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Case study of non-lethal sampling for plant-pollinator networks via barcoding and metabarcoding on bumble bees in Germany

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Abstract

1.) Insect decline is threatening ecosystem stability, making information about foraging preferences of pollinators a vital information to acquire. A powerful emerging tool to study pollinator foraging behavior is pollen-metabarcoding. This usually involves lethal sampling of insects.
2.) Here, we propose a new, non-lethal way of sampling DNA for the analysis of pollen loads of bumble bees as well as the pollinator. The new methodology does not significantly harm the insect and is easy to implement in a wide range of study designs. The tool is cheap and easy to acquire, can easily be used in the field, and has the potential to become a powerful tool in studying plant-pollinator interactions.
3.) To test its feasibility, plant-pollinator networks were analyzed using metabarcoding of the ITS2 region. Plants flowering at the time of collection were also recorded as a reference comparison.
4.) Bumble bees with ambiguous morphology were additionally identified based on COI barcoding.
5.) With the workflow developed here it is possible to gain knowledge about plants and their pollinators in a non-lethal way without reducing population sizes. This makes this method particularly suitable for endangered and protected species.

Keywords: Cytochrome c oxidase I (COI), endangered species, internal transcribed spacer (ITS), pollination, twinned pollinator-pollen analysis, species diversity

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Introduction

The ongoing decline of insect populations, both in terms of species numbers (Sánchez-Bayo and Wyckhuys 2019) and biomass (Hallmann et al. 2017), is concerning. This decline primarily affects pollinators, but it also has significant consequences for various plant species that they pollinate (Kluser and Peduzzi 2007, Althaus et al. 2021). Cascading effects of this loss can potentially destabilize ecosystems, leading to the collapse of pollination networks and jeopardizing overall ecosystem stability (Dicks et al., 2021; Lever et al., 2014; Rhodes, 2018). Among these vital pollinators, bumble bees (genus *Bombus*) stand out. Social bumble bees play a significant role by collecting nectar from plants and gathering pollen to nourish their offspring (Goulson 2010). They have unique characteristics, including adaptation to colder temperatures, enabling them to pollinate earlier as well as later in the year (Woodard 2017). Additionally, bumble bees possess the ability for buzz-pollination, a skill which is crucial for pollinating plants in the nightshade family Solanaceae (Pritchard and Vallejo-Marín 2020). However, the precise foraging behavior of bumble bees remains poorly understood. Though considered polylectic (Woodard 2017) variations in foraging and dietary preferences among species (Laverty and Plowright 1988) as well as individuals of the same species (Ruedenauer et al. 2016) have been reported. A better understanding is essential for the conservation of threatened species.

A promising tool for bridging this knowledge gap is pollen metabarcoding, a method to genetically analyze the pollen load of insects and identify the plant species they visit (Bell et al., 2022). However, until now, this method typically requires collecting and killing of insects (Bell et al., 2017). This also allows for genetic identification of bumble bees via DNA barcoding by using insect tissues, as certain species share similar morphology and can only be accurately distinguished by genetic means (Murray et al. 2008). Criticisms were raised regarding lethal collection of insect specimens (Miller et al. 2022), suggesting that this practice might further accelerate the decline of target species. Proposals were made to collect bee specimens only at specific times in the colony life cycle when the loss of workers is less detrimental (Camilo 2022). However, it is crucial to obtain information about foraging behavior during all life stages. A twinned approach of combining pollen metabarcoding and pollinator barcoding proved to be very promising (Ronca et al. 2023), but still depends on lethal sampling.

We here present a cost-effective, easy to implement, non-lethal alternative for the collection of genetic material from bumble bee pollen and insect tissue. The method ensures that the foraging ability of bumble bees is not significantly impaired. Using a sample dataset of bumble bees collected in Northern Germany and the pollen adhering to them, we demonstrate the successful analysis of bumble bee identification via barcoding, as well as pollen analysis via metabarcoding. Plant-pollinator networks are verified by observations of flowering phenology during insect collection.

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Methods

Sampling

Sampling took place on June 13th 2023 between 11:00 a.m. and 3:00 p.m in the nature protected area Cuxhavener Küstenheide (WDPA-ID: 329318) in northern Germany, with permission from the respective nature conservation authority. Along a 500 m north-west to south-east transect all plants flowering in a proximity of 30 m were recorded. Bumble bees were captured along the transect using a queen-marker-cage (Fig. 1A). This device was originally designed for beekeepers for tagging honey bee queens (e.g., Imgut®, article number 4756). It comprises a tube with a coarse mesh at one end, and a punch at the other, which was covered with sterile cling film before each sampling. Insects can be gently immobilized with this device (Fig. 1C). Pollen was collected from the corbicula of immobilized bumble bees using a fine art brush through the mesh. Cuticle scissors were then used to clip the tarsus from one middle leg of the insect (Fig. 1B). This tarsal-clipping of a middle leg does not affect the productivity of bumble bees (Holehouse et al. 2003) but provides sufficient material for the genetic analysis. To free the insect the mesh was cautiously opened. After the insect was released, the punch was lowered to the brim of the tube and the cling film was folded over the genetic material. This wrapped cling film was then placed into a 2 ml centrifuge tube until further processing in the laboratory. To prevent cross-contamination, all equipment, including queen-marker-cages, underwent a rigorous cleansing process with 10% bleach solution. Subsequently, the equipment was rinsed with 70% ethanol and dried off, before reuse in the field.

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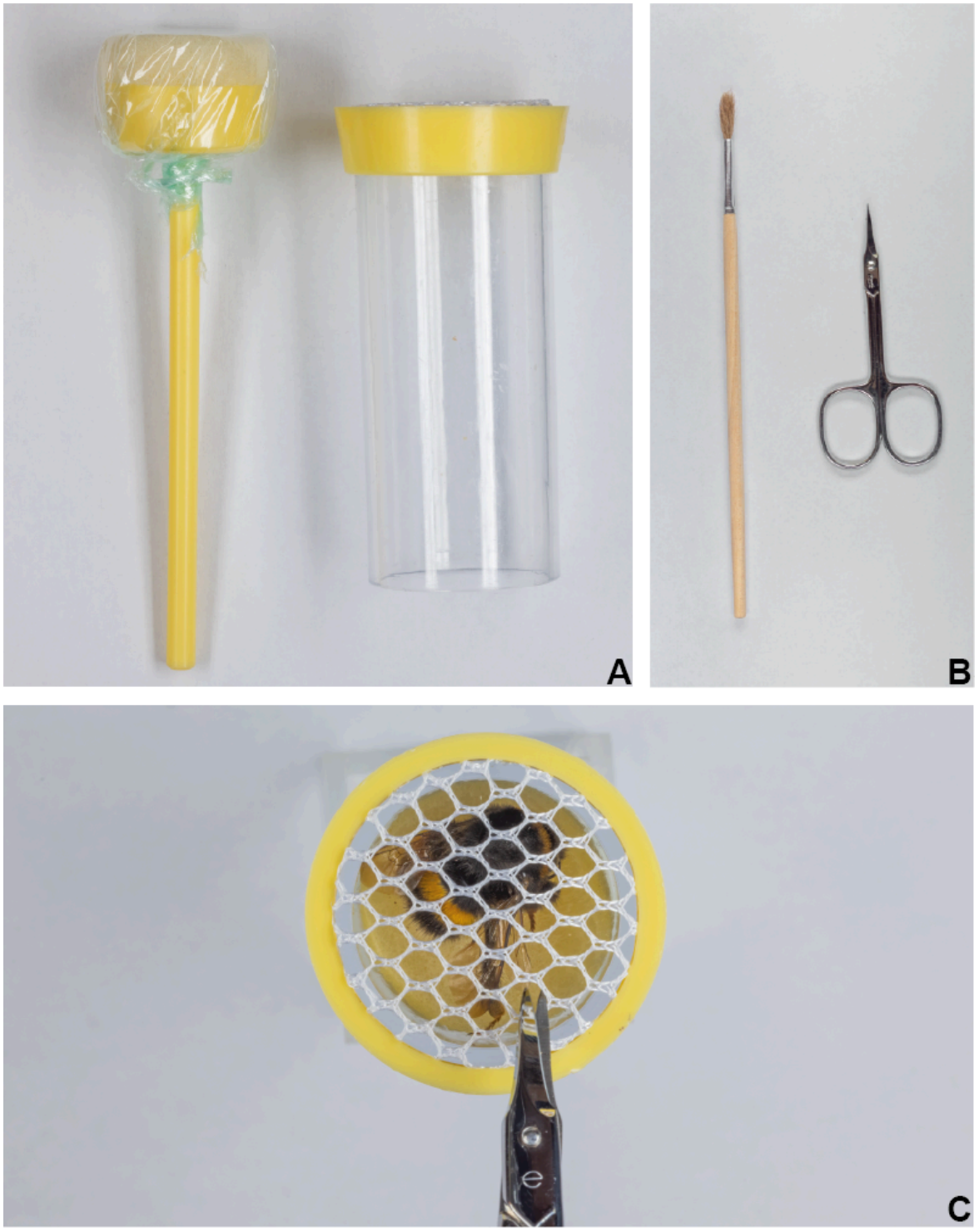

Figure 1: Equipment for non-lethal insect sampling in the field
A queen-marker-cage for immobilizing insects, **B** cuticle scissors and fine art brush, **C** captured bumble bee in the queen-marker-cage (top), displaying the mesh's large holes through which the equipment can be maneuvered to dislodge the pollen as well as remove the tarsus.

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Molecular laboratory work

Laboratory work for plant metabarcoding was carried out in a controlled clean room environment to minimize contamination. Quality control measures comprised controlled airflow, air filtration systems, and UV light cleaning. Using sterilized tweezers the tarsus was removed from the tube and the pollen was scraped from the cling film and transferred to a fresh 1.5 ml centrifuge tube using stainless-steel laboratory spatulas. Homogenization was achieved by bead milling the sample for 2.5 minutes at 30 Hz (Retsch MM400). Plant DNA extraction was performed using a modified protocol of the NucleoMag DNA extraction kit (Macherey-Nagel, REF744400.4; Kolter et al., 2023).

PCR targeting the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA followed (Kolter and Gemeinholzer 2021a) (See Supplementary file 1: PCR protocols). Positive and negative controls were added. Three PCR replicates per sample/control were conducted and subsequently pooled. Illumina NextSeq sequencing was carried out by LGC-Genomics (Berlin/Germany).
COI-barcoding analysis for bumble bee identification was performed in a standard molecular lab environment. For DNA extraction the DNeasy® Blood & Tissue kit (Quiagen, ID: 69506) was used with modifications (See Supplementary file 3: Tarsi DNA extractions). The LCO1490 and HCO2189 primers (Folmer et al. 1994) were used to target the COI gene. PCR details can be found in See Supplementary file 1: PCR protocols. Positive and negative controls were added.
Sequencing was carried out by Eurofins Genomics (Luxembourg City/Luxembourg).

Data analysis

Raw data from Sanger sequencing for the bumble bees underwent editing using MEGA X (Kumar et al. 2018). Taxonomic assignments were made using the BLAST search (Altschul et al. 1990) against GenBank (Sayers et al. 2021).

Raw Illumina MiSeq reads of pollen samples were analyzed via a bioinformatic pipeline using APSCALE (Buchner et al. 2022), VSEARCH (Rognes et al. 2016), cutadapt (Martin 2011), LULU (Frøslev et al. 2017) and R (See Supplementary file 2: Data analysis). A custom reference database was constructed, containing 583.533 vouchered plant and fungal ITS2 sequences obtained from GenBank (NCBI, data downloaded on 20.08.2023; Sayers et al., 2021). The search queries “ITS", "internal transcribed spacer", "voucher", "Streptophyta" and ”Fungi" were used and processed with the R package taxonomizr (Sherrill-Mix 2023).

The resulting Exact Sequence Variance (ESV) table used a 1% cutoff based on Peel et al. (2019), who found that false positives occur at a rate below 1%. ESV data were transformed into a presence/absence matrix to construct pollinator networks using the bipartite package in R (Dormann, 2019).

Manuscript preparation

During the preparation of this work, the authors used AI-assisted technology, including DeepL Write and ChatGPT (v4), to improve language and readability. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Results

In this study 12 out of 21 investigated bumble bee individuals could unambiguously be identified in the field based on their morphology (1 *B. hypnorum*, 8 *B. lapidarius* and 3 *B. pascuorum*). 9 bumble bees exhibited ambiguous morphological characteristics, identified as 4 *B. lucorum* agg. and 4 *Bombus* sp. Subsequent COI barcoding of these ambiguous individuals revealed 2 *B. cryptarum*, 4 *B. pascuorum* and 3 *B. terrestris*, resulting in the successful identification of all 21 bumble bee individuals, in total belonging to 5 distinct species (2 *B. cryptarum*, 1 *B. hypnorum*, 8 *B. lapidarius*, 7 *B. pascuorum* and 3 *B. terrestris*). The raw sequence data have been deposited in GenBank and the accession numbers as well as a full list species list is found in Supplementary file 4: Bumble bee species list.

Pollen metabarcoding generated 642.151 raw sequences, which are deposited in the Sequence Read Archive under the accession number PRJNA1120766. Quality filtered sequences were cross-referenced against the ITS2 database and resulted in an unambiguous identification of 18 plant species (Fig. 2). However, four ESVs could only be identified to genus level (*Lotus* sp., *Ranunculus* sp., *Rosa* sp. and *Trifolium* sp.). *Trifolium* was the most commonly detected genus, with *T. repens* found on all 21 bumble bees. In contrast, *Jasione montana, Lotus* spec., *Prunus persica*, *Ranunculus* spec., *Ranunculus sardous, Rubus caesius,* and *Rumex acetosella* were detected on only one bumble bee individual each.

The vegetation survey yielded 17 different plant species, 13 of which were also detected by pollen metabarcoding. *Achillea millefolium*, *Galium saxatile*, *Hieracium pilosella* and *Stellaria graminea* were flowering but did not show up in the metabarcoding analysis. *Prunus persica*, *Prunus serotina*, *Rosa* sp. and *Rubus caesius* were found in the pollen DNA analysis but were not detected on the sampling site. All plant species recorded through either the phenology survey or the pollen metabarcoding analysis are likely to occur on site, except *Prunus persica* (NLWKN, Lower Saxon State Department for Waterway, Coastal and Nature Conservation, und business section 4, 200; GBIF Occurrence Download from the 27th March 2024, https://doi.org/10.15468/dl.9sasyx).

Bumble bee individuals carried pollen from two up to seven different plant species, resulting in a plant-pollinator network with 28 interactions (Fig. 2). The most prominent plant genera were *Trifolium* and *Vicia*. All bumble bee individuals, regardless of species, were found to carry pollen of *Trifolium* sp. and *Trifolium repens*. Pollen belonging to the genus *Vicia* as well as pollen of *Trifolium pratense* were exclusively found on *B. pascuorum*. Pollen of *Jasione montana*, *Lotus* sp. and *Hypochaeris radicata* were only found on *B. lapidarius*.

B. cryptarum carried pollen of four different plant species (*Cerastium fontanum*, *Rumex acetosella*, *Trifolium* sp. and *Trifolium pratense*). *B. hypnorum* carried pollen from three plant species (*Rosa* sp., *Trifolium* sp. and *Trifolium repens*). The *B. lapidarius* specimens carried pollen from nine different plant species (*Cerastium fontanum*, *Hypochaeris radicata*, *Jasione montana*, *Plantago lanceolata*, *Prunus persica*, *Prunus serotina*, *Trifolium* sp., *Trifolium repens* and *Veronica officinalis*). Similarly, nine plant species were detected on *B. pascuorum* (*Cerastium fontanum*, *Ranunculus* sp., *Ranunculus sardous*, *Rubus caesius*, *Trifolium* sp., *Trifolium pratense*, *Trifolium repens*, *Vicia cracca* and *Vicia sativa*). Finally the *B. terrestris* individuals carried pollen from 7 different plant species (*Plantago lanceolata*, *Prunus persica*, *Prunus serotina*, *Rosa* sp., *Trifolium* sp., *Trifolium repens* and *Veronica officinalis*).

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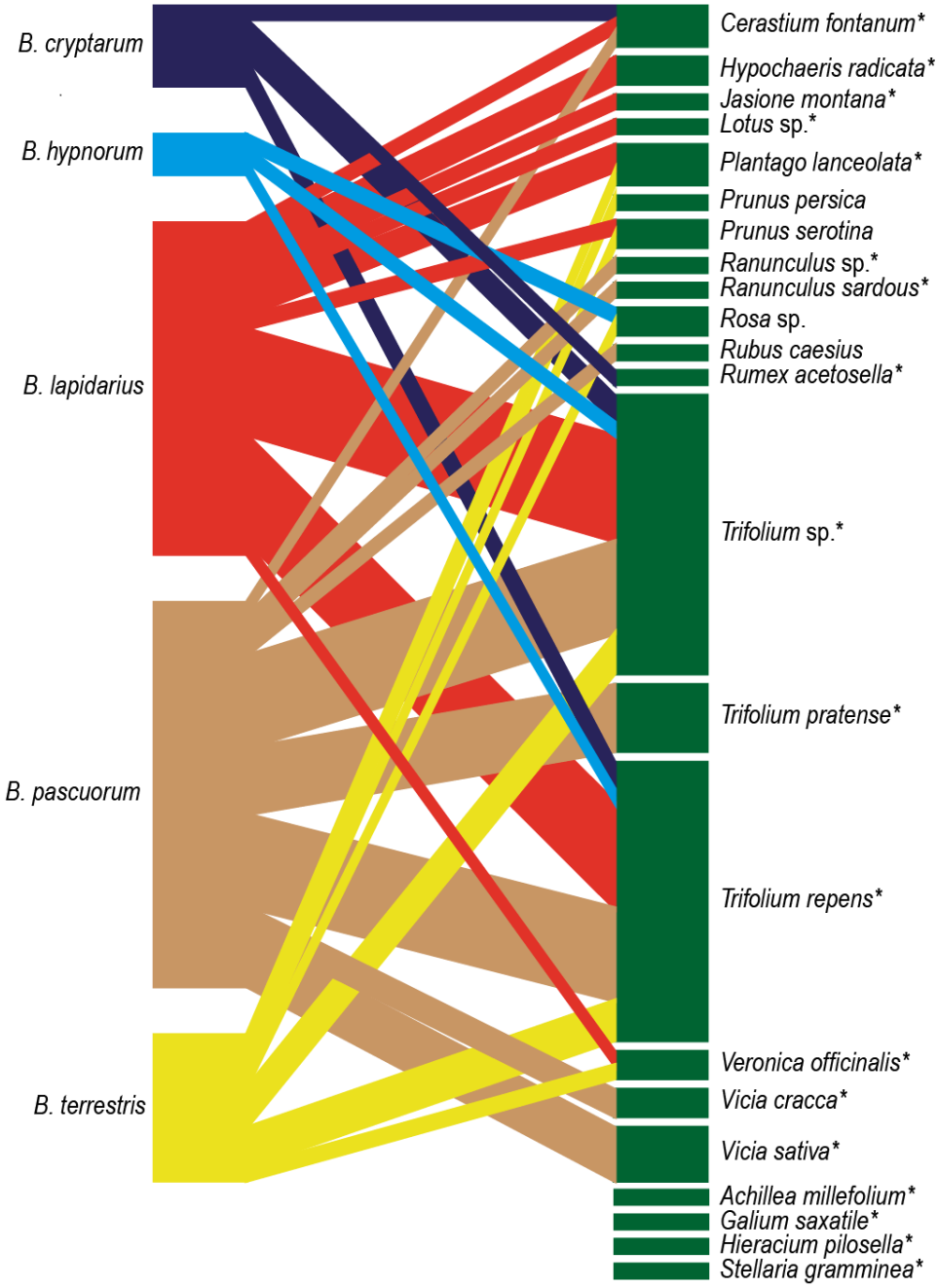

Figure 2: Plant-pollinator network of bumble bees collected in Cuxhavener Küstenheide
Interactions revealed by metabarcoding of pollen of 21 bumble bees and barcoding of insect tissue in a nature conservation area in Northern Germany on 13.06.2022. Detected plant species supported by phenology screening are marked with an asterisk.

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Discussion

The new, cost-effective, easy-to-perform and non-lethal alternative for obtaining DNA material from bumble bee pollen and insect tissue proved to be successful.

By conducting pollen metabarcoding, supported by phenological studies and non-lethal insect barcoding in addition to morphological identification we could deduce plant-insect relationships comparable to earlier studies on the same site (Kolter et al. 2023) and plant-pollinator networks in general (Memmott 1999, Dicks et al. 2002). As previously suggested, morphological identification of bumble bee species, particularly within the *B. lucorum* complex, proved challenging. However,the COI barcode region could resolve these uncertainties as well as complement correct *Bombus*-species level identification (Carolan et al. 2012, Williams et al. 2012, Ronca et al. 2023). The non-lethal sampling strategy presented here likely does not harm the insect population, as we observed bumble bees gathering pollen that lacked the mid-tarsus, indicating prior sampling. This echoes findings by Holehouse et al. (2003), suggesting minimal impairment of the insects foraging capabilities. The identified bumble bee species in our sampling align with those found earlier on this site (Sprichardt 2011). While *B. cryptarum* was observed in 2007, it was absent in 2009 (Spichardt 2011). Our study reaffirms the presence of this species at this site again in 2022.

Pollen metabarcoding successfully provided a species list of plants visited by bumble bees. For plant pollen species level identification conservative filtering is recommended (Tommasi et al. 2021). We chose a 1% cutoff in accordance with Peel et al. (2019), but if false positives pose a significant risk, a more conservative approach such as Receiver Operator Characteristics (ROC) should be considered (Serrao et al. 2018). Accurate species level identification is highly dependent on comprehensive reference databases (Kolter and Gemeinholzer 2021b, Keck et al. 2023). Here, we opted for an approach using publicly available sequences, which might have led to some plants being identified only to genus level. A database using regional plants could enhance the resolution.

Although four plants detected in pollen metabarcoding were absent in the flowering vegetation survey, this discrepancy likely stems from the small size of the nature protected area (<800 m^2) and the known foraging radius of bumble bees, surpassing the surveyed area (Westphal et al. 2006). All plants detected are common in the broader region, except *Prunus persica*, a popular ornamental garden plant. Its presence in our analysis likely stems from such planted trees in the nearby gardens. With the workflow developed here it is possible to gain knowledge about plants and their pollinators. The non-lethal sampling approach is not reducing bumble bees population sizes but allows for close examination of endangered bumble bees and their plant visits at different times throughout the colony's life cycle. This can mitigate the potential decline of pollinators, a goal also formulated by the IPBES report (Potts et al. 2016). With minor modifications, the method presented here could also be adapted to smaller pollinators, further expanding its applicability.

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Conclusion

In this study, we successfully demonstrate a cost-effective, easy-to-perform, and non-lethal method for obtaining DNA material from bumble bee pollen and insect tissue. By combining pollen metabarcoding and insect barcoding, we constructed plant-pollinator networks that align with previous research and the phenology on the collection day.

Pollen metabarcoding retrieved a comprehensive species list of plants visited by bumble bees, very similar to the vegetation survey and consistent with the occurrence data for the area. Employing barcoding for insect identification resulted in delimitation of *B. cryptarum* underscoring the necessity of genetic identification of bumble bees.

Our non-lethal sampling approach offers a valuable tool for studying plant-pollinator interactions without reducing bumble bee populations. It allows for the monitoring of endangered species and their foraging habits throughout the entire colony's life cycle, contributing valuable information to efforts aimed at mitigating pollinator decline. Future research could adapt this method for smaller pollinators, broadening its applicability and impact in the field of pollination ecology.

Additional Information

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Ethical statement

No ethical statement was reported.

Competing interests

The authors have declared that no competing interests exist.

Authors Contributions

Alexander Edwards and Birgit Gemeinholzer conceived the ideas and designed methodology; Alexander Edwards collected and analyzed the data; Alexander Ewards wrote the manuscript with feedback and editing from Birgit Gemeinholzer. All authors contributed critically to the drafts and gave final approval for publication.

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