



DNA metabarcoding of faecal samples reveals life-history traits in the critically endangered bush-cricket

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Abstract

Understanding the diet composition of herbivorous insects is crucial for the conservation of threatened species and their habitats. This study investigated the diet of the critically endangered Bei-Bienko's Plump Bush-cricket (*Isophya beybienkoi*) using DNA metabarcoding of faecal samples collected from 49 individuals. A total of 28 families and 52 genera of plants were identified, with an average of 9.0 (± 2.77) plant taxa per sample. The diet consisted exclusively of dicotyledonous plant species, dominated by herbaceous flowering plants, which accounted for 76.5% of all the species identified. The most abundant families were Fabaceae (34.9%), Apiaceae (19.8%) and Rosaceae (16.9%). Key species included *Securigera varia* (26%; Fabaceae), *Peucedanum cervaria* (16%; Apiaceae) and *Prunus spinosa* (11%; Rosaceae). A complementary vegetation survey recorded 178 vascular plant species—primarily herbaceous plants—in the bush-cricket's habitat. Of these, 31 taxa were found in both the faecal samples and vegetation plots. Notably, several plant families, including Fabaceae and Orobanchaceae, were significantly more prevalent in faecal samples than in vegetation plots. This study showed the selection of nitrogen-rich herbs with higher water content, indicating the insect's life-history traits and nutritional needs in the extreme environment of the Slovak Karst National Park. Overall, this study provides critical insights into the bush-cricket's ecological requirements and underscores the importance of specific habitat types and plant species for its conservation.

Implications for insect conservation

Our results show that Bei-Bienko's Plump Bush-cricket exclusively consumes dicotyledonous plants, with a strong preference for nitrogen-rich herbs with higher water content. Many of the plant species found in this study also serve as forage plants for ruminants in extensive meadows or pastures. Extensive grazing therefore appears to be the most appropriate conservation measure that could preserve the habitat of this critically endangered endemic, including the water-rich legumes important for its growth and reproduction.

Keywords Conservation · Diet · *Isophya* · Karst environment · Life-history traits · Orthoptera

Introduction

Diet composition is fundamentally important for ecology, evolutionary adaptations and trophic interactions among animals (Chambers et al. 1996; Behmer and Joern 2008; Rytönen et al. 2019; Tang et al. 2023). Knowledge on diet provides key information on conservation needs and extinction risk, which is important for many threatened species (Liu et al. 2018; Yamamoto and Uchida 2018; Goldberg et al. 2020).

Herbivorous insects are a highly species-rich group with extremely diverse feeding strategies that may change during

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their life cycle (Bernays 1998; Hochuli 2001; König et al. 2022). A substantial proportion of herbivores are specialists (Chambers et al. 1996; Bernays 1998; Behmer and Joern 2008), whose survival greatly depends on one or a few specific host plants. This significantly contributes to the risk of extinction, for example, when a food plant is scarce (Sánchez-Bayo and Wyckhuys 2019; Dong et al. 2020; Habel et al. 2022). Specialist herbivores are thus more susceptible to changes in the plant community composition than generalist herbivores, which can consume a diverse range of plant taxa (Yamamoto and Uchida 2018). However, generalists also exhibit dietary selection, for example, when balancing their nutritional requirements for growth or reproduction (Bernays 1998; Yamamoto and Uchida 2018). Therefore, some generalist herbivores are able to mix their diet in clearly defined, species-specific nutritional niches, depending on the level of macronutrients rather than discrete plant taxa (Behmer and Joern 2008; Unsicker et al. 2008; Franzke et al. 2010; Ibanez et al. 2013; Pitteloud et al. 2021; König et al. 2022). This may be complicated by dietary differences between males or females and different life-history traits of the given species (Hochkirch et al. 2000, 2007; Hochkirch and Gröning 2008; Clark et al. 2013, 2015; Lan et al. 2021). As a result, the extinction risk for generalists is not always directly linked to changes in plant species composition. Instead, population declines are more commonly driven by the loss of suitable habitats (Gröning et al. 2007; Čelik and Vreš 2018; Yamamoto and Uchida 2018), including the disappearance of entire species-specific nutritional niches, as discussed above. For instance, many flightless herbivorous generalists are unable to migrate between isolated habitat patches. They are thus more susceptible to extinction than species capable of flight (Weyer et al. 2012; Heller 2020; Nuhlíčková et al. 2021b, 2023b). Vegetation structure is crucial for many insect species that use their host plants not only as a food source, but also as a shelter or refuge from predators (Hochuli 2001; Bauer and Kenyeres 2006; Kenyeres and Bauer 2008, 2021).

Bei-Bienko's Plump Bush-cricket (*Isophya beybienkoi*, Mařan 1958), endemic to the Slovak Karst (southern Slovakia, Central Europe), is an example of a critically endangered flightless species (Chobanov 2016; Nuhlíčková et al. 2023b). Like other species of the genus *Isophya*, it is assumed to be herbivorous (Ingrisch and Köhler 1998). Almost nothing has generally been known about the biology of this insect, aside from very few studies on its morphology, distribution and bioacoustics (Mařan 1958; Orci et al. 2001; Heller et al. 2004; Kriřtín et al. 2009, 2020) and several general statements about the herbivory of this genus (e.g. Ingrisch and Köhler 1998; Grzywacz et al. 2010). Only recently has study on the movement patterns of *I. beybienkoi* revealed its very low mobility, indicating a strong

dependence on small, isolated patches of natural and semi-natural calcareous grasslands (Nuhlíčková et al. 2023a). A strong link to a particular habitat may indicate a narrow dietary pattern, as shown in many herbivorous insects (Forister et al. 2015). However, most Orthoptera species are considered to be generalist feeders, dependent on specific habitat types. They consume a greater variation of the plant taxa rather than one or few species, mixing their diets, for example, to increase fitness (Bernays 1998; Ingrisch and Köhler 1998; Hochkirch et al. 2000; König et al. 2022). Nevertheless, certain groups show distinct preferences for specific plant families. For example, while grasshoppers (Gomphocerinae) often prefer grasses (Poaceae), some species of the family Tettigoniidae prefer forbs (dicotyledons). Groundhoppers (Tetrigidae), instead, choose algae, lichens or mosses (reviewed König et al. 2022). Earlier results on the habitat requirements of *I. beybienkoi* showed that it occurs in ecotonal habitats at the edge of sparse vegetation, shrubs or fringes. No association with a specific plant community or taxon was shown (Nuhlíčková et al. 2021a, 2023a). Therefore, detailed information on dietary and microhabitat requirements is still poorly understood and crucial for the design of appropriate conservation measures for this critically endangered species.

In this study, DNA metabarcoding was selected as a suitable method to examine the diet of the target species (Taberlet et al. 2012). Compared to traditional methods, which are often time-consuming (Čížek 2005; Kaňuch et al. 2015) or harmful to the animal (Pérez-Cobas et al. 2015; Brunetti et al. 2022), DNA metabarcoding makes it possible to identify even small and highly digestible food items that cannot be detected using other methods (Taberlet et al. 2012). The entire procedure includes the non-invasive collecting of faeces, thus ensuring the full survival of the insects and their careful release back into the habitat. It thus represents an ideal tool for conservation purposes (Groom et al. 2017; Young et al. 2020).

The main objectives of this study were (1) to examine the plant composition in faecal samples collected from 49 individuals of *I. beybienkoi* in order to identify the diet composition of this threatened species, (2) to compare the plant composition found in faeces and vegetation plots sampled in situ to identify possible dietary preferences, and (3) to examine the life-history traits of plants found in the diet and habitats to gain a comprehensive picture of the bush-cricket's ecological requirements.

We expected the diet of the threatened bush-cricket will copy the overall composition of the vegetation found under in situ conditions, which may indicate the key importance of the habitat for a species with low mobility (Nuhlíčková et al. 2023b). We also expect DNA metabarcoding will provide more detailed information on the dietary preferences,

capturing plant taxa that may not be detected by vegetation plots. In this context, plant characteristics, such as height, leaf thickness or size, were described to understand whether a given plant is consumed or used as an important refuge, e.g., against predators, or as a shelter due to favourable vegetation architecture (Bauer and Kenyeres 2006; Kenyeres et al. 2017, 2018; Kenyeres and Varga 2023). Furthermore, the nutritional requirements of *I. beybienkoi* may include the consumption of specific plants, which would suggest a relationship between bush-cricket life-history traits (e.g., low locomotory skills, hidden way of life) and food preferences that require consuming a nutrient-rich diet (Lehmann 2012; Lewis and South 2012; Clark et al. 2013, 2015; Lan et al. 2021). Thus, context-dependent resource specialization below a certain plant taxonomic level might be expected, assuming that the bush-cricket mix its diet, for example, to enhance its fitness (Chambers et al. 1996; Bernays 1998; König et al. 2022). We therefore hope that this study will provide essential information to support the development of conservation strategies, specifically through the identification of priority habitats for the protection of this critically endangered species.

Methods

Study species

The genus *Isophya* is one of the most species-rich genera of bush-crickets in Europe (more than 90 species) (Chobanov 2009; Grzywacz-Gibała et al. 2010). Bei-Bienko's Plump Bush-cricket (*I. beybienkoi*, Mařan 1958) is endemic to the Slovak Karst National Park (NP) (southern Slovakia, Central Europe). It is a relatively large (up to 26 mm) species with well-developed acoustic communication (Orci et al. 2001). It reproduces from June to September (Chobanov 2016; Nuhlićková et al. 2023b). Like other species of this genus, *I. beybienkoi* is characterised by mostly crepuscular to nocturnal activity – an adaptation for protection against predators and to avoid overheating or dehydration (Varga and Kenyeres 2025). Similar to other closely related species, both females and males move mainly in the evening and at night, staying in the higher (40–60 cm) areas of the vegetation (Heller et al. 2004; Varga and Kenyeres 2025). The male complex song is highly characteristic and differs from all the songs that have been described in other species from the genus *Isophya*. Distinctive features of the song include the presence of two specific syllable types, a characteristic rhythm and sequence of syllable repetition, and the relatively short duration of the syllables, resulting in the species-typical song (Orci et al. 2001).

I. beybienkoi occurs in very small (up to 30 adults ha⁻¹) and isolated subpopulations with an occurrence area of only 24 km² in karstic grasslands up to 800 m a.s.l (Chobanov 2016; Kriřtín et al. 2020).

Study site

The study was conducted in the Slovak Karst National Park (48.5984806°N; 20.6351703°E). The karst environment is associated with rich xerophilous steppe vegetation on sunny slopes, rocky fields and plateaux edges (Dúbravková-Michálková et al. 2008). Prior to this study, all historical, potential and currently known sites had been intensively surveyed by slowly walking through the relevant habitats and sweeping the vegetation with a net to detect *I. beybienkoi* (Wagner 2015; Nuhlićková et al. 2023b). A total of 484 km of transects were carried out in this way in 2021 (254 km) and 2022 (230 km) in all potential habitats (Nuhlićková et al. 2023b). All eight subpopulations discovered throughout the entire Slovak Karst National Park were subsequently included in this study (Fig. 1). The locations were mostly in remote areas on karstic plains at elevations from 358 to 675 m a.s.l. (see Supplementary Material 1). This habitat was primarily characterised by fringe vegetation rich in broad-leaved flowering plants in transitional zones between forest edges, xerophilous grasslands and solitary trees (Dúbravková-Michálková et al. 2008; own unpublished data).

Collection of faeces

Faecal sampling was carried out in all eight subpopulations in 2022 (Fig. 1). The aim was to find 10 adult individuals (5 males and 5 females) in each subpopulation. Due to the very low numbers and rarity of the species in the field, a total of 49 individuals were collected (22 males and 27 females) (see Supplementary Material 1). The captured animals were very carefully placed into sterile 50 ml Falcon® tubes, where they typically produced one or two faecal pellets in a very short time (Fig. 2). After defecation, the insects were released back into their habitat. The faeces were then put into pure ethanol (95%) and frozen.

Vegetation survey and determination of life-history traits

While the faeces were being collected, vegetation surveys were also conducted on the same positive plots — that is, the plots where *I. beybienkoi* was present (F1–F8, see Supplementary Material 3, Fig. 1), using the standard methodology of the Zürich-Montpeliér school and the Braun-Blanquet cover-abundance scale (Braun-Blanquet 1964; Westhoff

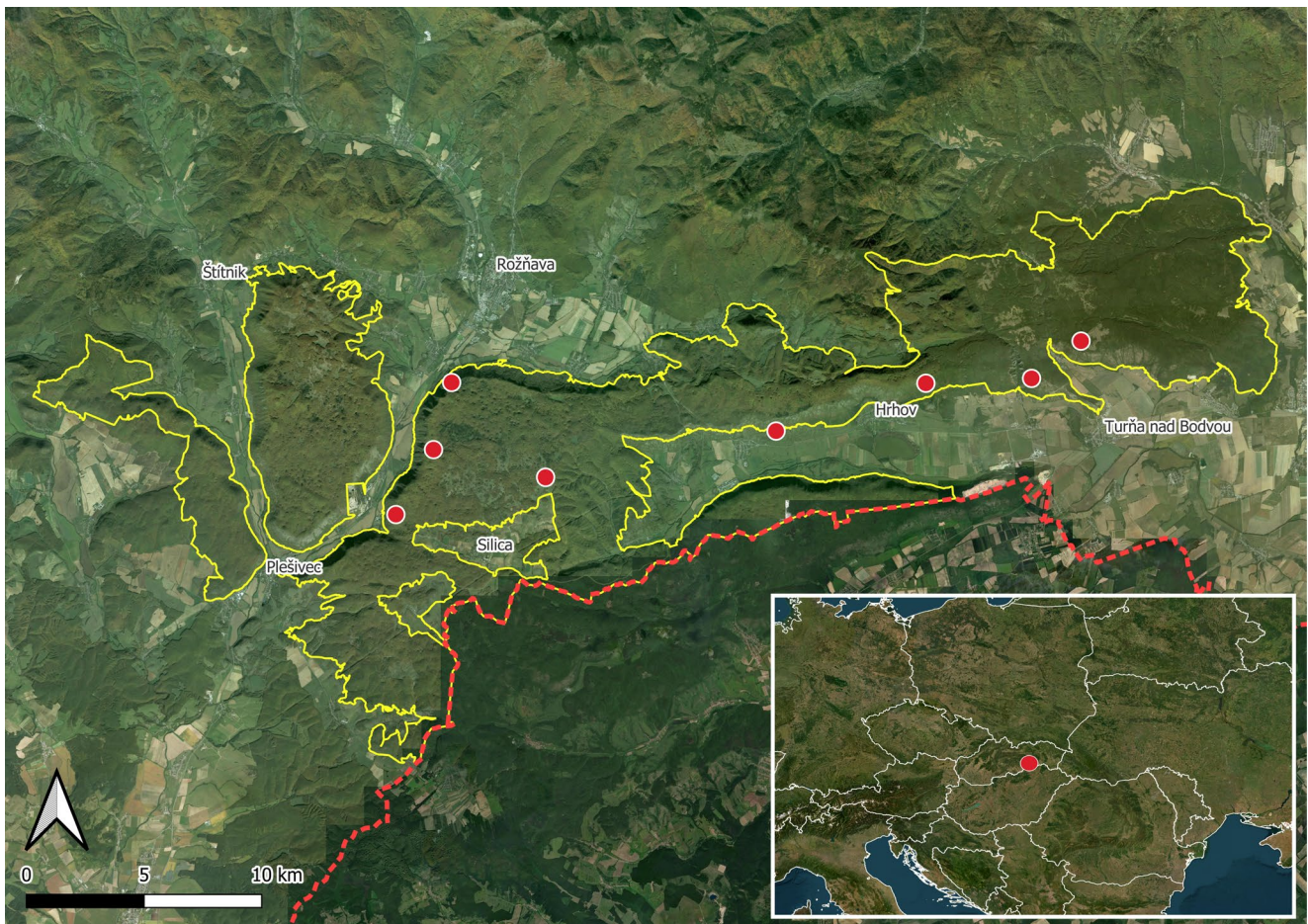


Fig. 1 The eight subpopulations of *I. beybienkoi* included in this study and their locations (in red) within the Slovak Karst National Park (southern Slovakia, Central Europe, right)

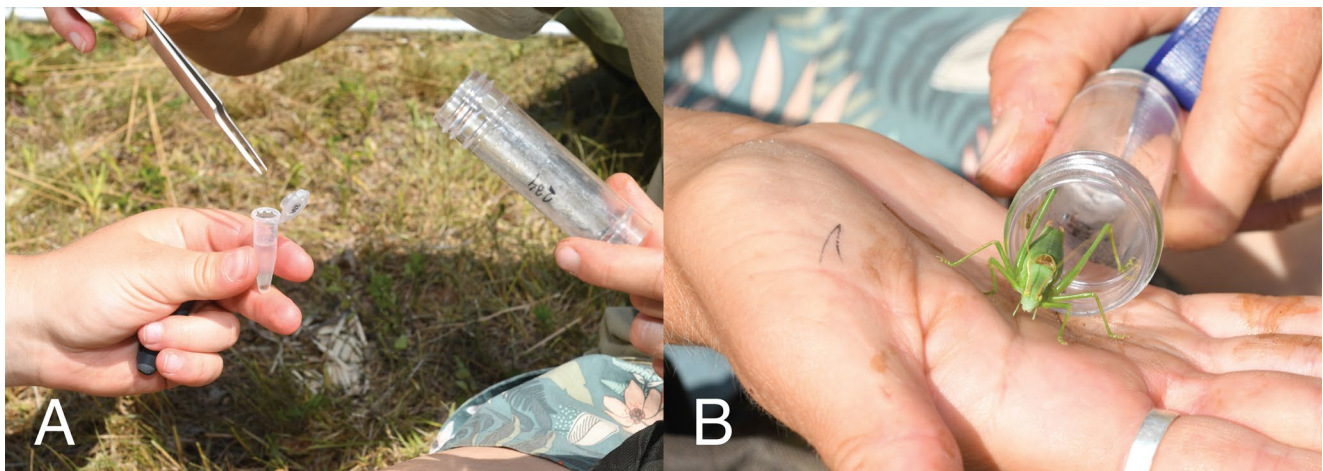


Fig. 2 Faecal sampling procedure: (A) faecal pellets collected in tubes; (B) return of the animal from the tube to its habitat. Photo: Ján Svetlík

and van der Maarel 1973). At each sampling site, vegetation data were sampled on 1–2 equal-sized squares (5 m x 5 m). All vascular plant species were recorded in each plot to determine their cover (%), which was estimated using

the extended 9-degree Braun-Blanquet cover-abundance scale (Barkman et al. 1964; van der Maarel 1979). The obtained vegetation data were stored in the TURBOVEG database (Hennekens and Schaminée 2001) and processed

in the Juice software ver. 7.0 (Tichý 2002). The number of plant species (n) and the total cover of herbs, grasses and shrubs (%), as well as dicotyledonous versus monocotyledonous species (%), were expressed to give information on the microhabitat requirements of the target species (Supplementary Material 3).

The life-history traits of all plants identified in positive vegetation plots as well as the faecal samples were obtained from the Pladias and BIEN databases. The Pladias database (Database of the Czech Flora and Vegetation, <http://www.pladias.cz/>), focused on the flora of the Czech Republic (which is very similar to our study locality), serves as a comprehensive resource for information on plant species, including their distribution, taxonomy, ecology and plant traits. The BIEN (Botanical Information and Ecology Network, <https://bien.nceas.ucsb.edu/bien/>) database provides access to data on plant species from various sources, including the LEDA traitbase (<http://www.leda-traitbase.org/LEDAportal/>) and the datadryad – a curated online repository designed to facilitate the sharing and preservation of research data (<https://datadryad.org/>). The single traits obtained from Pladias were plant height (cm), leaf shape and leaf arrangement. The traits leaf area (mm), leaf area per leaf dry mass ($\text{m}^2 \text{kg}^{-1}$), leaf dry mass (g), and leaf dry mass per leaf fresh mass (mg g^{-1}) (see Supplementary Material 4) were computed from the BIEN database as the mean values of all resources available using the r-BIEN package (Maitner et al. 2018). The computed traits were described as follows:

Leaf area per leaf dry mass ($\text{m}^2 \text{kg}^{-1}$) – is computed as the ratio of the leaf area (in square metres) to the dry mass (in kilograms) of the leaf. Plants with a high leaf area relative to their dry mass tend to have thinner leaves and more surface area for photosynthesis. These plants often grow quickly and are adapted to environments with ample resources, such as high light and moisture availability. In contrast, plants with a lower ratio typically have thicker, denser leaves that are more resistant to environmental stresses (e.g., drought, herbivory). They often prioritise resource conservation over rapid growth and are often found in harsher conditions.

Leaf dry mass (g) – represents the weight of leaves after all moisture content has been removed (by various drying methods).

Leaf dry mass per leaf fresh mass (mg g^{-1}) – is the ratio of the leaf dry mass to its fresh mass (which includes the water content). This ratio indicates the proportion of dry material in a leaf relative to its total weight when it is fresh. A higher leaf dry mass-to-fresh mass ratio suggests that a larger proportion of the leaf's weight is made up of structural and metabolic components (cellulose, lignin, etc.) rather than water. A lower ratio indicates that the leaf contains a higher proportion of water. In light of diet preferences, leaves with a

higher dry mass relative to fresh mass typically have thicker cuticles and denser tissues.

The traits were compared using two different datasets. In the first step, all species occurring in faeces and vegetation plots were assigned according to their presence in the diet or habitat, and the two groups were compared. In the second step, only the 31 species identified in both vegetation plots and in faecal samples were compared. For a complete overview of all vegetation traits, see Supplementary Material 4.

Laboratory work and diet identification

DNA was extracted at the molecular lab of the Leibniz Institute for Biodiversity Change Analysis (LIB), Hamburg, using the standard protocol of the Qiagen DNA Blood & Tissue kit, which has proven efficient in previous metabarcoding studies (Hawllitschek et al. 2017). All further steps were conducted by an external service provider (AIM – Advanced Identification Methods GmbH, Leipzig, Germany) (<https://www.metabarcoding.com/>).

The library was prepared using the provider's standard metabarcoding protocol, including amplification with primers of the ITS2 locus, a standard marker for plant DNA metabarcoding (Fahner et al. 2016; Banchi et al. 2020). The library was then sequenced on an Illumina MiSeq v3.0 machine with 2×300 bp read length.

The quality of the raw data was checked using FastQC 0.11.9 (Andrews 2019). Paired-end merging of the reads was performed using usearch v11.0.667 (Edgar 2010), with the parameters -fastq_maxdiffs 99 -fastq_pctid 75 -fastq_trunc-tail 0. Primers were removed using cutadapt 4.1 (Martin 2011). Sequences were then quality filtered using vsearch 2.21.1 (Rognes et al. 2016) and the parameters -fastq_min-len 100 -fastq_maxee 1. Clustering was carried out with the parameters -id 0.98 -iddef 0 -centroids, and chimeras (sequences generated through erroneous annealing during library preparation of DNA strands that are not each other's mirror) were detected and removed using the same tool with the parameters -uchime_denovo -nonchimeras (see Maidak et al. 1999).

We consider the remaining clusters to be Operational Taxonomical Units (OTUs). We identified them using the BLAST algorithm of the National Center for Biotechnology Information (NCBI) and the identification engine of the Barcode of Life Data Systems (BOLD) (Ratnasingham and Herbert 2007). We only included OTUs with 100 or more total reads in further analyses and considered all other OTUs to be either contamination or of only minor dietary importance. Illumina sequencing resulted in 1,656,300 paired-end raw reads, leaving 805,854 sequences after quality-filtering. After dereplication, 39,306 unique, non-singleton sequences were kept, resulting in 876 clusters (OTUs)

before and 315 after chimera removal, 265 of which found matches in BLAST and/or BOLD. Altogether, 69 OTUs were represented by 100 or more reads and were therefore included in the downstream analyses. Because many identification searches yielded several plant species with a high sequence identity and other reliability indicators for many OTUs, the results were subsequently refined, compared and verified using the Slovak Karst National Park vegetation database and botanical expert knowledge. After excluding all plant species not found in the study area, a total of 64 taxa were included in the study, with the highest sequence identity as the correct identification result (see Supplementary Material 2).

Data processing and statistical analysis

Data preparation involved compiling primary data sets that included: (1) the relative proportions (%) of plant items identified in faeces, and (2) percentages (%) of plant covers identified on the given vegetation plots. A series of generalised linear mixed models (GLMMs) were constructed to estimate the proportions of each plant species in the diet and given plot, accounting for random effects due to the site and individual variations. GLMMs extend generalised linear models (GLMs) by incorporating both fixed and random effects, making them suitable for handling hierarchical or grouped data (Bolker et al. 2009).

Each model employed a beta distribution to handle proportions bounded between 0 and 1. The beta distribution is well-suited for modeling proportions as it accounts for the continuous nature of the data within the unit interval. Models were fitted using the *glmmTMB* package in R (Brooks et al. 2017). The model structure included fixed effects to estimate the overall mean proportion of each plant species and random effects to account for variations between sites and individual bush-crickets.

Fixed effect estimates and 95% confidence intervals (CI) were extracted from the models. These estimates and CIs, initially on the logit scale, were transformed to the response scale using the logistic function to ensure interpretability as proportions. The transformed estimates and CIs were then multiplied by 100 to express them as percentages, facilitating easier comparison and interpretation.

The obtained estimates were visualised as bar plots with confidence intervals to compare the proportion of plant species volumes in faeces and coverage (%) for each plant taxa. Each plant species was represented by two bars: one for locality and one for faeces. Error bars were used to indicate the 95% confidence intervals. Significant differences between the proportions of plants in faeces versus locality were marked with asterisks (*). Confidence intervals that do not overlap indicate a preference for the given plant species.

Additionally, these significant differences were annotated with the factor of difference, such as 2.5, to denote the magnitude of preference or selection. All calculations were performed in the R programming environment (R Core Team 2023). The graphical outputs were edited in Inkscape (Inkscape_Project 2020). For comparison of vegetation traits found in *I. beybienkoi* faeces and habitat, sets of unpaired t-tests were performed (R Core Team 2023). The obtained results were visualised as boxplot graphs using the *ggplot2* package (Wickham 2016).

Results

Diet identified from faeces

In total, we found 64 plant species belonging to 52 genera and 28 families had been consumed by Bei-Bienko's Plump Bush-cricket (Supplementary Material 2). On average, 9.0 (± 2.77) plant species were identified in each faecal sample (range 6–16 taxa per sample). The diet consisted exclusively of dicotyledonous plant species (64 taxa). Herbaceous flowering plants predominated in the diet (49 taxa), accounting for 76.5% of all determined species (Supplementary Material 2). The rest (23.4%) was represented by woody plants, which comprised 15 species across all samples (Supplementary Material 2).

At the order level, the most abundant and prevalent species belonged to Fabales (35.0%), Rosales (20.4%), Apiales (19.8%) and Lamiales (11.9%). Around 0.7% of the reads were assigned to the category “other” due to very small proportions of plant taxa. At the family level, Fabaceae had the highest frequency (34.9%), accounting for more than one-third of all identified taxa, followed by Apiaceae (19.8%) and Rosaceae (16.9%) (Fig. 3). *Securigera varia* (26.0%; Fabaceae), *Peucedanum cervaria* (16.0%; Apiaceae), *Prunus spinosa* (11.0%; Rosaceae), *Lotus corniculatus* (4.0%; Fabaceae) and *Urtica dioica* (4.0%; Rosaceae) were the most important taxa found in the Bei-Bienko's Plump Bush-cricket diet (Fig. 3). For a complete picture of all plant taxa identified in faeces, see Fig. 3 and Supplementary Material 2.

Vegetation and its life-history traits in the bush-cricket habitat

A total of 178 species of vascular plants (42 families and 25 orders) were detected in the *I. beybienkoi* vegetation plots, with more than 30 species per site (range 31–56 species per plot) (Supplementary Material 3). Herbs represented the substantial body of identified taxa (163 species, 91.5%), followed by woody vegetation, such as shrubs or trees (14

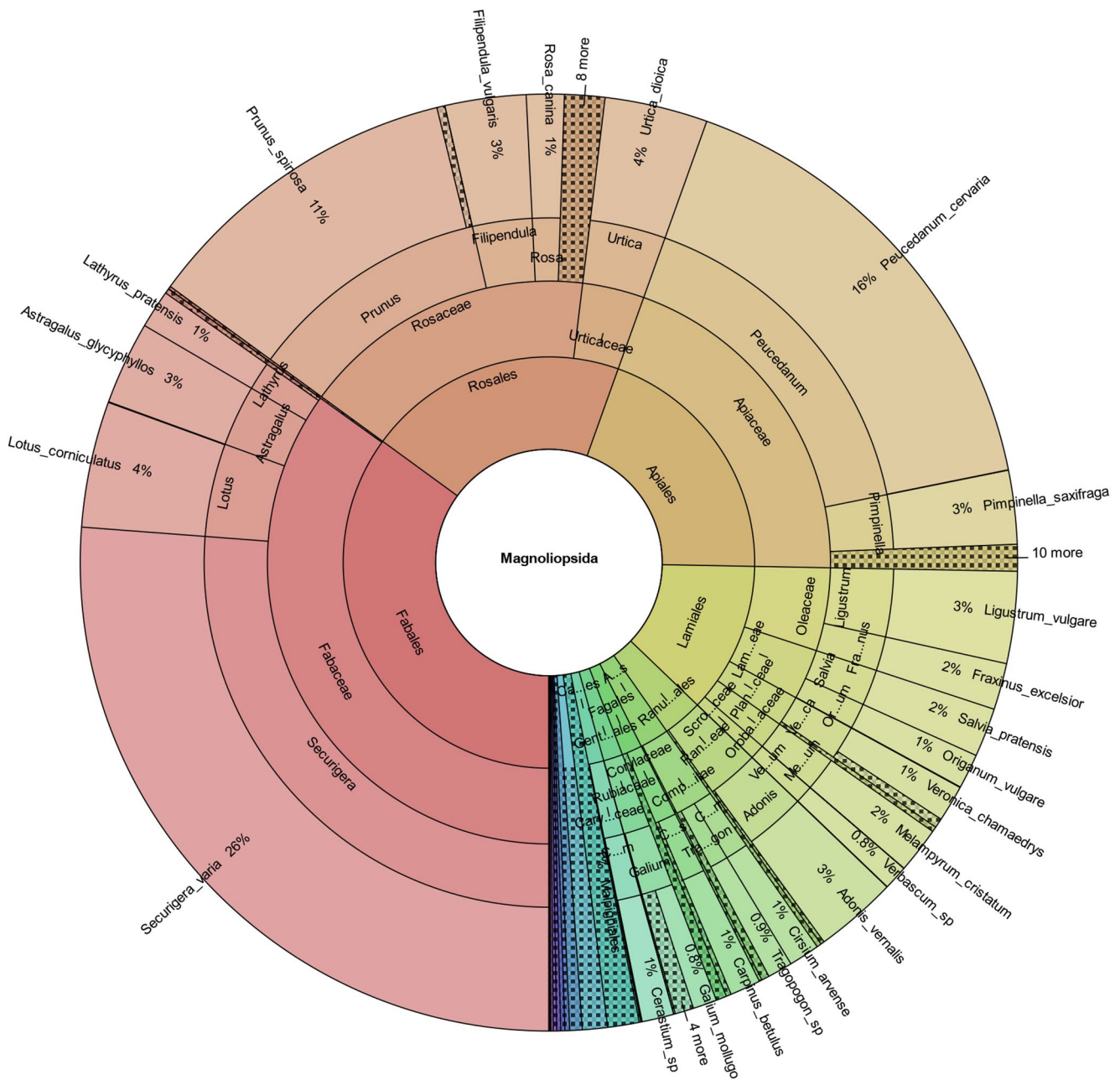


Fig. 3 Diet composition of Bei-Bienko's Plump Bush-cricket identified from faeces using DNA metabarcoding

species, 7.8%). The majority of taxa were dicotyledonous plant species (135 species, 75.8%). The rest was represented by grasses, grass-like (graminoids) flowering plants (41 species, 23.0%) and one species of polypodial ferns (Polypodiaceae).

At the order level, Poales, Asterales, Lamiales, Fabales and Rosales were the most common groups, accounting for more than 13–38 species per order. A very similar result was obtained at the family level. Poaceae, Compositae and Fabaceae were the groups with the highest number of species on vegetation plots (ranging from 14 to 32 species).

At the species level, the highest average coverage (10% or more) of the vegetation plots was found to be *Fraxinus excelsior*, *Ligustrum vulgare*, *Quercus pubescens*, *Potentilla reptans*, *P. cervaria* and *Origanum vulgare*. For a complete list of all plant species found in each study plot, see Supplementary Material 3.

For the life-history traits of plants, we found relevant information for a total of 195 plant taxa, specifically for 75.0% (48 species) found in faeces and 82.5% (147 species) found in the habitat. Among herbs (form H), the average height of the *I. beybienkoi* habitat was 48.0 (± 29.8) cm. The

habitat also included trees and shrubs (form W), with the average height $7.9 (\pm 8.9)$ m. The most common leaf shape in the bush-cricket habitat was the simple shape (a leaf consisting of only one blade), comprising a total of 81.0% (158 species) of all plant species, although multiple leaf shape combinations are also known for some species (see Supplementary Material 4). Most plants were characterised by alternate (66.6%; 130 species) or opposite (22.5%; 44 species) arrangement of leaves, with an average leaf area of 47.1 cm^2 . Neither comparison — the entire dataset nor the subset of 31 most common species confirmed by both methods — showed significant differences between the species found in the diet and those present in the habitat. However, these results did show a slight tendency for differences in the mean values of two parameters — leaf area per leaf dry mass (LA_LDM) and leaf dry mass per leaf fresh

mass (LDM_LFM) (see Fig. 4). Both parameters indicate that *Lathyrus pratensis*, *Galium mollugo*, and *Cerastium holosteoides*, which were consumed, had larger and thinner leaves than other plants present in the habitat, but not consumed. These plant species also showed higher values of water content, together with *Melampyrum cristatum* and *L. corniculatus*. For a complete overview of all plant taxa and their life-history traits, see Supplementary Material 4.

Comparison of vegetation taxa in faeces and habitat of the bush-cricket

Altogether, 31 plant taxa were identified in both faeces and vegetation plots (Fig. 5). Up to 64.5% of estimates (20 species) did not show any significant difference between the occurrence in faeces and habitat plots. At the family level,

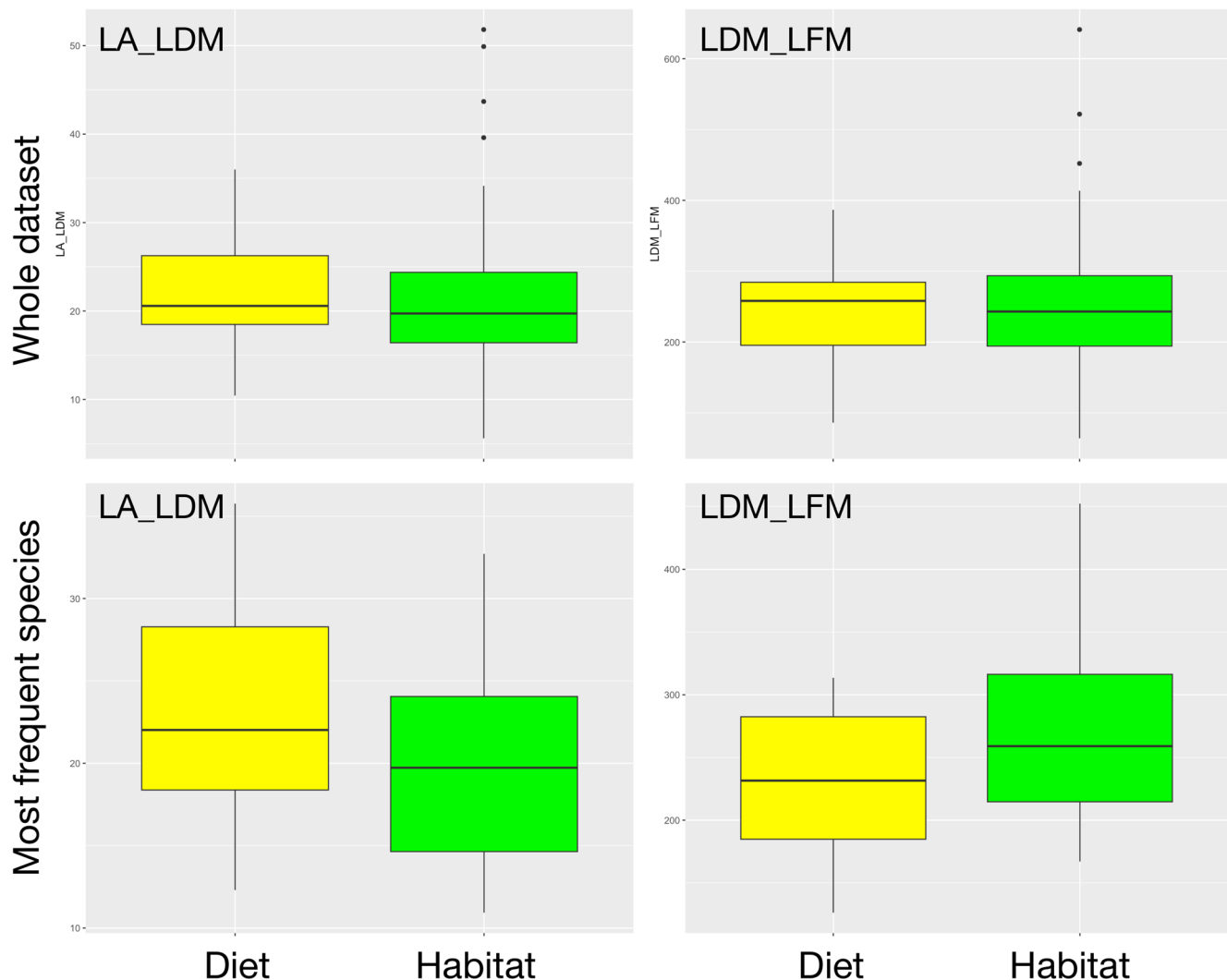


Fig. 4 Comparison of the mean values of two life-history traits that indicate water content in plants: leaf area per leaf dry mass (LA_LDM) and leaf dry matter per leaf fresh mass (LDM_LFM) detected in the diet and habitat of *I. beybienkoi*. Explanations: Whole dataset – com-

parison of life-history traits found in all 195 plant taxa; Most frequent species – comparison of life-history traits 31 plant taxa found in vegetation plots and faeces

Fabaceae ($f=4.381$; $z = -2.80$; $p=0.006$), Orobanchaceae ($f=13.841$; $z = -3.14$; $p=0.002$), Apiaceae ($f=6.313$; $z = -2.84$; $p=0.005$), and Ranunculaceae ($f=5.373$; $z = -2.75$; $p=0.006$) were the most important taxa, occurring significantly more frequently in faecal samples than in vegetation plots. Only the family Dipsacaceae was less represented in the faecal samples than in the vegetation plots ($f = -7.971$; $z=2.87$; $p=0.004$).

At the species level, we found nine of the 31 species estimates to be significantly more prevalent in faecal samples than in the vegetation plots (Table 1; Fig. 5). The most important herb for *I. beybienkoi* was *S. varia* (Fabaceae). It was much more prevalent (44 x higher estimate of occurrence) in the faeces samples than in the vegetation plots. It was followed by *M. cristatum* (Orobanchaceae) (37 x), *L. pratensis* (Fabaceae) (30 x) and *G. mollugo* (Rubiaceae) (28 x) (Fig. 5). Only two species (*Fragaria viridis* and *Trifolium repens*) were found less commonly in faecal samples than in vegetation plots (see Table 1; Fig. 5).

Discussion

This study provides the first DNA metabarcoding assessment of the diet of the endemic Bei-Bienko's Plump Bush-cricket. Our study showed that DNA metabarcoding provides high-resolution quantitative data comparable with field surveys obtained from the bush-cricket habitat. By investigating faecal pellets from 49 individuals, our results showed that *I. beybienkoi* fed on 28 families and 52 genera of plant taxa with more than 60 species, indicating a rather wide dietary range, which is typical for many Orthoptera species (Bernays 1998; Ingrisch and Köhler 1998; Hochkirch et al. 2000; Bellmann 2006; Fischer et al. 2016). We found that *I. beybienkoi* is a so-called forb-feeder (König et al. 2022). It consumes exclusively flowering dicotyledonous herbs and several woody plants such as *P. spinosa*, *L. vulgare* or *F. excelsior*. This result is consistent with the plant composition found in its habitat (see Results) and the diet partially known for several related species from the family Tettigoniidae (Ingrisch 1991; Ingrisch and Köhler 1998; Heller et al. 2004; König et al. 2022).

The plant species identified both in faecal samples and vegetation plots varied across subpopulations, probably in response to the different environments of the sites (Dúbravková-Michálková et al. 2008; Švada 2023). The average height of herbs in *I. beybienkoi* habitats was no more half a metre. These findings correspond well to the vegetation height used by the sister species *I. costata* (47 cm) (Berg et al. 1996; Nuhličková et al. 2021b; Varga and Kenyeres 2025) and to the plant species composition, comprised of many dicotyledonous herbs, which is in line

with the plant species composition typical for many *Isophya* species (Heller et al. 2004; Bauer and Kenyeres 2006). However, *I. beybienkoi* has never been observed in treetops, suggesting its limited mobility and thus a strong attachment to dicotyledonous herbs and lower shrubs, which provide critical requirements for both food and shelter (Berg et al. 1996; Bauer and Kenyeres 2006; Kenyeres and Bauer 2008, 2021; Nuhličková et al. 2021b). This was apparently the case for the white swallow-wort (*Vincetoxicum hirsutinaria*), which is commonly used as a host plant (own observations), but was not detected in the faeces, suggesting its unlikely consumption by *I. beybienkoi* due to its high toxicity to many insect species (Muola et al. 2010).

The most abundant taxa in faeces were legumes (Fabaceae), which made up more than one-third of the bush-cricket's diet. This pattern was even more evident upon comparing the prevalence of 31 species detected in both faecal samples and vegetation plots. Fabaceae, Orobanchaceae and Ranunculaceae were taxa with a strong prevalence in faeces, but rather lower coverage in the vegetation plots. Specifically, the crown vetch, *S. varia* (Fabaceae), was by far the most common taxon identified in faeces, suggesting that *I. beybienkoi* is highly selective for legumes. Legumes thus play an important role in the nutrition of this insect and are particularly crucial for the synthesis of proteins used in oogenesis and spermatogenesis (Zahran 2001; Spehn et al. 2002; Spreti et al. 2017). This is especially true for the family Tettigoniidae (Orthoptera), as its members, like *I. beybienkoi*, typically produce structures requiring significant protein investment, such as yolk-rich eggs in females and large nuptial gifts from males (Wedell and Ritchie 2004; Voigt et al. 2005; Lehmann 2012; Lewis and South 2012; Cueva del Castillo et al. 2024). For example, the crown vetch (*S. varia*), the most consumed plant species in the *I. beybienkoi* diet, contains 15% raw protein, 3% raw fat, 35% raw cellulose, 40% nitrogen-free extractive substances and 7% minerals (Țîței et al. 2016). The protein content in the grass pea, *L. sativus* (Fabaceae), can reach 25.6%, and protein digestibility in some legumes ranged from 76% in the meadow pea (*L. pratensis*) to 87% in the annual vetchling (*L. annuus*), which indicates a very rich source of proteins (Pastor-Cavada et al. 2013). This is also in line with our finding that *I. beybienkoi* mainly selected nitrogen-rich plants, such as legumes.

Flightless insects with low mobility, such as *I. beybienkoi*, spend most of their time in the habitat where they engage in reproduction, for which a protein-rich diet is crucial (Clark et al. 2013; Yamamoto and Uchida 2018; Lan et al. 2021). Therefore, the diet composition found in this study reflects the life-history traits of this bush-cricket, such as its limited movement abilities, low dispersal, isolated and patchy distribution and strong attachment to

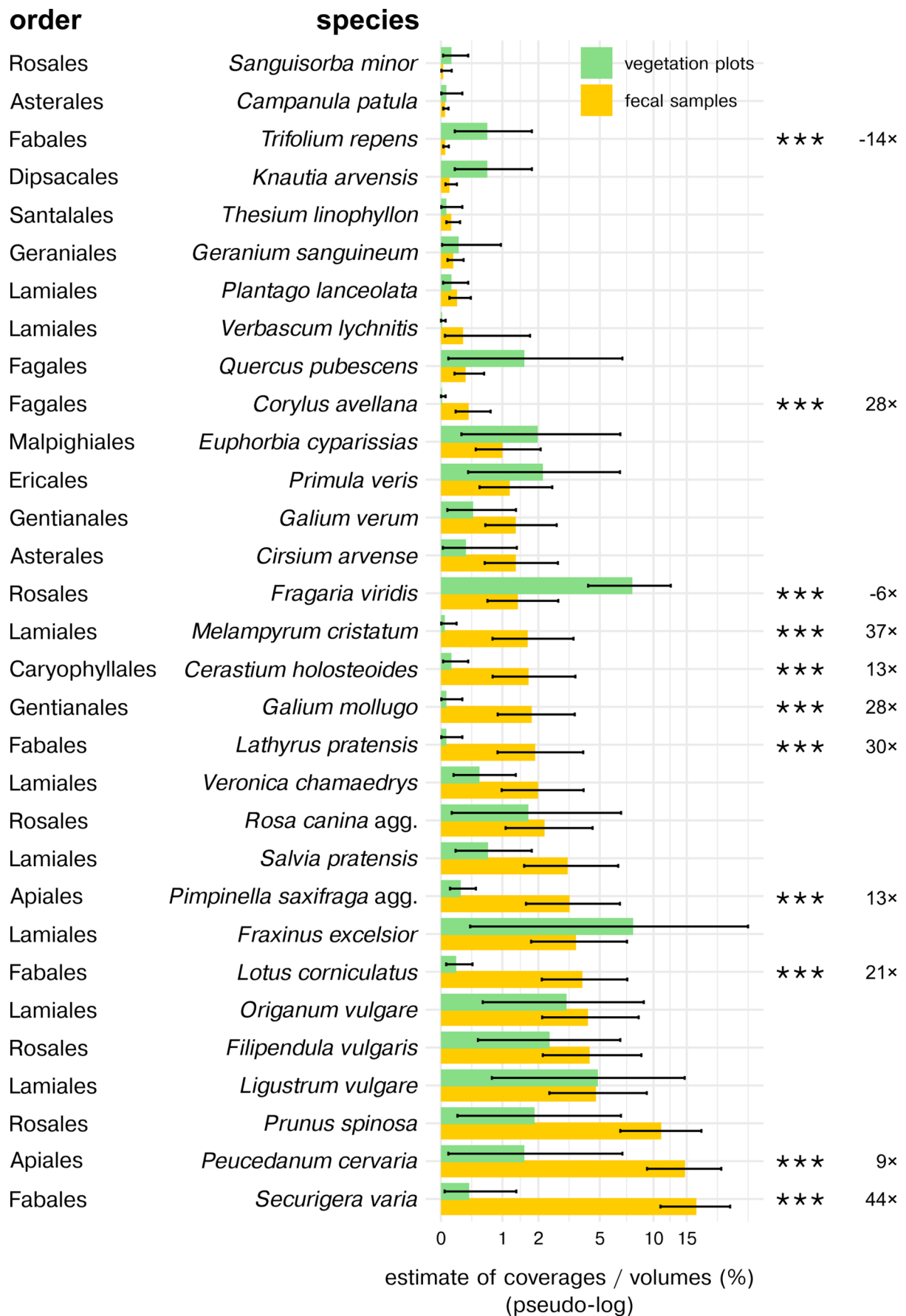


Fig. 5 Estimated values of CI confidence intervals with 95% confidence level for 31 common plant taxa identified as percent coverage (%) in (a) vegetation plots (green) and volumes (b) in faecal samples of *I. beybienkoi* (orange) sampled in the Slovak Karst NP (Central Europe, southern Slovakia). Explanations: a significant difference is indicated by asterix (***). Numbers represent a comparison of estimates in faeces and vegetation plots

existing habitats (Chobanov 2016; Nuhličková et al. 2021a, 2023a, b). Nitrogen-rich legumes are therefore likely to represent a key nutritional requirement for the growth and reproduction of *I. beybienkoi*. Another explanation of the preference for certain plant groups may be the association between the consumption of certain herbs and the specific microclimatic conditions found in karst habitats (see e.g., Bátori et al. 2019). A previous study showed that the largest number of bush-cricket individuals were found in ecotonal areas, which frequently bordered south-facing karst slopes (Nuhličková et al. 2021a). This environment generally has a very hot microclimate, characterized by high annual temperatures and increased evaporation (Dúbravková-Micháliková et al. 2008; Šuvada 2023). Therefore, our study also suggests that *I. beybienkoi* prefers a broader diet spectrum (see Results) and resource-dependent specialisation to certain plant families, which probably increases its fitness under specific karst environmental conditions (Chambers et al. 1996; Bernays 1998; Pitteloud et al. 2021; König et al. 2022). Although the life-history traits of tested plants showed no significant differences in the two datasets (occurrence of species found in the diet vs. species confirmed in the habitat), traits of leaf thickness (leaf area per leaf dry mass) and water content (leaf dry mass per leaf fresh mass) still indicated a slight tendency to favour larger and thinner leaves with higher water content. This was particularly true in the hemiparasite crested cow-wheat (*M. cristatum*), which generally has higher contents of water and minerals obtained from its hosts (Gauslaa 1990). This plant was found in amounts 37-times higher in the faeces than in the vegetation plots. After the crown vetch (*S. varia*), crested cow-wheat (*M. cristatum*) was found to be the second most abundant species in faeces compared to vegetation plots. A similar pattern was confirmed in other species, such as *G. molugo* (28 x), *L. corniculatus* (21 x) and *C. holosteoides* (13 x). This study reveals a preference for the listed plants, very likely due to their higher water content. Thus, one way the bush-cricket could survive in such environment is to be hidden in the fringes and to consume significantly more forbs with a higher water content, thus avoiding overheating or dehydration.

In conclusion, the identification of food plants using DNA metabarcoding could provide critical insight into the ecological requirements of the bush-cricket, highlighting the importance of specific habitat types and plant species for its conservation. We found that more than 170 plant species

occur in the habitat of this endemic species, with Fabaceae, Apiaceae, and Rosaceae being the most important families represented in its diet. However, upon comparing the vegetation identified in faeces and habitat, we found a strong selection of the bush-cricket for legumes and broad-leaved herbs rich in water. These results are likely to reflect the life-history traits of the bush-cricket and its nutritional needs in the extreme environment of the Slovak Karst National Park. Although this bush-cricket preferred a subset of plants (i.e., at least nine herb species were found more commonly as part of the diet than in the vegetation plots), the preferred plant species were also classified into diverse taxonomic groups (six families in total). Furthermore, among the dicotyledonous herbs consumed, several plant species identified in *I. beybienkoi* droppings were not detected in our study plots, suggesting a potential methodological bias. We found that these taxa (e.g., *Adonis vernalis*, *Berberis vulgaris* or *Geum urbanum*) are, in fact, commonly found in the study area. Therefore, given the very low mobility of the bush-cricket (Nuhličková et al. 2023b), we suggest that these plants were most likely not captured in vegetation plots due to methodological limitations of the vegetation sampling. Assuming the seasonal availability of plant species and different nutrition demands for growth and reproduction influence the herbal content in the diet of insects (Yamamoto and Uchida 2018; König et al. 2022), more faecal samples than we were able to collect, as well as more extensive vegetation sampling, are needed to reach reliable conclusions. In this study, due to the rarity of the endangered bush-cricket studied, our findings on the diet of *I. beybienkoi* were based only on faecal samples collected from 49 individuals. Furthermore, faecal sampling was limited to only one season and was sporadic and opportunistic rather than following a thorough, well-designed methodology. Faeces were often only collected from a few different individuals found in certain subpopulations. Hence, future research will be needed to understand in more detail the dietary needs and habitat requirements of the species. In the case of vegetation, it will be necessary to include negative controls (i.e., plots without any presence of bush-crickets) in order to compare the vegetation composition between positive and negative plots, along with measurements of microclimatic conditions. Such information would help to better understand the species' habitat requirements, especially in the highly heterogeneous environment of the Slovak Karst National Park (Bátori et al. 2019; Šuvada 2023).

Finally, many of the plant species found in this study also serve as forage plants for ruminants in extensive meadows or pastures. This applies particularly to crown vetch (*S. varia*), crested wheat (*M. cristatum*) and meadow pea (*L. pratensis*), which were highly selected by the bush-cricket. Therefore, this study strongly supports the importance of

Table 1 Estimated values of CI confidence intervals with 95% confidence level for 31 common plant taxa identified as percent coverage (%) in (a) vegetation plots (VP_est) and volumes (b) in faecal samples (FS_est), with lower and upper CI values for vegetation plots (VP_low; VP_upp) and faecal samples (FS_low; FS_upp)

Taxon name	VP_est	VP_low	VP_upp	FS_est	FS_low	FS_upp	factor	z	p
<i>Securigera varia</i>	0.376	0.042	1.340	16.840	10.898	25.110	44.787	-4.54	<0,001
<i>Melampyrum cristatum</i>	0.045	0.003	0.193	1.661	0.789	3.462	36.911	-4.19	<0,001
<i>Lathyrus pratensis</i>	0.063	0.004	0.273	1.896	0.893	3.978	30.095	-3.88	<0,001
<i>Galium mollugo</i>	0.063	0.004	0.273	1.786	0.897	3.525	28.349	-3.80	<0,001
<i>Corylus avellana</i>	0.013	0.001	0.054	0.368	0.180	0.751	28.307	-3.79	<0,001
<i>Lotus corniculatus</i>	0.188	0.061	0.427	4.013	2.188	7.249	21.331	-3.46	0.001
<i>Cerastium holosteoides</i>	0.126	0.025	0.360	1.687	0.792	3.556	13.388	-3.11	0.002
<i>Pimpinella saxifraga agg.</i>	0.251	0.108	0.483	3.270	1.610	6.531	13.027	-3.10	0.002
<i>Peucedanum cervaria</i>	1.563	0.087	6.743	14.704	9.226	22.623	9.405	-2.99	0.004
<i>Fragaria viridis</i>	7.667	4.262	12.366	1.384	0.690	2.757	-5.539	2.76	0.006
<i>Trifolium repens</i>	0.688	0.169	1.789	0.050	0.027	0.093	-13.818	3.13	0.002
<i>Campanula patula</i>	0.063	0.004	0.273	0.048	0.026	0.089	–	1.04	0.298
<i>Cirsium arvense</i>	0.326	0.022	1.354	1.328	0.638	2.742	–	-1.19	0.234
<i>Euphorbia cyparissias</i>	1.982	0.260	6.563	1.004	0.481	2.080	–	1.03	0.303
<i>Filipendula vulgaris</i>	2.409	0.518	6.560	4.353	2.156	8.593	–	-1.09	0.276
<i>Fraxinus excelsior</i>	7.743	0.390	30.972	3.590	1.766	7.159	–	1.03	0.303
<i>Galium verum</i>	0.438	0.075	1.331	1.327	0.650	2.690	–	-1.14	0.254
<i>Geranium sanguineum</i>	0.222	0.013	0.967	0.149	0.077	0.291	–	1.04	0.298
<i>Knautia arvensis</i>	0.688	0.169	1.789	0.102	0.053	0.197	–	1.01	0.312
<i>Ligustrum vulgare</i>	4.876	0.776	14.639	4.752	2.398	9.199	–	1.05	0.294
<i>Origanum vulgare</i>	3.126	0.602	8.858	4.254	2.132	8.307	–	-1.07	0.285
<i>Plantago lanceolata</i>	0.126	0.025	0.360	0.200	0.100	0.401	–	-1.08	0.280
<i>Primula veris</i>	2.159	0.359	6.537	1.175	0.546	2.510	–	1.03	0.303
<i>Prunus spinosa</i>	1.876	0.204	6.622	11.006	6.564	17.879	–	-1.27	0.204
<i>Quercus pubescens</i>	1.563	0.087	6.743	0.322	0.165	0.627	–	1.01	0.312
<i>Rosa canina agg.</i>	1.682	0.129	6.631	2.220	1.074	4.533	–	-1.07	0.285
<i>Salvia pratensis</i>	0.701	0.179	1.785	3.180	1.556	6.389	–	-1.21	0.226
<i>Sanguisorba minor</i>	0.126	0.025	0.360	0.026	0.005	0.129	–	1.01	0.312
<i>Thesium linophyllum</i>	0.063	0.004	0.273	0.124	0.063	0.241	–	-1.09	0.276
<i>Verbascum lychnitis</i>	0.013	0.001	0.054	0.284	0.046	1.733	–	-1.97	0.049
<i>Veronica chamaedrys</i>	0.545	0.153	1.327	1.994	0.984	3.999	–	-1.17	0.242

traditional management in the Slovak Karst National Park. Extensive grazing appears to be the most appropriate conservation measure for preserving the habitat of the Bei-Bienko's Plump Bush-cricket, including legumes rich in water content important for its growth and reproduction.

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Author contributions All authors contributed to the study conception and design. Material preparation and data collection were performed by SS, JS, RŠ, SP, MŠ, JŠ and IJ. Plant species collection and determi-

nation was performed by RŠ. MŠ, JŠ and IJ performed data processing of vegetation, including the analysis of plant life-history traits. DNA barcoding and data analysis were prepared by OH. Data interpretation of plants found in faeces was conducted by SS together with all co-authors. RŠ improved the data using of vegetation database from Slovak Karst NP and prepared Fig. 1. Fig. 2 was compiled by JS. The first draft of the manuscript was written by SS and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Code availability Not applicable.

Declarations

Ethical approval All authors declare that we did not collect threatened arthropods from study areas, and all individuals collected were carefully returned into their habitat.

Consent to participate Not applicable.

Consent for publication All authors declare that we agree to publish this manuscript in the *Journal of Insect Conservation*.

Competing interests The authors declare no competing interests.

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