performed to index each amplified product and attach Illumina adaptors (Illumina, San Diego, CA, USA). Amplification was carried out according to Cabodevilla *et al.* (2022). The resulting fragments were checked in a Bioanalyzer before pooling in equimolar amounts. The library was further purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA). The final library was sequenced in an Illumina MiSeq NGS platform with a MiSeq Reagent Kit v3 for 600 cycles, following the manufacturer's

instructions. Sequenced samples that did not achieve the minimum sequence coverage requested (50 000 reads per sample) were re-sequenced in order to increase the number of initial sequences. Amplification, amplicon pooling, library preparation and sequencing were conducted by the Genomics Unit of the Fundación Parque Científico de Madrid (Spain).

### Bioinformatic analysis

Analysis of the sequencing data was performed following the MJOLNIR pipeline (Metabarcoding Joining Obitools and Linkage Networks In R; <https://github.com/uit-metabarcoding/MJOLNIR>; Appendix S1), using the R package *mjолnir* (Wangensteen 2020). This pipeline allows processing metabarcoding data, including quality filtering with *OBITools* (Boyer *et al.* 2016), chimaera removal with *VSEARCH* (Rognes *et al.* 2016), molecular operational taxonomic unit (MOTU) clustering with *swarm* (Mahé *et al.* 2015), taxonomic assignment with *ecotag* (*OBITools*; Boyer *et al.* 2016) and pseudogene removal with *LULU* (Frøslev *et al.* 2017). The taxonomic assignment of MOTUs was performed using a reference database in ecoPCR format including sequences for the metabarcoded fragment (miniB18S\_81), obtained from the download of all 18S sequences from the NCBI database. All sequences identified to species and genus were manually confirmed, as well as their presence in Spain.

Only MOTUs considered as potential prey items were taken into account for further analysis (Table S2). We excluded fungi, algae, protists, all vascular and non-vascular plants, birds, mammals (mostly human), and possible internal parasites (phyla Nematoda and Platyhelminthes). We also removed MOTUs belonging to mite taxa (orders Sarcotiformes and Trombidiformes) and tick taxa (order Ixodida) because of the high probability that they were parasites of other arthropods or of the birds themselves, and the product of secondary detection (da Silva *et al.* 2019). The class Collembola was also removed because of the tiny size of the individuals present in the study area (our pers. obs.), which were probably not directly preyed on by the bird. Finally, we discarded plant MOTUs as possible dietary items because the high diversity of MOTUs obtained made it difficult to discriminate between environmental contamination, secondary

detection or items that were actively consumed by the birds. This problem had already been described in other studies of omnivorous animals (e.g. da Silva *et al.* 2019, Tercel *et al.* 2021), and also seemed to affect our results. Nevertheless, we believe that considering only the invertebrate component of the diet of the six targeting species should not affect their overall niche overlap because these species are known to feed mainly on invertebrates during the sampled period (Table S1; Billerman *et al.* 2020) and we did not observe any plant material in the faecal pellets. As such, plant consumption during the sampled period should be non-significant or non-existent.

All samples with fewer than 100 dietary reads were discarded because they were considered to have failed. This happened with the negative PCR control and some samples of different bird species. We further removed 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 and low-abundance MOTUs that probably resulted from previous meals. MOTUs were identified with the most resolved taxonomic assignment possible, and following da Silva *et al.* (2020), when multiple MOTUs were assigned to the same taxon at the same taxonomic level they were identified with the same name, but with an added number (e.g. Julidae 1, Julidae 2; Table S2).

### Statistical analysis

To perform the analyses, we built presence/absence matrices of each sample analysed at three different identification levels: MOTU, family and order. We also calculated the frequency of occurrence of each prey taxon for each bird species at the three mentioned identification levels, defined as the number of faecal samples in which an MOTU, family or order was detected divided by the total number of samples per bird species.

All analyses were performed in the R statistical environment (R Core Team 2020). To test for differences in prey average richness between steppe bird species, we carried out generalized linear mixed models with a Poisson error distribution using the *glmer* function of the R package *lme4* (Bates *et al.* 2015). The variable 'locality' was considered as a random factor in order to take into account the possible geographical effect. The significance of the generalized linear mixed models



was tested using the function *Anova* of the package *car* (Fox & Weisberg 2011). We also compared the niche breadth, at the three identification levels, between the six bird species by estimating richness values using Hill numbers for the double of species with the lower sample (Chao *et al.* 2020). This analysis was performed using the *iNEXT* function of the R package *iNEXT* (Hsieh *et al.* 2016). We determined significant differences between estimates if the 95% confidence intervals (CIs) around the richness estimate did not overlap.

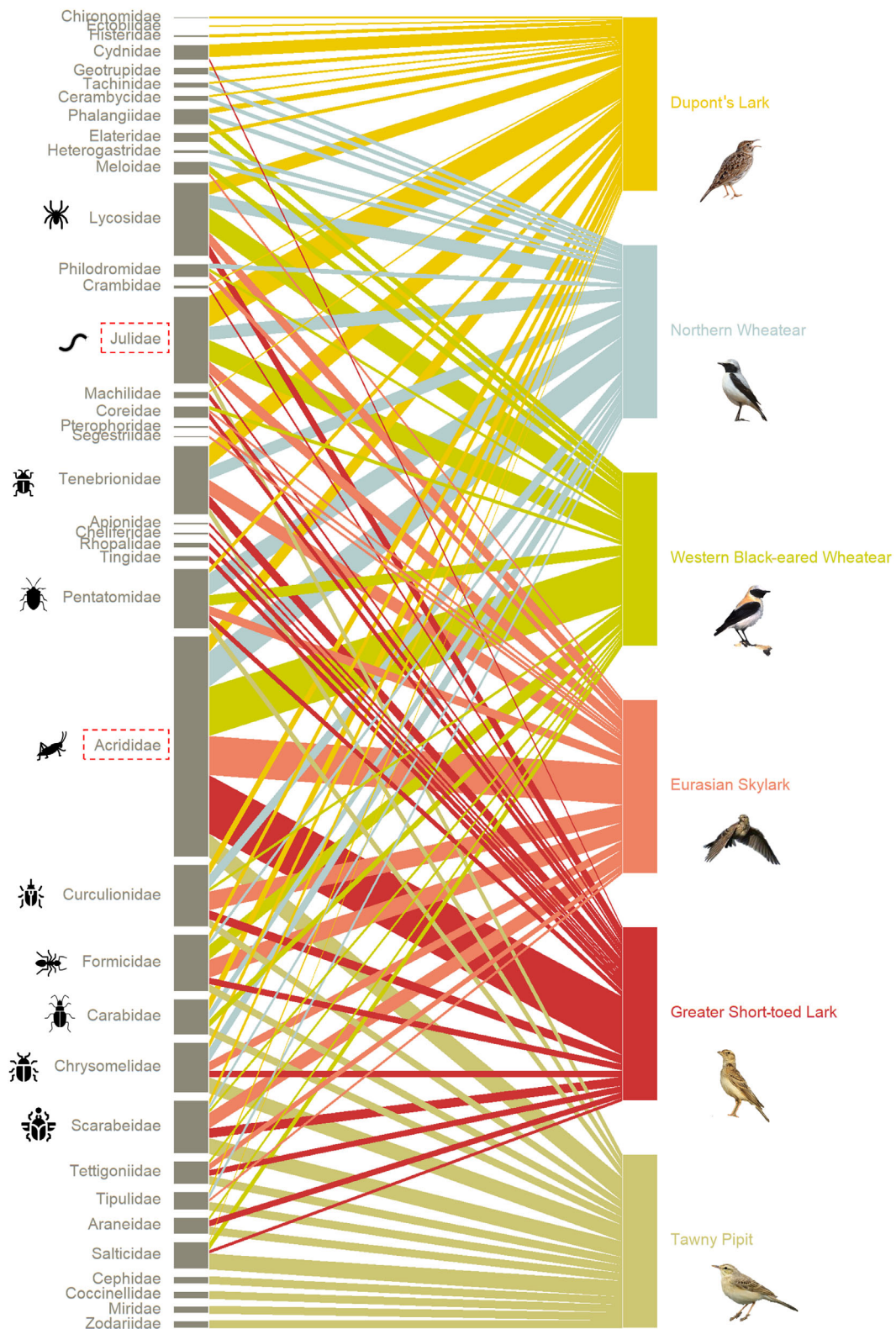
To assess trophic niche overlap among the six bird species, we calculated dietary niche similarity using Pianka's index (Pianka 1973) based on the frequency of occurrence of the prey families. This index ranges from 0 to 1, where 1 indicates complete overlap. We performed a null model analysis to test whether the observed niche overlap differs from what would be expected by chance, using the R package *EcoSimR* (Gotelli *et al.* 2015). Null models were calculated based on the randomization algorithm RA3, which reshuffles zero and non-zero row values (Lawlor 1980, Gotelli & Ellison 2013), and 10 000 simulated resource utilization matrices were generated to compare with observed resource utilization data. Dietary niches were visualized using non-metric multidimensional scaling (NMDS) and Jaccard distance with the *metaMDS* function of the R package *vegan* (Oksanen *et al.* 2018). The kernel probability density function of NMDS axis 1 and axis 2 for each steppe bird species was also plotted, calculated with the R base function *density*, and using the Gaussian smoothing kernel and the Silverman smoothing bandwidth (Silverman 1986).

Finally, statistical differences in prey composition between species at the MOTU, family and order levels were tested using a generalized linear model for multivariate abundance data, implemented in the function *manyglm* of the R package *mvabund* (Wang *et al.* 2012) and using binomial distribution. We used the function *anova.manyglm* of the same package to test model significance and to perform pairwise comparisons, with the argument *pairwise.comp*. After testing for multivariate effects, we carried out univariate tests to identify which response variables, i.e. prey items, were behind the differences in diet composition between the bird species. For this, we used the above function (*anova.manyglm*) with the argument *p.uni* = 'adjusted'.

## RESULTS

We recovered a total of 9.4 million sequencing reads from the faecal samples of the six steppe bird species. After bioinformatics processing, we kept 700 267 diet reads, with a mean of  $3586 \pm 1849$  standard deviation (sd) diet reads for Dupont's Lark samples ( $n = 39$ );  $1312 \pm 898$  sd diet reads for Eurasian Skylark samples ( $n = 19$ );  $11\,336 \pm 10\,688$  sd diet reads for Tawny Pipit samples ( $n = 14$ );  $5453 \pm 4004$  sd diet reads for Greater Short-toed Lark samples ( $n = 24$ );  $4221 \pm 3395$  sd diet reads for Northern Wheatear samples ( $n = 18$ ); and  $6795 \pm 4073$  sd diet reads for Western Black-eared Wheatear samples ( $n = 25$ ). Of the total 3407 MOTUs obtained, only 112 were considered as potential prey items (Table S2). Most of these dietary MOTUs, 111, represented three classes from the phylum Arthropoda (Insecta, Arachnida and Diplopoda), and one MOTU represented one class from the phylum Mollusca (Gastropoda). We identified 13 orders, 39 families, 27 genera and 14 species (Table S2), all of them belonging to the phylum Arthropoda, because the single Mollusca MOTU detected was identified only to the class level.

We found, on average, three dietary MOTUs per sample (sd =  $\pm 1.92$ , range 1–11), and no differences in MOTU richness among bird species (LR  $\chi^2 = 8.067$ , df = 5,  $P = 0.15$ ). We also found no difference in the number of families (LR  $\chi^2 = 7.438$ , df = 5,  $P = 0.19$ ) or orders (LR  $\chi^2 = 3.869$ , df = 5,  $P = 0.57$ ) detected per bird species. The most frequent MOTU was an unidentified Acrididae species (43% of the samples). Acrididae, Julidae, Tenebrionidae and Lycosidae were the most frequent preyed families (Fig. 2), with frequencies of occurrence of 43, 21, 16 and 16%, respectively. Other highly preyed families were Curculionidae (13%), especially for the Eurasian Skylark (32%), the Northern Wheatear (17%) and Dupont's Lark (15%), and Formicidae (12%), mainly for the Eurasian Skylark (32%) and the Western Black-eared Wheatear (16%). Figure 2 shows the trophic interactions between bird species and the consumed arthropod families based on the frequency of occurrence at the family level. Only two bird species preyed on arthropod families not present in other species: Dupont's Lark (Histeridae, Ectobiidae and Chironomidae) and Tawny Pipit (Cephalidae, Coccinellidae, Miridae



**Figure 2.** Network of the six passerine species' dietary niche showing the frequency of occurrence of the prey families detected in each bird sample. Red squares indicate arthropod families that were significantly consumed by birds in the univariate tests.

and Zodariidae; Fig. 2). At order level, Coleoptera was the most commonly identified order preyed upon across all bird samples (Table 1), but regarding each bird species independently, the most frequent order differed, Orthoptera being the most common for the Eurasian Skylark, the Greater Short-toed Lark and the Western Black-eared Wheatear, and Coleoptera for Dupont's Lark, the Tawny Pipit and the Northern Wheatear (Table 1).

We found no differences between species in the estimated niche width, at any taxonomic level, as shown by the overlapping confidence intervals in Figure 3, except between Dupont's Lark (10.42, 95% CI 9.22–11.61) and Western Black-eared Wheatear (8, 95% CI 7.49–8.51) at order level, and between Eurasian Skylark (48.94, 95% CI 38.92–58.95) and Western Black-eared Wheatear (32.63, 95% CI 27.47–37.80) at MOTU level.

Niche overlap analysis returned a mean value of Pianka's index of 0.74 for arthropod families, greater than the null expectation (Fig. S1;  $P < 0.001$ ), indicating a higher overlap in the consumption of arthropod families than expected by chance. In pairwise comparisons (Table 2), all Pianka's index values were greater than 0.54 and statistically significant ( $P < 0.05$ ). Higher dietary

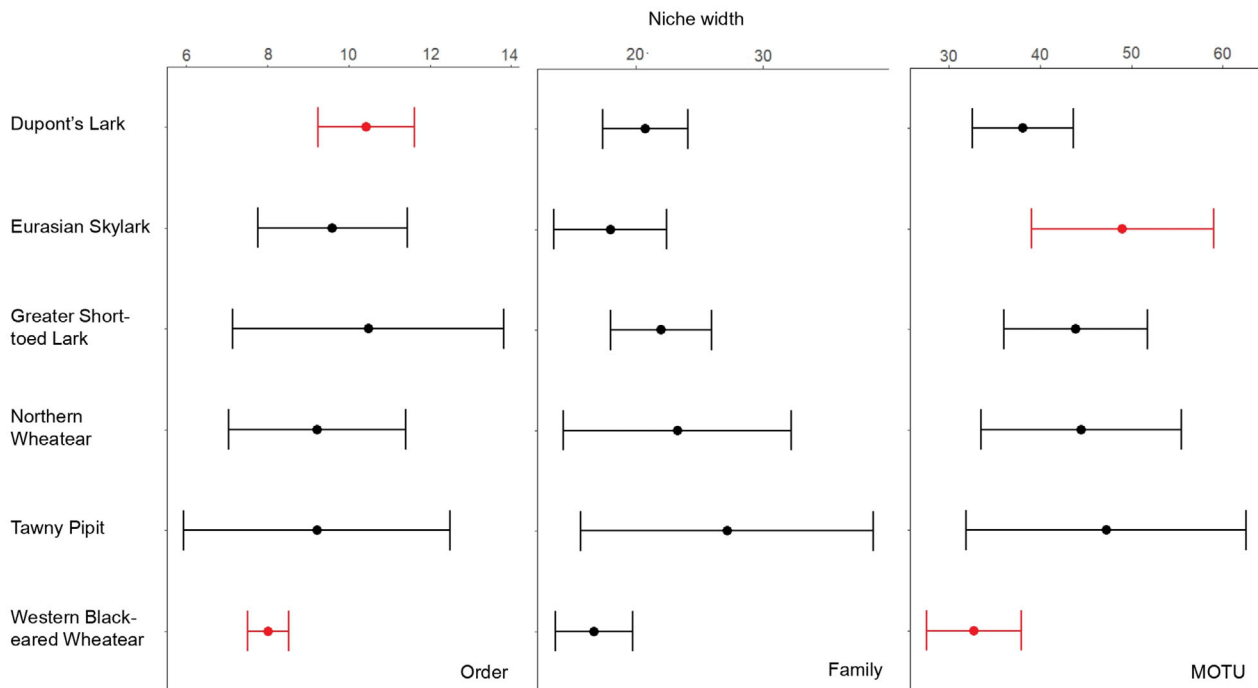
niche overlap values were found between the Eurasian Skylark and the Greater Short-toed Lark and the Northern Wheatear (0.87 in both comparisons), whereas the lowest values were observed between Dupont's Lark and the Greater Short-toed Lark and Tawny Pipit (0.54 and 0.57, respectively). NMDS showed a generally high diet overlap between most bird species, but a higher segregation was observed between Dupont's Lark samples and those of Greater Short-toed Lark and Tawny Pipit (Fig. 4), which is consistent with the results of the dietary overlap analysis (Pianka's index). The kernel distribution estimation plot on NMDS axis 1 showed that Dupont's Lark samples were the least overlapping of all species (Fig. 4).

In line with the above results, we found differences in prey composition between bird species considering the three identification levels (Table 3). At MOTU level, all bird species presented different dietary compositions compared with each other, except the Northern Wheatear with the Eurasian Skylark, Tawny Pipit and Western Black-eared Wheatear (Table 3). At the family level, we found differences in prey composition between Dupont's Lark and four other passerines, Eurasian Skylark, Greater Short-toed Lark, Western Black-eared Wheatear and Tawny Pipit, and at order

**Table 1.** For each arthropod order and for the gastropod clade, frequency of occurrence is presented for overall samples and for each passerine species. Number of samples per bird species is indicated in parentheses. The three most frequently preyed orders by each species are in bold type. Orders that were not found within the diet of a species are marked with '–'.

	Frequency of occurrence (%)						
	All (139)	Dupont's Lark (39)	Eurasian Skylark (19)	Tawny Pipit (14)	Greater Short-toed Lark (24)	Northern Wheatear (18)	Western Black-eared Wheatear (25)
<b>Insecta</b>							
Coleoptera	50	<b>74.4</b>	<b>63.2</b>	<b>57.1</b>	<b>50</b>	<b>55.6</b>	24
Orthoptera	44.2	23.1	<b>68.4</b>	28.6	<b>83.3</b>	<b>44.4</b>	<b>56</b>
Hemiptera	29.2	23.1	36.8	<b>35.7</b>	<b>50</b>	<b>27.8</b>	<b>28</b>
Hymenoptera	17.5	12.8	<b>47.4</b>	14.3	12.5	11.1	24
Diptera	11.7	12.8	21.1	21.4	12.5	16.7	–
Lepidoptera	11	17.9	5.3	–	4.2	11.1	24
Archaeognatha	1.9	2.6	–	–	8.3	–	–
Phasmatodea	1.3	–	5.3	7.1	–	–	–
Blattodea	0.6	2.6	–	–	–	–	–
<b>Arachnida</b>							
Araneae	28.6	<b>25.6</b>	31.6	<b>42.9</b>	25	22.2	<b>48</b>
Opiliones	3.9	7.7	–	–	–	5.6	8
Pseudoscorpiones	0.6	–	–	–	4.7	–	–
<b>Diplopoda</b>							
Julida	20.8	<b>43.6</b>	21	7.1	4.2	16.7	24
<b>Gastropoda</b>							
Panpulmonata	4.55	5.13	5.26	–	8.33	5.56	4





**Figure 3.** Niche width estimates (taxon richness) along with the 95% lower and upper confidence intervals for the six steppe bird species at each taxonomic level. Red colour indicates significantly different pairs.

**Table 2.** Pairwise Pianka's index values among steppe bird species based on the frequency of arthropod families consumed. All values indicate statistically significant niche overlap (i.e. greater than expected by chance based on comparison with 10 000 null models; \* $P = 0.05$  to  $P = 0.01$ , \*\* $P = 0.01$  to  $P = 0.001$ , \*\*\* $P < 0.001$ ).

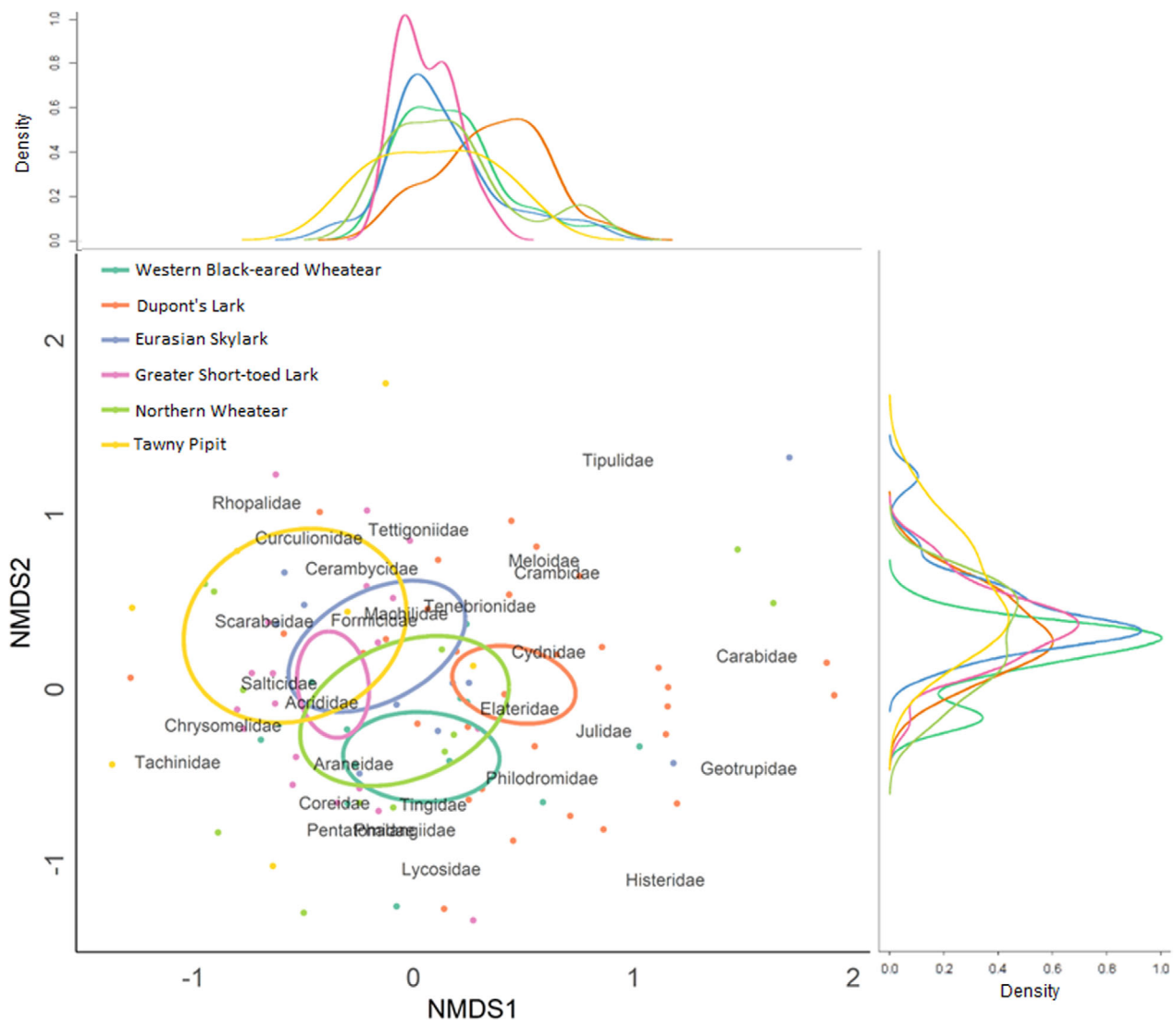
	Dupont's Lark	Eurasian Skylark	Tawny Pipit	Greater Short-toed Lark	Northern Wheatear
Eurasian Skylark	0.70**				
Tawny Pipit	0.57*	0.74*			
Greater Short-toed Lark	0.54*	0.87***	0.68**		
Northern Wheatear	0.75***	0.87***	0.70***	0.81***	
Western Black-eared Wheatear	0.66**	0.83***	0.62**	0.84***	0.82***

level also between the Greater Short-toed Lark and Western Black-eared Wheatear (Table 3). Univariate tests showed that differences in prey composition were mainly due to the MOTUs Acrididae 1 and Julidae 1 (Table S3). Acrididae was the most frequent MOTU across all samples but was more often detected in Greater Short-toed Lark, Eurasian Skylark and Western Black-eared Wheatear samples, while Julidae 1 was more often detected in Dupont's Lark and Western Black-eared Wheatear samples (Table S3). The families Acrididae and Julidae were the main drivers of the differences at the family level

(Table S4) and differences at the order level were mainly due to the orders Orthoptera, Coleoptera and Julida (Table S5).

## DISCUSSION

This study represents the first application of faecal DNA metabarcoding to assess interspecific trophic niche relationships in a shrub-steppe passerine community. Our findings highlight the power of faecal metabarcoding to provide accurate diet assessments and to resolve unknown ecological patterns of food niche overlap and partitioning



**Figure 4.** Non-metric multidimensional scaling (NMDS) of steppe bird prey composition, calculated using the Jaccard index on family-level prey identified in the faecal samples and visualized in two-dimensional space ( $k = 2$ ; stress = 0.126). Points closer to each other correspond to individuals with a more similar dietary composition. Proximity of points to the name of an arthropod family indicates the likelihood of individuals to have consumed that family. Ellipses represent the standard error of the bird species diet centroid at 95% confidence. Each axis shows the kernel probability density function of NMDS axis 1 and axis 2 among all the steppe bird species. Density plots might not align perfectly with the points inside the NMDS plot because of scale differences between the axes.

among small passerine birds. We found that the shrub-steppe bird species analysed in this study consumed a diverse array of arthropod prey, including 39 families from 13 different orders, supplemented by occasional gastropod prey, probably small snails (Cramp 1988). At the community level, beetles (Coleoptera) of the families Tenebrionidae, Curculionidae, Scarabeidae, Carabidae and Chrysomelidae, and grasshoppers (Orthoptera,

Acrididae) were the most frequently consumed taxa across bird species.

Beetles have been described as the most important invertebrate taxa in the diet of a wide variety of birds of different habitats (Rotenberry 1980, Holland *et al.* 2005, Buchanan *et al.* 2006), representing a clear preference in prey selection by insectivorous species, probably due to their high levels of macronutrients and nutritive quality

**Table 3.** Results of the generalized linear models for multivariate abundance data considering three taxonomic levels: molecular operational taxonomic unit (MOTU), family and order. Pairwise comparisons between each bird species are also shown. Significant *P* values are in bold type.

	MOTU		Family		Order	
	Deviance	<i>P</i> value	Deviance	<i>P</i> value	Deviance	<i>P</i> value
Multivariate test	597.5	<b>&lt;0.001</b>	254.2	<b>&lt;0.001</b>	129.7	<b>&lt;0.001</b>
<b>Pairwise comparison</b>						
Dupont's Lark – Eurasian Skylark	147.54	<b>&lt;0.001</b>	67.67	<b>0.004</b>	32.96	<b>0.02</b>
Dupont's Lark – Tawny Pipit	124.23	<b>0.002</b>	60.04	<b>0.02</b>	22.70	0.23
Dupont's Lark – Greater Short-toed Lark	158.69	<b>&lt;0.001</b>	86.92	<b>&lt;0.001</b>	55.65	<b>&lt;0.001</b>
Dupont's Lark – Northern Wheatear	88.24	<b>0.04</b>	33.75	0.36	11.02	0.78
Dupont's Lark – Western Black-eared Wheatear	122.64	<b>0.002</b>	65.58	<b>0.005</b>	39.19	<b>0.005</b>
Eurasian Skylark – Tawny Pipit	98.10	<b>0.04</b>	42.50	0.23	12.56	0.76
Eurasian Skylark – Greater Short-toed Lark	100.52	<b>0.04</b>	40.08	0.23	18.48	0.45
Eurasian Skylark – Northern Wheatear	75.35	0.12	27.91	0.36	11.47	0.78
Eurasian Skylark – Western Black-eared Wheatear	99.89	<b>0.04</b>	44.59	0.17	26.43	0.11
Tawny Pipit – Greater Short-toed Lark	91.87	<b>0.04</b>	42.39	0.23	20.35	0.35
Tawny Pipit – Northern Wheatear	78.19	0.12	33.84	0.36	8.87	0.78
Tawny Pipit – Western Black-eared Wheatear	103.93	<b>0.03</b>	40.77	0.23	26.34	0.11
Greater Short-toed Lark – Northern Wheatear	98.99	<b>0.04</b>	46.07	0.17	16.38	0.58
Greater Short-toed Lark – Western Black-eared Wheatear	100.72	<b>0.04</b>	50.06	0.11	34.80	<b>0.01</b>
Northern Wheatear – Western Black-eared Wheatear	68.25	0.12	35.32	0.32	16.37	0.58

(Razeng & Watson 2014). At our study site, beetles are very abundant and diverse (Traba *et al.* 2022), with more than 15 different families. Grasshoppers are also abundant at the study site (Traba *et al.* 2022), primarily diurnal (Ingrisch & Rentz 2009) and a prey with a high nutritive value (Ueckert *et al.* 1972, Razeng & Watson 2014), representing an important food source for numerous insectivorous and omnivorous birds (Sullins *et al.* 2018, Hebda *et al.* 2019, Cabodevilla *et al.* 2021).

Other important arthropod taxa for the overall shrub-steppe bird community were hemipterans (Hemiptera) and spiders (Araneae), also essential prey for insectivores in terms of fat and protein supply (Razeng & Watson 2014). In this study, the plant component of samples has not been analysed (see Bioinformatic analysis section), but it could represent a non-negligible fraction of the diet of some of the study species, as pointed out by some authors (Green 1980, Donald & Vickery 2000). For instance, the Eurasian Skylark in Iberian shrub-steppes has been reported to consume a high percentage of green material and seeds during autumn and winter (Suárez *et al.* 2009). Another study conducted in the shrub-steppes of southern Spain also reported occasional

consumption of fleshy fruits by the Western Black-eared Wheatear in the post-breeding period (Hóðar 1998). However, these studies concur with the predominance of invertebrate prey in the diet of these species during the breeding season, which provides confidence that our dietary niche results are as complete as possible with the applied methodology for the sampled period.

The endangered Dupont's Lark most frequently consumed beetles, followed by spiders (mainly Lycosidae), millipedes (Julida, Julidae), grasshoppers, hemipterans (primarily burrower bugs, Cydnidae) and lepidopterans (Lepidoptera). Within Coleoptera, the families Tenebrionidae, Carabidae and Curculionidae were the most preyed on by this passerine. The scarce previous literature concerning the trophic ecology of Dupont's Lark also points out the importance of beetles in its diet (Cramp 1988, Aragüés 1992, Talabante *et al.* 2015). In another study, beetles appear to be a key factor in determining the intensity of space use by this species (Gómez-Catasús *et al.* 2019). Likewise, spiders of the family Lycosidae were also reported as one of the most important groups in the diet of the nestlings of Dupont's Lark (Herranz *et al.* 1993), which is evidence of its relatively specialized behaviour in the capture

of this group of spiders. However, this is the first time that a high consumption frequency of millipedes of the family Julidae by Dupont's Lark has been reported, with a Julidae MOTU being the most frequently detected in its faeces. Such new insights into the diet of a critically endangered passerine may be of great importance for the implementation of future conservation measures to influence the availability of those arthropods most often consumed by this species.

Eurasian Skylark individuals analysed in this study more often preyed on grasshoppers, beetles of the families Curculionidae, Tenebrionidae and Scarabeidae, and ants (Hymenoptera, Formicidae), followed by hemipterans and spiders. Ants are not usually listed as one of the main food resources of this species (Green 1980, Cramp 1988, Ottens *et al.* 2014), and are among the least energetically valuable prey items (Razeng & Watson 2014), but they are a dominant arthropod group in Mediterranean semi-arid terrestrial ecosystems (Piñero *et al.* 2011), suggesting that they were probably consumed because of their high abundance and ease of capture. Tawny Pipit in our study more frequently consumed beetles (principally Scarabeidae and Chrysomelidae), spiders, hemipterans and grasshoppers. Several dietary studies compiled by Cramp (1988) conducted in the Palaearctic also included these arthropod prey as among the most frequent in this species' diet. Grasshoppers were the main prey item consumed by the Greater Short-toed Lark, followed by beetles and hemipterans. This high grasshopper consumption is not reported in the scarce information on the food ecology of the Greater Short-toed Lark (de Juana *et al.* 2020). The diet of the Northern Wheatear in our study was based mainly on beetles, grasshoppers and hemipterans (primarily stink bugs, Pentatomidae), with an important contribution of spiders and millipedes, in line with previous studies (Dunn *et al.* 2020). Finally, the Western Black-eared Wheatear more frequently preyed on grasshoppers, spiders (mainly Lycosidae) and stink bugs, consuming beetles, ants, millipedes and lepidopterans to a lesser extent. These results are consistent with previous studies demonstrating that grasshoppers and hemipterans were positively selected by this passerine in two Iberian shrub-steppes (Hódar 1998).

Based on resource partitioning theory (MacArthur & Levins 1967, Chesson 2000), we predicted that the space-sharing passerines in the

shrub-steppe would prey on different food resources to limit interspecific competition. However, we reported significant community-level overlap, primarily driven by a high degree of dietary similarity between the Northern Wheatear and all the other bird species, and between the Eurasian Skylark and the species Greater Short-toed Lark and Western Black-eared Wheatear (Table 2). This dietary overlap may represent the similarity of feeding strategies, as these shrub-steppe bird species forage mainly on the ground, by walking or running and picking up prey from the soil surface (de Juana *et al.* 2004, Serrano & Astrain 2005), though the Western Black-eared Wheatear, and occasionally the Northern Wheatear, also forage from perches (shrubs, rocks or fences; Santos & Suárez 1985, Dunn *et al.* 2020, Collar 2021). On the other hand, the trophic niche overlap detected may reflect a peak period of arthropod abundance and availability, coinciding with these bird species' breeding season, and indicate shared exploitation of abundant food resources by bird species during the breeding season (see, for example, Rotenberry 1980, Orłowski & Karg 2013). In periods when food is in short supply, differences between species in diet composition are likely to be more evident (MacArthur & Pianka 1966, Schoener 1971, Bumelis *et al.* 2021, Mansor *et al.* 2021).

The least degree of trophic niche overlap was found when comparing the diet of Dupont's Lark with the diets of the other shrub-steppe passerines, especially with the Greater Short-toed Lark and the Tawny Pipit (Table 2). Moreover, diet composition analysis showed the same pattern, indicating differences in prey composition between Dupont's Lark and the other species at order, family and MOTU level, with some exceptions (Table 3). It should be noted, however, that differences in prey composition among bird species were conditioned by the level of identification reached and, hence, by the taxonomic resolution provided by the primer set used. Therefore, Dupont's Lark appeared to be the species with the most distinct dietary niche within the community. Focusing on the family level, a good surrogate of species guilds and diversity in arthropods (Cardoso *et al.* 2011, Slimani *et al.* 2019), Dupont's Lark differs from the other species studied mainly in its higher preference for millipedes (Julidae), ground beetles (Carabidae) and burrower bugs (Cydnidae), and the exclusive consumption of histereid beetles



(Histeridae), chironomids (Diptera, Chironomidae) and cockroaches (Blattodea, Ectobiidae). In addition, as mentioned briefly above, Dupont's Lark exhibited greater resource partitioning with the Tawny Pipit and, particularly, the Greater Short-toed Lark. These three species are passerines with very similar ecological traits and foraging habits that seem to be partitioning to some extent the available resources in the shrub-steppe environment to relax the effects of potential interspecific competition, although a sharing of the most abundant resources also occurs, as competition occurs basically for the most limiting resources. The bill is the most distinctive morphological trait of Dupont's Lark (curved and long, exceeding 2 cm in length; Gómez-Catasús *et al.* 2016, García-Antón *et al.* 2018). Given the close relationship of bill size and shape with feeding substrates and foraging methods (Lederer 1975, Remsen 2003), our results suggest that the differential evolution of this trait, an ecological phenomenon known as character displacement (Dayan & Simberloff 2005), might be a potential explanation for differences in prey composition and resource segregation of this bird species with other passerine species. Hence, we detected in Dupont's Lark samples greater consumption of prey favoured by its bill morphology: large and worm-like arthropod prey hidden or living in soil or under stones, such as millipedes, ground beetle larvae, burrower bugs or spiders.

To the best of our knowledge, this study is the first to use faecal DNA metabarcoding to investigate the trophic niche of a steppe passerine assemblage. Previous studies dedicated to the diet of these species used conventional methodologies, especially microscopic examination of prey remains from faeces (Hódar 1998, Donald & Vickery 2000, Talabante *et al.* 2015) or stomach contents (Cramp 1988, Aragüés 1992). However, these techniques are more time-consuming and, in addition to the difficulty of identifying taxa from masticated and semi-digested fragments, are unable to identify soft-bodied animals that leave few or no hard remains (Pompanon *et al.* 2012, da Silva *et al.* 2019). DNA metabarcoding typically provides a more accurate identification of taxonomic composition of degraded samples, such as faeces, representing a powerful tool for ecologists. However, some drawbacks of DNA-based dietary assessments have also been reported, such as the inability in most cases to obtain reliable

quantitative information of dietary prey items, due to differences in digestion rates for DNA from different species, and DNA extraction, amplification and sequencing biases (Piñol *et al.* 2018). For that reason, our study has provided a dietary niche assessment based on presence/absence data. Furthermore, the results of dietary DNA-based analyses are dependent on the primer set used as well as on the completeness of the taxonomic reference database. In addition, DNA metabarcoding does not allow distinction of arthropods at different life stages. Therefore, our molecular analysis was incapable of determining whether arthropods were consumed as adults, larvae or eggs. This missing information also plays an important role in resolving the dietary resource partitioning of the community, because different bird species may prey on the same arthropod group but with different life-stage preferences (Sherry *et al.* 2016), which could indicate smaller diet overlap than suggested by our results.

Our molecular approach used a single 18S primer set designed for the detection of diet and parasites of birds (Cabodevilla *et al.* 2022), but as it is a universal eukaryotic marker, the sequencing results provided a large number of reads assigned to non-target taxonomic groups (fungi, algae, internal parasites, etc.), yielding a small per-sample number of reads of the target taxa (mainly arthropods). Increasing the sequencing depth when using such generalist primers would be desirable to ensure that sufficient dietary reads are obtained per sample. This primer limitation might also be addressed using arthropod-specific primer sets, such as those targeting the cytochrome *c* oxidase subunit I (COI) gene, e.g. ZBJ (Zeale *et al.* 2011), Fwh2 (Vamos *et al.* 2017) or ANML (Jusino *et al.* 2019), which has the most extensive reference library for arthropods (BOLD Systems, Ratnasingham & Hebert 2007). However, several studies have highlighted that some COI primers might provide biased taxonomic results (Clarke *et al.* 2014, da Silva *et al.* 2019). Therefore, the choice of primer sets to use is a critical decision in DNA metabarcoding, and can even affect the results obtained (Alberdi *et al.* 2019). In our study, the inclusion of COI markers and/or other techniques (e.g. visual examination of faecal contents) might have provided additional dietary information that would enable a different assessment of trophic niche overlap among steppe passerines, probably leading to a decrease in overlap.

In our approach, we only included one type of control (negative PCR control). Despite the increased cost and work hours, incorporating more controls for different processing steps, for example a field sampling negative control or a positive control (DNA extracts from known taxa), may also be useful to address other potential sources of contamination. Nevertheless, the most relevant sources of contamination in the field have been identified and/or controlled (e.g. human manipulation; or interspecific contamination in collecting bags, avoided through the use of bleach washing) and, because microorganisms, plants and very small arthropods have not been analysed, we are reasonably confident that relevant biases have been avoided.

Our primer set showed an excellent taxonomic resolution capacity at the ordinal level, and good resolution at the family level. Although prey identification at higher taxonomic resolution (genus and species) remained unresolved in some cases, our faecal DNA metabarcoding results make a significant contribution to the knowledge of shrub-steppe bird diets, for some of which the dietary information was very scarce. For instance, we described here for the first time the high frequency of millipede consumption by the critically endangered Dupont's Lark. In addition, the dietary niche overlap observed in the overall community reflected the similar foraging behaviours and shared abundant food resources, such as grasshoppers, beetles or spiders, which could lead to negative biotic interactions (Barrero *et al.* 2023). However, we detected differences in prey composition, indicating to some extent resource partitioning among species, notably Dupont's Lark and Greater Short-toed Lark. Regarding endangered species, these findings provide valuable ecological insight for the design of biodiversity and conservation programmes in the increasingly scarce and threatened steppe habitats. Further research with higher taxonomic resolution primer sets, including arthropod-specific COI markers, as well as covering the non-breeding season, other regions and less natural habitats, may describe species' diets in greater detail and reveal trophic relationships that were undetected in this study.

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## AUTHOR CONTRIBUTIONS

**Julia Zurdo:** Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Adrián Barrero:** Conceptualization; investigation; writing – review and editing. **Luís Pascoal da Silva:** Conceptualization; data curation; formal analysis; methodology; resources; writing – review and editing. **Daniel Bustillo-de la Rosa:** Conceptualization; investigation; writing – review and editing. **Julia Gómez-Catasús:** Conceptualization; investigation; writing – review and editing. **Manuel Morales:** Conceptualization; methodology; project administration; supervision; writing – review and editing. **Juan Traba:** Conceptualization; funding acquisition; methodology; project administration; supervision; writing – review and editing. **Vanessa A Mata:** Conceptualization; data curation; formal analysis; methodology; resources; writing – review and editing.

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## CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

## ETHICAL NOTE

None.

## DATA AVAILABILITY STATEMENT

Data used in this study are provided as Table S2. Sequence reads can be found at NCBI (BioProject PRJNA928438).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Appendix S1** Bioinformatic pipeline description.

**Figure S1** Graphical results of the niche overlap analysis.

**Table S1** Descriptive information about the six shrub-steppe passerine species studied.

**Table S2** Prey items detected in 139 faecal samples from six steppe bird species captured in Soria, central Spain.

**Table S3** Frequency of occurrence and univariate tests from the generalized linear models for multivariate abundance data at the molecular operational taxonomic unit (MOTU) level.

**Table S4** Frequency of occurrence and univariate tests from the generalized linear models for multivariate abundance data at the family level.

**Table S5** Univariate tests from the generalized linear models for multivariate abundance data at the order level.