

Snacking during hibernation? Winter bat diet and prey availabilities, a case study from Iskar Gorge, Bulgaria

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Abstract: Better empirical knowledge of how bat and insect phenology are influenced by seasonal environmental conditions and how this may affect fitness is essential in the face of changing climatic conditions. We examined the winter diet of the Schreibers' bent-winged bat *Miniopterus schreibersii* (Kuhl, 1817) from Razhishkata Cave, Balkan Mountains, Bulgaria during four sampling periods in the winter of 2021 (end of January – end of March). We used a combination of DNA metabarcoding and a microscope-based morphological analyses of bats droppings. Additionally, we tracked prey availability in the studied area using insect flight interception traps (FIT). The species was actively feeding outside the cave and did not use the cave fauna. Our samples indicate a shift in the winter diet of the bats throughout the studied periods. Even though Diptera was the most abundant order of insects during two of the study periods, bats were preying predominantly on Lepidoptera and Hymenoptera. The metabarcoding also supported this with data for the presence of insects with diurnal activity in the samples. The temperature loggers showed a strong correlation between the outside temperature and the temperature in the cave where the colony was located, probably impacting the activity patterns of the bats. To our knowledge, this is the first study of winter bat activity and diet in Bulgaria. Our research can serve as a potential framework for studying winter bat activity and insect activity during this sensitive period.

Keywords: bat guano, DNA metabarcoding, insects, *Miniopterus schreibersii*, winter activity, winter diet

Introduction

With winter's onset and the lowering of ambient temperatures, mammals face serious energetic challenges. Small insectivorous mammals like bats need to cope with two key challenges. First, during the winter, there is a reduction in insect abundance, and second, they need to minimise heat loss due to their high surface-to-volume ratio (Speakman & Thomas, 2003). Some species of bats migrate to warmer regions, while others use hibernation to survive. Hibernation is a condition in which the body temperature drops, leading to a decrease in metabolic rate and increased energy savings for an extended period. It is crucial for survival during periods of limited resources (Buck & Barnes, 2000; Geiser, 2004). The hibernation period consists of frequently interrupted torpor bouts. These periodic arousals can cost bats up to 80% of their energy reserves (Thomas et al., 1990). Rising temperatures in the hibernacula (Humphries et al., 2006) and changes in airflow are expected to play a significant role in the arousals. Additionally, frequent extreme temperature changes during the winter can cause premature arousal (Thomas & Geiser, 1997), which can deplete critical energy reserves to the extent that the bat cannot survive the winter (Jones et al., 2009). This emphasises the need to increase our understanding of bat activity and the need for remote monitoring systems that cause less disturbance in the roosts and are low-cost and efficient (Revilla-Martín et al., 2020). The importance of remote monitoring systems capable of continuous data collection on the abundance and activity of bats has been even more pronounced since the COVID-19 pandemic. Additionally, non-invasive studies on bat populations are essential for understanding the natural activity patterns and can be used to set a baseline against which aberrant behaviour can be detected.

Water demands have been considered the main reasons for activity (Speakman & Racey, 1989), but research has been proposing multiple explanations for the winter activity, like activation of the immune system, sleep, and foraging (Hays et al., 1992; Boyles et al., 2006; Zahn & Kriner, 2016). Bat winter activity has been frequently observed in Bulgaria, due to milder winters. However, their feeding activity and dietary preference during hibernation has been poorly studied (Kanuch et al., 2005; Sano, 2006; Willams et al., 2011; Miková et al., 2013; Hope et al., 2014; Lino et al., 2014). Disturbance related to more frequent extreme

temperature changes during winter can cause premature arousal (Thomas & Geiser, 1997), which can deplete critical energy reserves to the extent that the bat cannot survive the winter (Jones et al., 2009).

A special complex of invertebrates is active during the winter (Taylor, 1963). In Europe, the most abundant invertebrate order during the winter is Diptera. Flies (Diptera), springtails (Collembola), snow scorpionflies (Mecoptera: Boreidae), spiders (Araneae), beetles (Coleoptera), psyllids (Psylloidea), bugs (Heteroptera), earwigs (Dermaptera) and many species of moths (Lepidoptera) belong to the assemblage of snow-active invertebrates (Aitchison, 2001; Hågvar, 2001, 2007, 2010; Hågvar & Greeve, 2003; Hågvar & Aakra, 2006; Hågvar & Krzeminska, 2007; Soszyńska-Maj, 2008; Jaskuła & Soszyńska-Maj, 2011; Soszyńska-Maj & Buszko, 2011; Langourov, 2004; Pavlova & Stoyanova, 2020). Some aquatic insects like stoneflies (Plecoptera), caddisflies (Trichoptera) and non-biting midges (Chironomidae) (Bouchard & Ferrington, 2009; Hågvar, 2010) have also been observed during the winter and on snow. This complex of invertebrates active during the winter can provide hunting opportunities for bats (Park et al., 1999, 2000).

The winter activity and diet of bats in Bulgaria have not been studied yet and little is known about the winter insect activity (Langourov, 2004; Pavlova & Stojanova, 2020). Considering that the overwintering bat populations in Bulgaria are some of the largest European bat aggregations, more research and conservation efforts are needed. The aim of our study was to investigate the winter activity patterns and trophic interactions of Schreiber's bent-winged bat in the Lakatnik region, using two techniques: a microscope-based morphological analysis and DNA metabarcoding analysis. We studied the insect abundance and how insect availability will shape the dietary preference of the studied bat species. In addition, we investigated if there is a correlation between the outside temperature and the cave temperature at the location of the studied bat colony.

Materials and methods

Study site and species

The study was conducted in the middle of the Iskar Gorge, Balkan Range, Bulgaria. The canyon walls of



Fig. 1. The study area with rocky complex above the entrance of Razhishkata Cave.

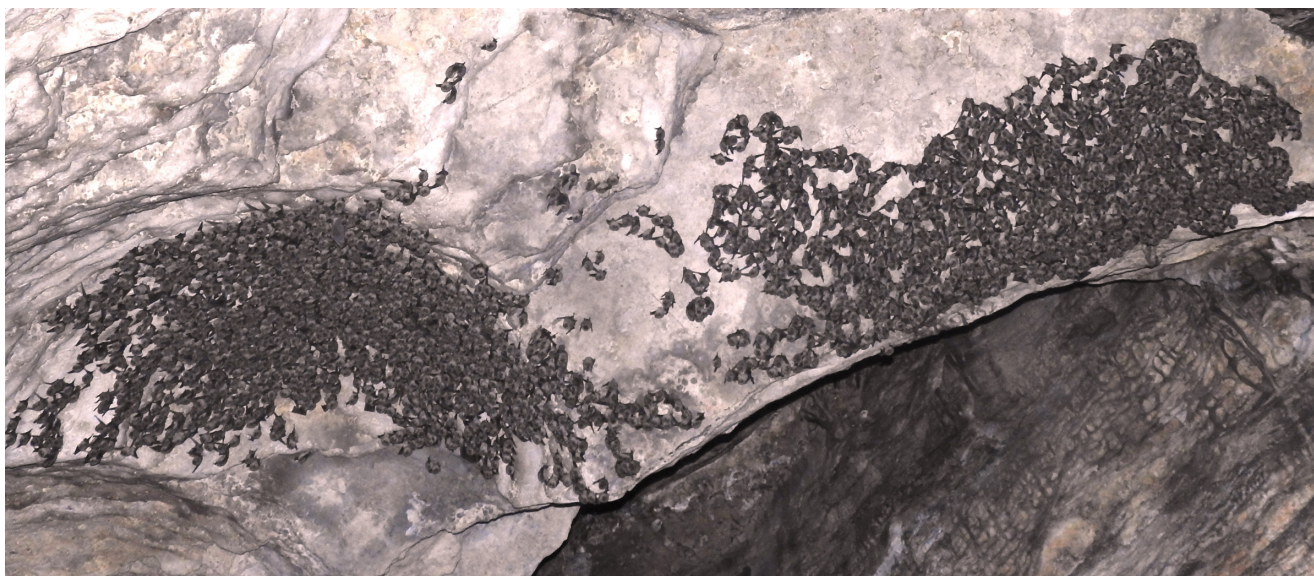


Fig. 2. The winter colony of Schreiber's bent-winged bat (*Miniopterus schreibersii*) from Razhishkata Cave.

limestone were carved down by the Iskar River over thousands of years, resulting in rugged crags, caves and other typical karst reliefs. The studied region is included in several protected territories: Vrachanski Balkan Natural Park, Lakatnishki Skali Protected Area and four Natura 2000 zones (BG0002053; BG0001042; BG0002005; BG0001040). Razhishkata Cave (43.09008°N; 23.38507°E; 575 m a.s.l.) (Fig. 1) hosts one large winter colony of Schreiber's bent-winged bat (*Miniopterus schreibersii* (Kuhl, 1817)) and several smaller clusters of five different bat species (*Rhinolophus ferrumequinum* (Schreber, 1774), *Rhinolophus hipposideros* (Bechstein, 1800), *Nyctalus noctula* (Schreber, 1774), *Pipistrellus pipistrellus* (Schreber, 1774) and *Pipistrellus pygmaeus* (Leach, 1825)). We focused our research only on *M. schreibersii* as it is the main resident species in the cave. The species is included as Vulnerable A2c in the Red Data Book of the Republic of Bulgaria and in the International Union for Conservation of Nature (IUCN) Red List (Popov & Ivanova, 2015; Gazaryan et al., 2020). Recent unexplained mass mortality events have been documented in Europe (Roué & Némoz, 2002). It is a specialist predator, feeding mainly on moths, but can opportunistically switch to other prey that are more seasonably abundant (Preselnik & Aulagnier, 2013). Forage near the vegetation and over woodland habitats (Dietz et al., 2009; Vincent et al., 2011). The size of the Schreiber's bent-winged bat winter colony in Razhishkata Cave (Fig. 2) varies from about 600 individuals at the beginning of the winter (January) to a maximum of 2000 individuals towards the end of the winter (March). The cave is among the important hibernacula for bats in Bulgaria (Ivanova, 2005).

Sample collection

Bat guano samples were collected during the following periods: 8.i–12.ii.2021 (period I); 12–23.ii.2021 (period II); 23.ii–5.iii.2021 (period III); 5–26.iii.2021 (period IV). White polythene sheeting (3 x 4 m) was laid down under the Schreiber's bent-winged bat cluster, which is located approximately 300 m from the cave entrance. Each cluster was checked, and we documented the species composition and density during the roost survey. During collection, the guano samples were placed on a paper sheet in a plastic box. After sample collection, the sheeting was changed to

avoid mixing material between survey periods. Samples were stored at room temperature. Due to heavy rain, water dripped from the cave ceiling where the sheet was placed, and the guano collected on 12.ii.2021 was liquefied. Therefore, it was collected in a zip-lock bag and stored in the freezer. After heavy snowfalls during the last sampling period and flooding in the cave, the guano was practically washed from the plastic sheet. As a result, the colony changed its location to a drier place, and no guano was collected from this period (5–26.iii.2021). We followed all ethical requirements for working with bats. The research was carried out under permit by the Bulgarian Biodiversity Act (No 830/19.09.2020).

Microscope-based morphological analysis

Often, insectivorous bats do not chew their prey entirely. Because of their fast digestion, parts of insects can be traced in the guano, which can be used for further identification (Kovtun & Zhukova 1994). Insect fragments were identified with the help of an entomological key (Shiel et al., 1997) and identification guidelines (Whitaker et al., 2009; Whitaker & Castor, 2009) to the lowest possible taxonomic level. We selected only whole guano pellets with still preserved cylindrical shapes for the analysis. In order to avoid biased choice by picking only pellets that look more interesting than others, the suitable pellets were selected randomly. In total, 254 faecal samples were analysed. Of all morphologically analysed droppings, 126 (49.6%) were collected in February and 128 (50.4%) in March 2021.


Each pellet was pre-soaked in a small Eppendorf tube with ethyl alcohol (70%) for at least one hour to soften. Once a pellet had softened, it was placed on a microscope slide, and two drops of glycerol were added. The slide was then put under a low-power binocular microscope (x20–40), with graph paper sellotaped to the bottom of the microscope's stage. Using two fine dissecting needles, each sample was separated and examined. After tearing apart, more glycerol was added if needed. The remains were compared with whole insects caught in the area with the interception traps. For the liquified sample, the procedure was the same as stated above. The only exception was that after the sample was taken from the fridge, it was left for around 15 min at room temperature to unfreeze itself. After that, almost equal

proportions (31 in number) were taken from the sample using a small teaspoon until everything was analysed. The relative importance of different prey orders in the diet was quantified by calculating the percentage frequency (%F), defined as the number of occurrences of an order divided by the total occurrences of all orders x 100.

Molecular analysis

From each sample period, we created pools, each containing 100 pellets selected randomly, and for the liquified sample, a pool containing a similar volume of sample material to the other one. Species identification from the guano samples was performed using DNA metabarcoding following the protocol published in Hausmann et al., 2020. The samples (n=3) were dried in a 60°C oven for at least eight hours. Then we homogenised them in a FastPrep96 machine (MP Biomedicals) using sterile steel beads to generate a homogeneous mixture of guano and submitted them for subsequent metabarcoding (conducted by AIM GmbH). Before the DNA extraction, 1 mg of each homogenate was weighed into sample vials. The samples were then processed using adapted volumes of lysis buffer and the Zymo faeces kit (Zymo), following the manufacturer's instructions. We performed a 2-step PCR using mlCOIintF with jgHCO2198 primers to amplify the COI-5P target region and prepare the MiSeq libraries. First, a 313 bp long mini-barcode region was amplified by PCR (Leray et al., 2013; Morinière et al., 2016), using forward and reverse HTS primers equipped with complementary sites for the Illumina sequencing tails. In a subsequent PCR reaction, index primers with unique i5 and i7 inline tags and sequencing tails were used to amplify the indexed amplicons. Afterwards, equimolar amplicon pools were created, and size was selected using preparative gel electrophoresis. The pooled DNA was purified using 92 MagSi-NGSprep Plus beads (Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany). A bioanalyser (High Sensitivity DNA Kit, Agilent Technologies) was used for a final check of the bp distribution and concentration of the amplicons before creating the final library. High-throughput sequencing (HTS) was performed on an Illumina MiSeq using v2 (2*250 bp, 500 cycles, maximum of 20 min reads) chemistry (Illumina).

Metabarcoding library preparation

The bioinformatics processing of raw FASTQ files from Illumina was carried out using the VSEARCH suite v2.9.1 (Rognes et al., 2016) and Cutadapt v1.18 (Martin, 2011). Forward and reverse reads in each sample were merged using the VSEARCH program “fastq_mergepairs” with a minimum overlap of 10 bp, yielding approximately 313 bp sequences. Forward and reverse primers were removed with Cutadapt, using the “discard_untrimmed” option to discard sequences for which primers were not reliably detected at ≥90% identity. Quality filtering was done with the “fastq_filter” in VSEARCH, keeping sequences with zero expected errors (“fastq_maxee” 1). Sequences were dereplicated with “derep_fulllength”, first at the sample level and then concatenated into one FASTA file, which was subsequently dereplicated. Chimeric sequences were filtered out from the FASTA file using the VSEARCH program “uchime_denovo.” The remaining sequences were then clustered into OTUs at 97% identity with “cluster_size,” a greedy centroid-based clustering program. OTUs were blasted against a custom Animalia database downloaded from BOLD, including taxonomy and BIN information, through Geneious (v.10.2.5 – Biomatters, Auckland – New Zealand) and following methods described in Morinière et al. (2016). The resulting CSV file included the OTU ID, BOLD Process ID, BIN, Hit-%-ID value (percentage of overlap similarity (identical base pairs) of an OTU query sequence with its closest counterpart in the database), length of the top BLAST hit sequence, phylum, class, order, family, genus, and species information for each detected OTU was exported from Geneious and combined with the OTU table generated by the bioinformatics pipeline. The combined results table was then filtered by Hit-%-ID value and total read numbers per OTU. All entries with identifications below 97% and total read numbers below 0.01% of the summed reads per sample were removed from the analysis. OTUs were then assigned to the respective BIN. Additionally, the API provided by BOLD was used to retrieve BIN species and BIN countries for every OTU, and the Hit-%-IDs were aggregated over OTUs that found a hit in the same BIN and shown in the corresponding column as a % range. To validate the BOLD BLAST results, a separate BLAST search was carried out in Geneious (using the same parameters) against a local copy of the NCBI nucleotide database downloaded from <ftp://ftp.ncbi.nlm.nih.gov/blast/db/> .

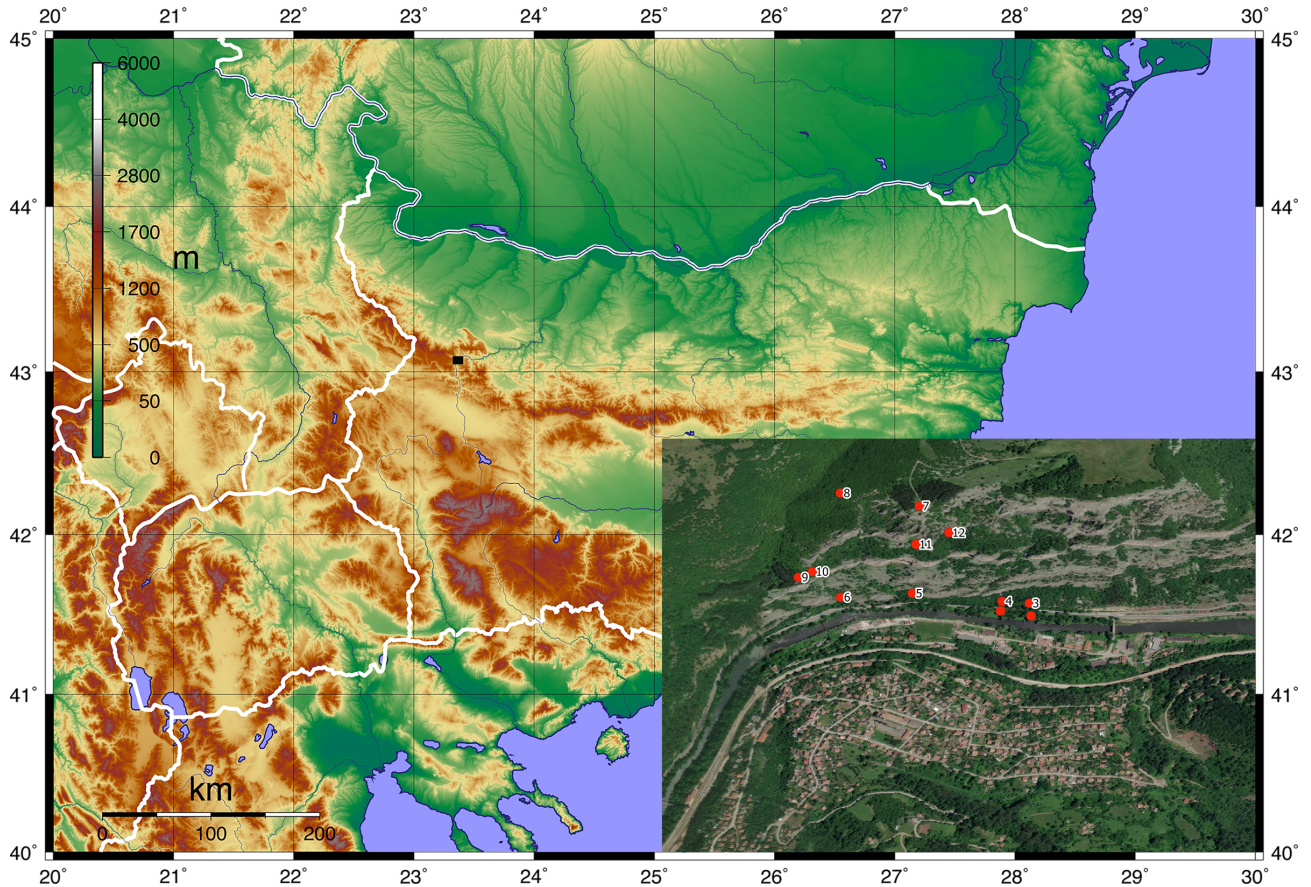


Fig. 3. Map of the locations of the 12 flight interception traps in the area of Iskar Gorge.



← Fig. 4. Photo of one of the flight interception traps in the area of Iskar Gorge.

Species identification in bulk samples was based on high-throughput sequencing (HTS) data grouped to genetic clusters (OTUs), blasted, and assigned to barcode index numbers (“BINs”: Ratnasingham & Hebert, 2013) which are considered to be a good proxy for species numbers (Hausmann et al., 2013; Ratnasingham & Hebert, 2013).

Insect survey

To determine the winter activity of the insects in potential feeding places in the area of Iskar Gorge (Lakatnishki Skali Protected Area), 12 flight interception traps (FIT) were placed in different habitat types and at various altitudes at a distance of no more than 1 km from the cave (Fig. 3). We selected this type

Table 1. Flight interception traps in the studied area in Iskar Gorge, Lakatnishki Skali Protected Area.

Trap number	Decimal geographic coordinates		alt.	EUNIS Habitat types	Tree species
	N	E			
1	43.08805	23.38756	384 m	T1-2116: Dacio-Moesian ash-alder forests T1-1112: Eastern European poplar-willow forests	<i>Juglans regia</i>
2	43.08790	23.38845	384 m	T1-2116: Dacio-Moesian ash-alder forests T1-1112: Eastern European poplar-willow forests	<i>Alnus glutinosa</i>
3	43.08828	23.38840	390 m	T1-2116: Dacio-Moesian ash-alder forests	<i>Fraxinus excelsior</i>
4	43.08834	23.38761	392 m	T1-9B8: Sub-Mediterranean and Pannonic mixed forests H3.2A13: Balkan Range calcicolous chasmophyte communities	<i>Quercus</i> sp.
5	43.08858	23.38500	406 m	H3.2A13: Balkan Range calcicolous chasmophyte communities H3.2E: Bare limestone inland cliffs	<i>Prunus domestica</i>
6	43.08846	23.38291	408 m	H3.2A13: Balkan Range calcicolous chasmophyte communities H3.2E: Bare limestone inland cliffs T1-9B8: Sub-Mediterranean and Pannonic mixed forests T1-1112: Eastern European poplar-willow forests	<i>Juglans regia</i>
7	43.09120	23.38520	671 m	T3-M2: Native pine plantations	<i>Pinus sylvestris</i>
8	43.09159	23.38290	633 m	T1-9B222: Moesian oriental hornbeam forests	<i>Carpinus orientalis</i>
9	43.08906	23.38168	530 m	H3.2A13: Balkan Range calcicolous chasmophyte communities T1-9B8: Sub-Mediterranean and Pannonic mixed forests	<i>Quercus</i> sp.
10	43.08923	23.38209	530 m	H3.2A13: Balkan Range calcicolous chasmophyte communities T1-9B8: Sub-Mediterranean and Pannonic mixed forests	<i>Quercus</i> sp.
11	43.09005	23.38510	570 m	H3.2A13: Balkan Range calcicolous chasmophyte communities H3.2E: Bare limestone inland cliffs	<i>Robinia pseudoacacia</i>
12	43.09040	23.38606	608 m	H3.2A13: Balkan Range calcicolous chasmophyte communities H3.2E: Bare limestone inland cliffs T1-9B8: Sub-Mediterranean and Pannonic mixed forests	<i>Fraxinus excelsior</i>

of trap because it does not use any attractants, making it ideal for our research purposes. Each FIT (our modification of Wilkening et al., (1981) model) with a collecting surface of 0.5 m² was filled with a collecting fluid (propylene glycol (C₃H₈O₂)) (Fig. 4). They were

deployed on mid-section of tree crowns, at a 7–9 m above the ground, in order to collect as many ecologically different invertebrates as possible. In Table 1, we have presented the exact locations, main habitat types, and data of the tree species around each

trap. The trap collection period was synchronised with the guano collection periods II and III. After each sampling period, the caught insects from each trap were identified to order and stored for future reference and species identification.

Air temperature data

The temperature and precipitation data were extracted from the European Center for Medium-Range Weather Forecast (ECMWF) fifth-generation Reanalysis (ERA5). The ERA5 is a global reanalysis of weather data, spanning from 1901 to the present, with assimilated data from the surface, upper-air and satellite observations with a spatial resolution of 0.1 degrees (10 km) and temporal resolution of 1 hour (Muñoz-Sabater et al., 2021). The data is extracted as 2 m temperature and daily accumulated precipitation from the surface ERA5-L and datasets with coordinates (N 43.1, E 23.4) and 814 m above sea level altitude of the selected nod. The temperature data is not interpolated to the altitude of 575 m above sea level, which is the altitude of the Razhiskata Cave entrance. Additionally, we recorded the cave temperature with temperature and humidity logger iButtons (model DS1923-F5, Maxim Integrated Products, Inc., Sunnyvale, California) situated close to the colony. They were set to record air temperature and relative humidity twice a day (at 0000 and 1200 hrs) for the whole study period.

Results

Microscope-based morphological analysis

The microscope-based morphological analysis of the guano revealed fragments from 6 taxonomic orders of arthropods (Araneae, Lepidoptera, Diptera, Trichoptera, Coleoptera, and Hemiptera). Lepidoptera was the most frequently occurring order measured by % frequency (%F) (Fig. 5), though many of these remains were attributed to lepidopteran scales. Diptera was the second most frequently encountered order in the pellets, followed by Trichoptera. Coleoptera and Araneae were the least abundant food items identified in the diet during the winter season (<5 %F). Parts from Hymenoptera were traced from the February guano samples but were less than 1 %F. The percentage

frequency of Lepidoptera was higher during February compared to March. Moreover, the percentage of frequency of Trichoptera was almost doubled during March (15 %F in March, 8 %F in February).

The liquid guano sample revealed Lepidoptera was the most frequently occurring order, followed by Diptera, Coleoptera, and Trichoptera. Class Arachnida was the least frequently occurring of invertebrates. The data was skewed because the sample was in liquid form. Thereafter, the interpretation is not as accurate. All raw data is available in [Supplementary material 01 \[* .xlsx\]](#) [\[* .xlsx\]](#) [\[🔗\]](#).

Molecular analysis

From the molecular analyses of the three pooled samples of bat faecal pellets, we identified a total of 215 Operational Taxonomic Units (OTU). The data was further approved by taxonomists and only possible prey items were used for further analyses. The criteria for inclusions were: 1) the taxon is possibly prey for bats; 2) The insect species have been recorded in Bulgaria/Europe. We excluded: fungal taxa, which are potentially growing on the guano in the cave; Amoebozoa or other unicellular organisms which are probably part of the cave fauna; slugs, earthworms, nematodes which are taxa that are not potential prey for bats; skin and internal parasites; taxa with strict extra Palaearctic distribution which are probably contamination or mistake in the reference database, some examples of this are: *Bertmainius tingle* (Main, 1991), *Celatoblatta laevispinata* Johns, 1966, *Declivitata hamata* (Thunberg, 1808) and *Essigella eastopi* Sorensen, 1994. After this initial data elimination, the number of OTUs accepted as potential bat prey was reduced to 66 taxa for the three sampling periods together. Even though some species were amplified with a few reads (<10 repeats), we included them because they were detected in the outside traps, are common in the region, and could potentially be food sources for the bat species. The molecular results show that in the bat guano samples from Razhishka Cave, 5 orders of insects can be distinguished (Fig. 6).

The most abundant taxa revealed by the metabarcoding analyses were *Ypsolopha ustella* (Clerck, 1759), which was recorded during all periods, but the number of reads were 3 times greater during period III; *Emmelina monodactyla* (Linnaeus, 1758) – during all periods, but the number of reads were around

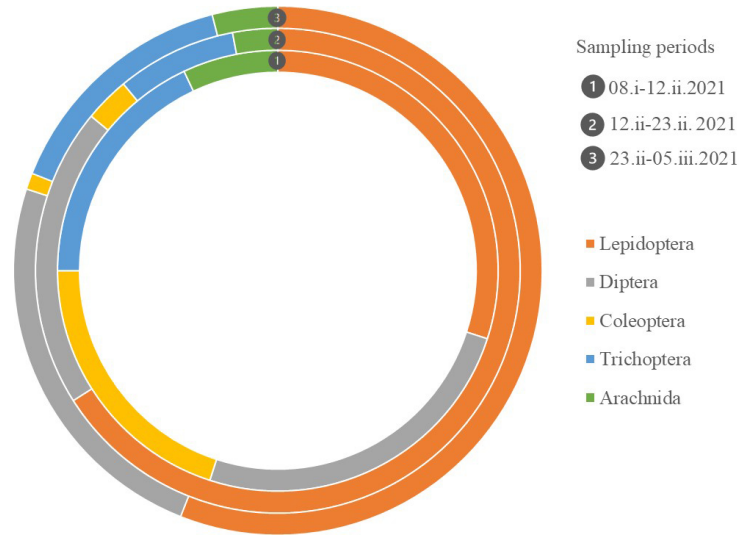


Fig. 5. Percentage frequency of prey orders encountered within bat guano pellets collected from three sample periods in 2021 from the Razhishka Cave – microscope-based morphological analysis.

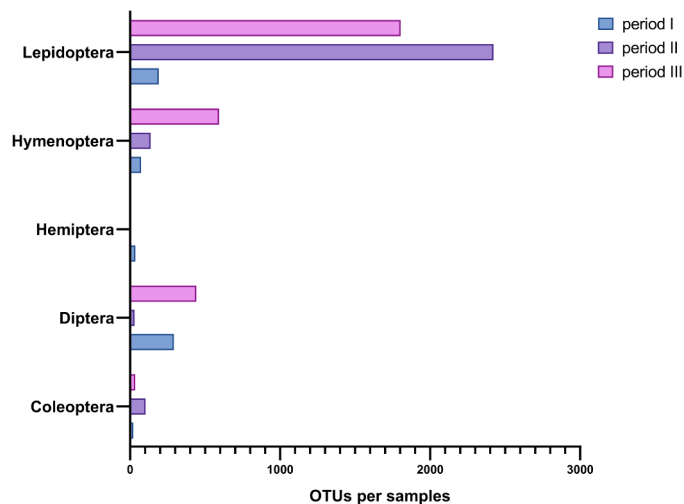


Fig. 6. Summarised results from the metabarcoding sequencing analysis of bat guano samples collected in 2021 from three time periods from the Razhishkata Cave.

200 times greater during period II; *Conistra vaccinii* (Linnaeus, 1761) – during all periods, but the number of reads were 100 times greater during period III; *Eupsilia transversa* (Hufnagel, 1766) – during periods II and III; followed by chalcid wasps and hyperparasitoids of the family Perilampidae – during all periods, but the number of reads were greater during period III; flies *Limnophora tigrina* (Am Stein, 1857) recorded in periods I and III; *Heteromyza rotundicornis* (Zetterstedt, 1846) recorded only in period III; and two

species of the genus *Poecilus* recorded in periods II and III. Raw data is available in [Supplementary material 02](#) [[*.xlsx](#)] [↗](#).

Insect activity

A total number of 2921 arthropod specimens were collected by FIT during the study. Diptera was the most abundant order recorded during the winter, followed by

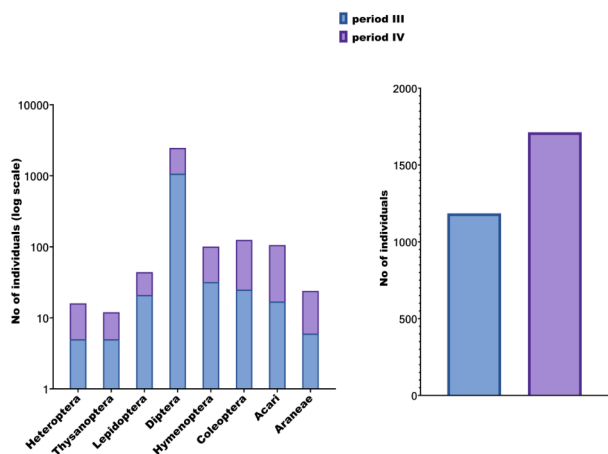


Fig. 7. Summarised results of the insect abundance, collected with FIT during the two sampling periods in 2021 in the studied area of Iskar Gorge.

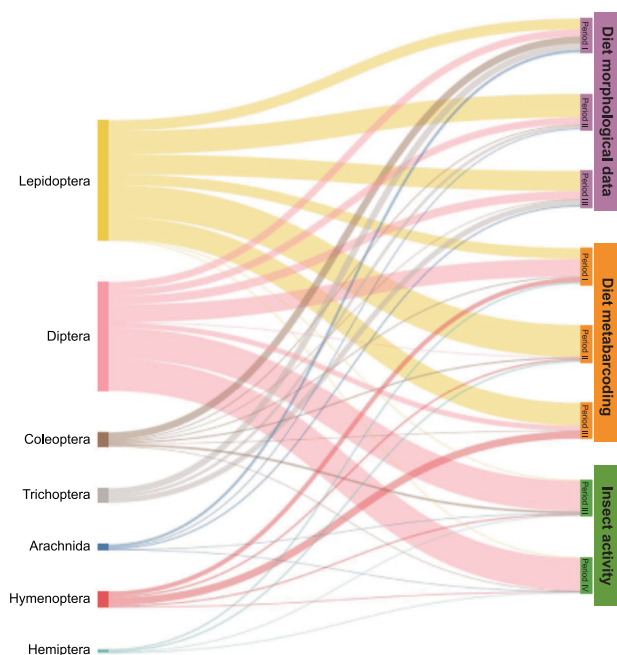


Fig. 8. Summarised results from the relative abundance of prey items in the metabarcoding sequencing analysis and the morphological analyses of the bat guano samples collected in 2021 from the three time periods from the Razhishkata Cave, together with the data for insect activity in the study region.

Hymenoptera and Coleoptera (Figs 7, 8; raw data is available in [Supplementary material 03 \[* .xlsx\]](#)). During our study, we observed temporal changes in insect activity. More Diptera and Lepidoptera were

found in February compared to March. Blattodea was recorded only in February, while Neuroptera was recorded in March. For the whole study period, only 44 individuals from the order Lepidoptera were collected in the traps, compared to 2323 individuals from Diptera. The majority of the collected Lepidoptera species are nocturnal, and only 3 of them are known to be active during the day. Thus, almost all of the recorded moths are potential prey for the bats in the region. The activity pattern of beetles is unclear, but some might be active around dusk. Most Coleoptera species are rarely recorded in flight.

The insect orders Lepidoptera, Hemiptera, Coleoptera, Hymenoptera, Diptera (partly) were identified to the lowest possible taxonomic level ([Supplementary material 04 \[* .xlsx\]](#)). During the first sampling period (23.ii–5.iii.2021) Lepidoptera are presented with 11 species of 9 genera. During the second sampling period (5–26.iii.2021), we collected 9 species of 8 genera. Regarding Coleoptera, for the first period, we recorded 18 species of 17 genera; for the second period – 8 species from 8 genera respectively. Diptera: first period 20 species from 7 genera and second period – 21 species from 8 genera. Hymenoptera: first period 21 species from 12 genera and second period – 13 species from 7 genera. Hemiptera: first period 9 species from 8 genera and second period – 5 species from 5 genera. As all specimens of order Araneae collected in the traps were juveniles, their species identification based on morphology was not feasible.

Air temperature data

We performed a Pearson r correlation analysis to compare the effect of the outside temperature on the cave temperature at the colony's location. For this purpose, we used the daily average temperature from the surface and the daily average from the cave. The r value was 0.53, and the R squared value was 0.28. There is a significant positive correlation between the outside temperature and the cave temperature, with a P -value of 0.0001. These values suggest that the temperature inside the cave is influenced by the air temperature outside the cave. Only 30% of the temperature variance inside the cave can be attributed to internal cave factors. Since the temperature data from outside the cave is representative of 814 metres above sea level and the entrance of the cave is at 575

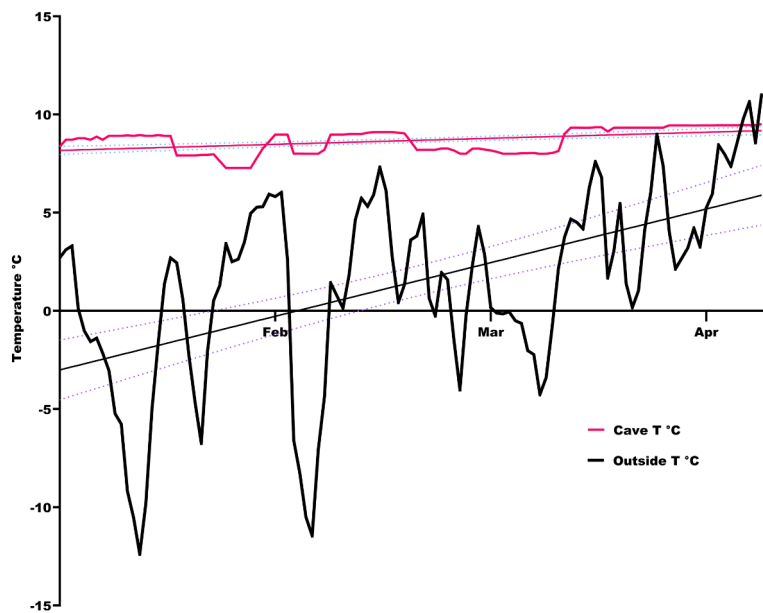


Fig. 9. Average daily temperature fluctuations in Razhishkata Cave and outside the roost.

metres above sea level, the altitude difference of 239 metres introduces a negative bias of between 1.4–1.8 K. This is one of the contributing factors to the temperature bias between the temperature outside and inside the cave. The graphic representation of the data is presented in Fig. 9. All raw data is available in [Supplementary material 05 \[* .xlsx\]](#).

Discussion

Our study is the first regarding the winter activity and dietary preference of bat diet of Schreiber's bent-winged bat. The winter diet of only four European bat species has been studied so far: the Mediterranean horseshoe bats (*Rhinolophus euryale* Blasius, 1853) from Slovakia and Hungary (Miková et al., 2013), the lesser horseshoe bat (*Rhinolophus hipposideros* (Bechstein, 1800)) from Great Britain and Ireland (Williams et al., 2011), the Natterer's bat (*Myotis nattereri* (Kuhl, 1817) from Great Britain (Hope et al., 2014) and noctule bat (*Nyctalus noctula* (Schreber, 1774)) from Slovakia and the Czech Republic (Kanuch et al., 2005). Three of the studies used only morphological diet identification. In the study from 2014, Hope et al. used molecular and morphological diet approaches to determine the winter diet of *Myotis nattereri*. Our study was the first to combine both approaches with an intensive insect survey in the

studied area and the first to look at the winter diet of the Schreiber's bent-winged bat (*Miniopterus schreibersii*). In addition, we also collected data for correlation of the temperature fluctuation in and outside the roost with an ultimate goal to see how this is affecting the bat activity patterns. Even though we could not measure the total guano accumulated in the guano traps during our study, we could observe differences between the studied periods. The coldest winter period was also followed by the least droppings gathered on the guano sheet. This also corroborates other European studies showing that outside temperatures and wind speed strongly affected bat foraging behaviour during the winter (Avery, 1986; Barros et al., 2017; Mas et al., 2022). Thus, we argue that the accumulation of guano can be used to measure Schreiber's bent-winged bat activity during the winter. This gives information on the activity outside the roost and samples for their trophic interactions.

Given the limitations of our study, we could only assess the most frequently occurring (FO) prey item in the diet. Metabarcoding on pooled samples leads to high error rates in estimating the FO rate of prey items (Mata et al., 2018). Furthermore, common prey species will become much more abundant but less common prey will be in an even smaller proportion in the pool samples (Mata et al., 2018). The analysis of a single pellet is as likely to detect abundant species as is the analysis of a pooled sample (Mata et al., 2018). Thus,

pooled and small sample-size metabarcoding research can be used for low-cost and preliminary research, especially during periods when prey availability and overall prey diversity are scarce. Additionally, the molecular data used in our study was under rigid validation by experienced taxonomists. This validation was crucial as a fraction (5%) of the assessed prey items were species and genera that occur neither in Bulgaria nor in the Palaearctic. Their presence in the studied species' diet is probably a mistake from the taxonomic assignment or initial lab contamination. Hence, we argue that combining metabarcoding with specialist checks for the taxa and insect surveys in the region which provide a reference “menu” of the available food resources, is essential for higher levels of confidence in the results and better interpretation of the bat diet.

With the metabarcoding, we could identify insects that were not detected in the traps, like the common moth species *Emmelina monodactyla* (Linnaeus, 1758), which is indeed active in this region and has been frequently observed during the winter (our data with UV traps). All of the most abundant moth species detected via barcoding are nocturnal. According to our observations in the region, they are active all winter if the temperature is above 0°C in the evening. A lot of the detected in metabarcoding bees (*Andrena helvola* (Linnaeus, 1758), *Bombus* spp.), wasps (*Vespa crabro* Linnaeus, 1758, *Cerceris rybyensis* (Linnaeus, 1771), *Sphex funerarius* Gussakovskij, 1934)), flies (*Acemya* sp., *Atherigona soccata* Rondani, 1871, *Chrysomya* sp., *Cheilosia soror* (Zetterstedt, 1843), *Eristalis pertinax* (Scopoli, 1763), *Hebia flavipes* Robineau-Desvoidy, 1830, *Heteromyza rotundicornis* (Zetterstedt, 1846), *Hydrotaea basdeni* Collin, 1939, *Limnophora tigrina* (Am Stein, 1860), *Phaonia valida* (Harris, 1780), *Silba adipata* McAlpine, 1956), butterflies (*Maniola jurtina* (Linnaeus, 1758)) have a diurnal activity. Rarely *Bombus* spp. or some of the above-mentioned flies are active at dusk, but only in warmer periods of the year (our observations). Presence of prey with obligatory diurnal activity in the identified diet items, suggests that the bats might also be feeding during the day. We could only speculate that bats have a diurnal activity or an activity shifted towards twilight during the winter period (our previous observations of flying bats at noon in similar habitats in the winter). This confirms some observations for other areas and periods of the year (Russo et al., 2011; Mikula et al., 2016; Malmqvist et al. 2018). Some studies have claimed that bats might feed

on the cave fauna during the winter (Kanuch et al., 2005; Sano, 2006). Nine non-parasitic insect species (6 Lepidoptera, 1 Hymenoptera and 2 Diptera) are recorded in the well-studied Razhishkata Cave (Beron, 2015). In the metabarcoding analysis, none of the detected prey items were cave dwellers and the above-mentioned species were also missed. We argue that the densities of their populations are very low and cannot be considered a substantial food resource for bats during any period of their life. Our results showed that winter insect activity in the region near the roost provides the bats with enough food opportunities.

The flight interception traps give a good and attractant-independent picture of the number of flying insects. This together with the easy maintenance were the reasons for choosing them for this study. On the other hand, our results indicate that the FITs are not the best alternative for estimating the species diversity of potential Schreibers' bent-winged bat prey. A lot of common noctuid moth species in the Lakatnik region, collected by UV light (our observations) were missed in our material. The reasons could be the smaller population density of the winter active moths in comparison to other insect groups. The light traps with UV-rich spectra are the best methods for collecting nocturnal moths, but relying on attraction, their use can produce unrealistic assessment of the population density compared to other insects that are not attracted to artificial light. In general, for future studies, the combination of different trap techniques (light traps with UV-rich spectra, Malaise traps, pitfall traps, Langourov type tree traps) would be a good solution.

Many factors affect the temperature inside caves and the underground temperature is strictly connected with the external climate. Consequently, the global climatic changes will somewhat influence the cave climate (Badino, 2004). Our data shows that the temperature in Razhishkata Cave, at the location of the bat colony, positively correlates with the temperature fluctuation outside the cave. This might have a strong influence on the activity patterns of the bats. Many of the winter colonies of the Schreibers' bent-winged bat in the important bat underground habitats in Bulgaria (Ivanova, 2005; Deleva et al., 2022) are situated at a similar distance from the roost entrance as in the Rezhishkata Cave (own observations). It could be speculated that bats choose sites for hibernation in the winter roosts that are relatively close to entrances and temperature fluctuations could be felt. This may give them an advantage and opportunity to be active in

winter with good weather and presumed winter activity of the insects. *Miniopterus schreibersii* selects warmer caves to hibernate (Barbu, 1958; Pandurska, 1993). During the first sampling period, strong snow melting led to significant water dripping from the ceiling of the studied cave. As a result, the bat cluster was more spread out which could lead to decreased advantages from the social thermoregulation (Willis & Brigham, 2007). After the heavy snowfalls, during the last sampling period and flooding in the cave, the colony changed its location to a drier place close to the entrance. More data regarding temperature fluctuation in caves at the locations of the bat colonies, and how this is affecting winter bat activity is needed. Additionally, more information on the flooding vulnerability in the important winter underground bat roosts will be key for identifying sites under risk and ultimately can lead to better conservation planning.

Lastly, we want to elaborate on some practical tips for future research. The selection of the study cave should consciously consider easy to access and survey winter roost; a flat floor under the colony is preferable and the size of the guano trap should be larger than the colony's size. Considering that winter is sensitive period of the life cycle of the bats and frequent visits to the roost could lead to disturbance following all the rules and recommendations is essential. Good knowledge of the potential spatial positions of the colonies will help to predict other locations for traps in the event of in-roost activity of the colony. Samples should be measured and collected weekly for better resolution. Including acoustic data for the bats exiting the roost, together with data on the temperature and humidity fluctuations could provide a good picture for understanding the winter activity patterns of bats of the Balkan Peninsula and Europe. Our main goal was to establish a low-cost and easy-to-replicate research framework which we plan to repeat once every few years. Ideally in the future, a long-term dataset could potentially shed light on the effect of climate change on hibernating bats in temperate regions. Hence, publishing this first season of data collection was a way to propose this strategy on a broader scale and inspire similar studies in different locations.

Availability of data and material

All datasets generated and analysed during the current study are available as supplementary materials.

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Conflicts of interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.






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Supplementary materials

01

Document title: Raw data collected during the microscope-based morphological analysis used in the study

Kind of document: Microsoft Excel (OpenXML)

MIME type: application/vnd.openxmlformats-officedocument.spreadsheetml.sheet

Document name: [000513000452023-01.xlsx](#) 

02

Document title: Validated and raw metabarcoding data used in the study

Kind of document: Microsoft Excel (OpenXML)

MIME type: application/vnd.openxmlformats-officedocument.spreadsheetml.sheet

Document name: [000513000452023-02.xlsx](#) 

03

Document title: Flight interception traps data (February-March) presenting all the invertebrate orders recorded in the study

Kind of document: Microsoft Excel (OpenXML)

MIME type: application/vnd.openxmlformats-officedocument.spreadsheetml.sheet

Document name: [000513000452023-03.xlsx](#) 

04

Document title: Flight interception traps data (February-March) listing the model groups recorded in the study

Kind of document: Microsoft Excel (OpenXML)

MIME type: application/vnd.openxmlformats-officedocument.spreadsheetml.sheet

Document name: [000513000452023-04.xlsx](#) 

05

Document title: Air temperature data from Razhishkata Cave recorded in the study and ERA5 temperature data for Lakatnik region

Kind of document: Microsoft Excel (OpenXML)

MIME type: application/vnd.openxmlformats-officedocument.spreadsheetml.sheet

Document name: [000513000452023-05.xlsx](#) 