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SHORT COMMUNICATION

Insect Conservation and Diversity

DNA barcoding resolves quantitative multi-trophic interaction networks and reveals pest species in trap nests

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Abstract

1. Insects, as one of the most species-rich taxa with enormous taxonomic, behavioural and functional diversity, are in decline. Bees and wasps are especially crucial for ecosystems as pollinators or to control populations of other insects. To understand population drivers, comprehensive knowledge about top-down and bottom-up interactions, including all interaction partners, is needed.
2. Nests of trap-nesting bees and wasps include multi-trophic interactions between bees, wasps, their food resources and natural enemies, simultaneously, however, up to today, all trophic interactions are not yet included in trap nest research because of challenges to identify the food used by nesting bees and wasps.
3. Here, we reconstructed quantitative three- and four-trophic interaction networks of species in three apoid wasp families using DNA barcoding. The obtained tripartite and quadripartite networks encompassed natural enemy-wasp-spider and natural enemy-wasp-herbivore-plant interactions. Moreover, we identified so far undescribed Hymenoptera-prey interactions, including prey species known as agricultural and forest pests.
4. More extensive research on bee and wasp multitrophic interaction networks will provide valuable insights to better understand responses to environmental and biodiversity change, to investigate ecological theory and to reveal so far unknown feeding links.

KEYWORDS
bipartite, cavity-nesting, food resources, quadripartite, tripartite, trophic cascade

INTRODUCTION

Within insects, bees and wasps play a fundamental ecological and economic role, particularly bees as crucial pollinators, and wasps as predators controlling populations at lower trophic levels such as agricultural pest species (Harris, 1994; Ollerton et al., 2006; Klein et al., 2007). Together with other insects, most wild bee and wasp species are in decline, for example, due to the use of agrochemicals or habitat loss (Dicks et al., 2021; Goulson, 2019; Hallmann et al., 2017; Powney et al., 2019; Senapathi et al., 2015; Trapp et al., 2017; Zattara & Aizen, 2021). To identify direct and indirect drivers of species decline, not only the focal species but also its trophic interactions acting bottom-up or top-down on its populations need to be considered. However, sampling of multi-trophic interactions from primary producers to natural enemies of predators at

species level identification of all organisms involved is challenging (Blanchet et al., 2020; Hochkirch et al., 2022).

To study effects across multiple trophic levels, analysis of observed trophic interactions should be more informative than analysing co-occurring communities (Blanchet et al., 2020). A unique option to study multi-trophic, quantitative interactions of individual insects across multiple trophic levels are nests of cavity-nesting bees and wasps. These trap nests include bee and wasp larvae, food resources, and natural enemies simultaneously (Krombein, 1967; Staab et al., 2018; Tscharntke et al., 1998; Turčinavičienė et al., 2016). The application of trap nests for monitoring populations, diversity and multi-trophic interactions, as well as natural history observations, has a long history and is now an established and increasingly used method in ecological research (Staab et al., 2018).

However, most trap-nesting studies focus only on bees and wasps or on bipartite interactions (e.g., Dürrbaum et al., 2023; Fornoff et al., 2021; Klein et al., 2004; Mayr et al., 2020; Staab et al., 2018; Tscharntke et al., 1998; Tylianakis et al., 2007). Studies investigating responses of species observed in trophic interactions should provide detailed information, for example, to study effects across multi-trophic levels like damping of bottom-up effects (Scherber et al., 2010; Schuldt et al., 2017) or dependence of network robustness across trophic levels (Fornoff et al., 2019). This is because interactions can be quantified and assigned to species–species interactions rather than trophic levels only.

Three-trophic interaction networks can be accomplished by inclusion of food resources in nests to create natural enemy-nesting Hymenoptera-food interactions. For example, to study bottom-up effects, the tripartite interaction network of natural enemy-bee-pollen is directly connected to primary producers. Also, herbivores such as aphids are mostly plant specialists (oligophagous/monophagous). Therefore, the interaction network around herbivore-hunting wasps can be extended from natural enemy-wasp-prey interactions to plants, by the inclusion of plant species that are consumed by herbivores acting as prey for wasps. To the best of our knowledge, there has not been any study on trap-nesting Hymenoptera that connects natural enemies to nesting Hymenoptera and these further to their food resources, especially due to challenges in food resource identification.

We use DNA barcoding to identify food resources and resolve multi-trophic interaction networks of cavity-nesting wasps that link natural enemies to nesting wasps, their food resources, and the plant level precisely. With this, we aim to reveal so far unknown feeding interactions, show that DNA barcoding is a crucial identification tool for the trap-nesting overall community and provide the first examples of three- to four-trophic interaction networks of trap-nesting wasps.

MATERIALS AND METHODS

Sample collection

Studied nests were obtained from trap nests exposed for colonisation at 13 sites across Germany from March to November 2019 (for coordinates see Table S1). Trap nests consisted of 120 hollow plant internodes (*Phragmites australis* and *Pseudosasa* sp.) providing about 240 cavities for nesting, with 20 cm length and a diameter range of 1–18 mm, placed in 10 cm diameter plastic tubes. All cavities were manually opened, and tubes containing Hymenoptera nests were stored at 4°C in glass tubes with a cotton plug. Within these, nests of the apoid wasp families Crabronidae, Pemphredonidae, and Psenidae were recognised by their nest closing plug, nesting architecture and collected prey arthropods. From these, 52 nests with developing prepupae and prey or prey remains were randomly chosen. The number of nests sampled from each site ranged from 1 to 11 (Table S1).

Sample preparation

Of each nest, all remaining prey individuals or remaining body parts were transferred to 96% pure ethanol. Of each nest, if available, three randomly chosen spiders or Hemiptera, or spider body parts were used for DNA barcoding. However, to capture the highest diversity of prey items, the available intact individuals of spiders and Hemiptera were sorted morphologically into morphotypes and, if present, distinct morphotypes were used for DNA barcoding. Wasps and natural enemies were reared to imagoes at room temperature in the glass tubes. Some wasps and natural enemies were identified morphologically using Jacobs (2007) and Kunz (1994). Subsequently, all wasps and natural enemies were barcoded.

DNA barcoding

Genomic DNA (gDNA) was extracted from 52 wasps (one from each nest), 98 prey items, and 10 natural enemies (Table S1), using the DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) by following the manufacturers' Protocol 'Purification of Total DNA from Animal Blood or Cells (Spin-Column Protocol)' with the following modifications: DNA was eluted in 30 µL of nuclease-free water. The quantity of the extracted gDNA was assessed with a Qubit 2.0 Fluorometer (Thermo Fisher, Waltham, USA). DNA samples were stored at −20°C until further processing.

Polymerase chain reactions (PCR) of the cytochrome C oxidase I (COI) gene fragment were conducted using the standard primer pair HC02198 and LCO1490 (Folmer et al., 1994). In cases where no amplicon has been yielded, we tested the following four primer pairs: LEP-F1 and LEP-R1 (Hebert et al., 2004), RonMWASPdeg_t1 and LepR1 (Hebert et al., 2004), LCO1490-JJ and HCO2198-JJ (Astrin & Stüben, 2008) and LCO1490 and C1-N-2191 (Simon et al., 1994) (Table S2), which also target COI. The Taq DNA Polymerase, recombinant (5 U/µL), was used to set up PCR reactions according to the manufacturer's protocol adapted with a total reaction volume of 20.0 µL. Depending on the sample quantity, the amount of gDNA and DEPC-treated water was adjusted. More information on the PCR conditions can be found in Table S2. Purified PCR products were sequenced on an ABI 3730xl system by Macrogen Europe B.V. (Amsterdam, Netherlands).

Raw DNA sequences were manually edited using Geneious Prime 2021.1.1 (https://www.geneious.com) and searched against the National Center for Biotechnology Information (NCBI) database using the Nucleotide collection (nt/nr) database of the Basic Local Alignment Search Tool BLAST with the following option: highly similar sequences (megablast; Altschul et al., 1990; Camacho et al., 2009). All findings were further verified with the BOLD Identification Engine for Animal Identification using the option 'Species Level Barcode Records' (Ratnasingham & Hebert, 2007).

Quantitative tripartite networks

To visualise interactions between the wasps species, their prey species, and their natural enemy species, quantitative bipartite networks were plotted using R version 4.2.2 (R Core Team, 2022) with the R package 'bipartite' (Dormann et al., 2009). Regardless of origin of the sample location, wasps were pooled by their trophic position, separating herbivore-hunting wasps (3rd trophic level) from spider-hunting wasps (4th trophic level), and their interaction partners.

For quantification of interaction networks, we used the number of individuals that were sequenced (maximum three per nest) for prey individual abundance (spider and Hemiptera). For wasp quantification, we used the number of nests from which prey was sampled in the wasp-prey network and the number of brood cells that were available for natural enemy attack for the wasp-natural enemy network. Natural enemy abundance was the count of brood cells that they attacked.

Hemipteran host plant species were extracted from the aphids on world's plants database (Blackman & Eastop, 1994). If species had no unique host preference all, species sharing host plant species/genera were associated with the plant taxon they had in common. In the Hemiptera-host plant network, abundance of host plants equals the abundance of Hemiptera, as we assume aphids to be sessile.

RESULTS

The studied cavity-nesting wasps belonged to two species of spider-hunting wasps (*Trypoxylon figulus* and *T. clavicerum*) that collected 16 spider species as larval food and to six species of herbivore-hunting wasps from the genus *Psenulus* (*P. fuscipennis*, *P. pallipes*), *Passaloecus* (*P. corniger*, *P. insignis*) and *Pemphredon* (*P. lugens*; Table S1) that collected 21 Hemiptera species as larval food. The wasps were attacked by 21 natural enemy individuals, including cuckoo wasps (*Omalus aeneus* and *Pseudomalus triangulifer*), ichneumon wasps (*Perithous septemcinctorius*, *Perithous* sp., *Pimplinae* sp.), the eulophid wasp *Melittobia acasta* and the bee fly *Anthrax anthrax* (Table S3). Hemiptera were associated with 16 plant genera or families.

Quantitative bipartite networks

Of the 52 analysed, nests 34 belonged to the spider-hunting wasp genus *Trypoxylon*, and the remaining 18 nests belonged to Hemiptera-hunting wasps. *Trypoxylon* nests contained 135 brood cells, of which 104 *Trypoxylon* imagoes developed, while in two natural enemies, imagoes developed and 25 brood cells did not develop but contained 52 spider individuals as prey (Figure 1a). Hemiptera-hunting wasp nests comprised seven nests *of Passaloecus* with 22 brood cells of which nine imagoes emerged and 12 cells remained undeveloped containing Hemiptera prey, nine nests *of Psenulus* with 80 brood cells of

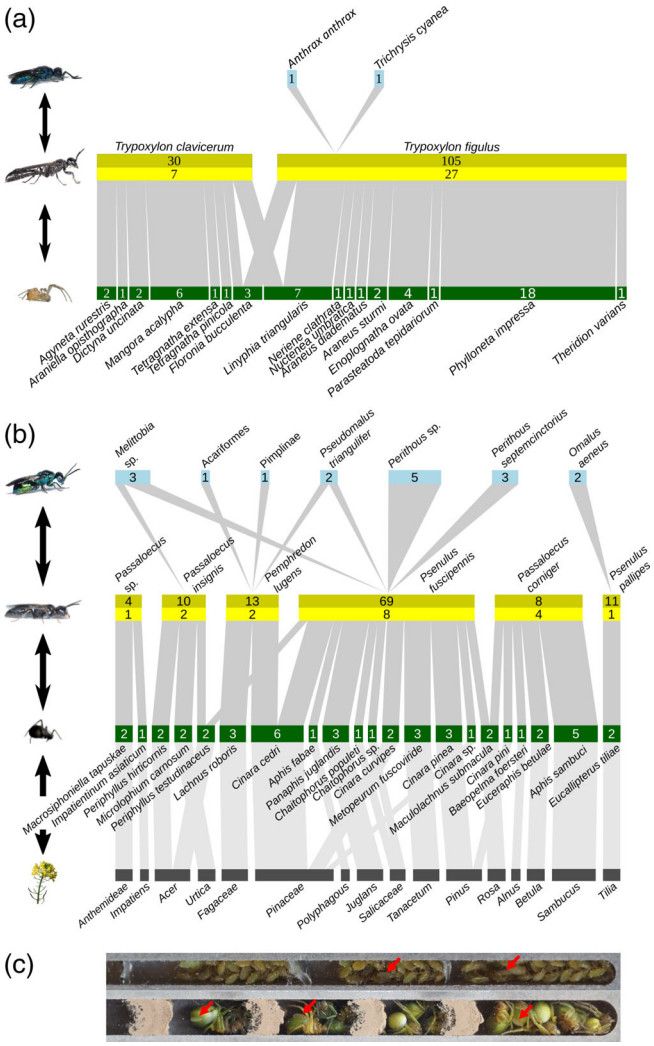

FIGURE 1 Tri- and four-trophic interaction network of directly observed interactions: (a) Natural enemies (blue boxes) of wasps (yellow boxes) and Araneae species (green boxes) collected as larval food. (b) Natural enemies (blue boxes) of cavity-nesting wasps (yellow boxes), their Hemiptera species collected as larval food (green boxes) and the literature-based addition of host plant species of Hemiptera (grey boxes). Grey bars (links) represent the recorded frequency of interactions. Numbers represent, for green boxes the number of individuals that were sequenced (maximum three per nest), for wasps, the number of nests (bright yellow) from which prey was sampled and the number of brood cells (dark yellow) that were available for natural enemy attack. Numbers in blue boxes represent the number of brood cells that natural enemies attacked out of all available brood cells. (c) Example brood cells of *Passaloecus* (top) and *Trypoxylon* (bottom) before egg hatching. Wasp eggs are indicated by red arrows between aphids and at spider abdomen.

which 31 imagoes and 15 natural enemies emerged and 34 cells remained undeveloped containing Hemiptera prey, and two nests of *Pemphredon* with 13 brood cells of which two imagoes and three natural enemies emerged and three cells remained undeveloped containing Hemiptera prey. Hemiptera species belonged to the families Aphididae and Psyllidae (Figure 1b).

DNA barcoding

We amplified DNA barcode sequences of 49 cavity-nesting individuals (94% of all attempts) comprising the three apoid wasp families Crabronidae, Pemphredonidae, and Psenidae, 98 prey individuals (100% of all attempts), and 10 parasites (100% of all attempts) (Table S3). All other individuals were identified morphologically. The DNA barcode sequences exhibited an average length of 966 bp (Min.: 551, Med.: 693 bp, Max.: 2064 bp; Table S3). After trimming, the DNA barcode sequences exhibited an average length of 597 bp (Min.: 108, Med.: 614 bp, Max.: 676 bp; Table S3). All amplified DNA barcode sequences were assigned to species level. All generated COI sequences were deposited in Mendeley (doi: https://doi.org/10.17632/9sbx5rpmy6.1). Morphology vouchers are available at the Institute of Biology at the University of Hohenheim, as listed in Table S3.

DISCUSSION

DNA barcoding revealed highly resolved tripartite and quadripartite trophic interaction networks, ranging from plants to herbivores (bees or food of wasps), predators and natural enemies of herbivore-predating wasps. These long interaction chains open up a vast spectrum of in-depth analysis. Moreover, we identified hitherto unknown predator–prey interactions, including agricultural pest species collected as larval food of wasps. With this, investigation of food resources of cavity-nesting bees and wasps widens the potential of trap nests as a standardised monitoring tool to study ecological processes and the drivers of species declines across levels of direct trophic interaction.

Quantitative tri-trophic interaction networks

Linking the identity and quantity of interaction partners across three to four trophic levels using individual-based interactions of cavity-nesting bees and wasps allows the analysis of interaction network indexes and feeding links across all trophic levels or in multiple connected bipartite interactions. Multitrophic interactions can be viewed from multiple angles and may be analysed in various ways, as summarised by Abdala-Roberts et al. (2019), Kawatsu et al. (2021), or García-Callejas et al. (2018), for example, for predicting extinction debt (Blanchard & Munoz 2023). Data on directly observed interactions across trophic levels may also allow for an in-depth description of the dependence of higher trophic levels on all levels below. For example, the damping of diversity-diversity associations with increasing trophic level (Scherber et al., 2010; Schuldt et al., 2017), but using observed interactions instead of co-occurring populations, could be studied. Multitrophic interaction data should be especially valuable when plants are part of the interaction network, as they can be effectively manipulated, for example, in BEF experiments (e.g., Bruelheide et al., 2014; Roscher et al., 2004). Moreover, connecting trophic interaction networks to the plant level should be valuable for species conservation, as plants are the foundation of many food chains but are comparatively easily managed. For example, reduced plant interaction partner diversity of herbivores should indicate their susceptibility to co-extinction (Aizen et al., 2012; Brodie et al., 2014), which also indicates changes in the interaction robustness of connected trophic levels that rely on these herbivores (e.g., Fornoff et al., 2019).

Sampling to resolve multitrophic interactions

Our study provides an example of how natural enemy-Hymenoptera-food resource networks can be attained and constructed. We processed nests and remaining food resources after imago development, which allows all natural enemies to develop and hence their detection. Especially parasitoids can be rare, cryptic, and difficult to identify at the larval or egg stage (Winterhagen, 2015). Alternatively, sampling food items before nesting Hymenoptera larvae develop (Figure 1c) increases DNA quality and quantity and allows the identification and quantification of all food arthropod species. Besides, gut content may also be analysed in fresh specimen, enabling the reconstruction of one further trophic level (Eitzinger et al., 2021; Hausmann et al., 2020; Jurado-Rivera et al., 2009). For each study, a balance between reared imagoes and fresh sampled food resources needs to be found to fit the purpose of the study.

DNA barcoding and metabarcoding

We applied DNA barcoding using traditional Sanger sequencing in combination with morphological identification and quantification of interacting individuals. The application of NGS for massively parallel sequencing of bulk samples (commonly referred to as DNA metabarcoding) allows the simultaneous detection of prey, nesting Hymenoptera and natural enemy species without prior separation of samples or even nests, but these separations are key for quantitative analysis of interactions. Therefore, individual-based or nested metabarcoding, as described by Evans et al. (2016), in which either the content of each nest (one cavity) or each individual can be traced back (Dürrbaum et al., 2023), seems to be the only feasible option to reveal the true potential of trap nests as monitoring tool for interaction network analyses.

The reliability of network analyses increases with network size. However, parasitoids are commonly much less frequent than nesting bees and wasps, but food items of the latter are commonly more frequent than those from their predators. Therefore, sampling intensity and workload increase with increasing trophic level, to produce reliable network indexes at all trophic levels.

Species identification and natural history observations

Information on food resources of cavity-nesting bees and wasps also has the potential to provide new insight into their feeding ecology and to identify potential biocontrol species. For example, in the nests of *P. fuscipennis* and *P. lugens*, we found *Aphis fabae*, known as a major pest of beans, beets, or potatoes (Blackman & Eastop, 2000), and *Cinara cedri*, a known pest of diverse *Cedrus* species (Ji et al., 2021). For a complete list of host plants and their known economic importance, see Table S4. Some trap-nesting bee species, for example, the Alfalfa-leafcutting bee, are reared in trap nests and used for crop pollination. In the same way, the studied generalist wasp species could potentially be reared to control pest species.

Besides, we found new records on aphid provisioning by several apoid wasp species. For example, *Psenulus fuscipennis* was known to provision their nests with aphids of the genus *Cinara* (Blösch et al., 2000), but half of all collected prey species belong to different genera, which shows that the prey spectrum is broader than previously described. This highlights how investigation of trophic interactions reveals top-down dependencies, which can be crucial information for conservation of species.

Furthermore, we found the spider species *Dictyna unicata* (Dictynidae) in a nest of *Trypoxylon clavicerum*, so far known to collect spiders from the spider families Araneidae, Linyphiidae, and Tetragnathidae. All of these new wasp-prey interactions were found by investigating only 52 nests belonging to eight different wasp species; therefore, much broader feeding niches can be expected with more extensive sampling efforts. These new insights into feeding preferences of common wasps will help explain their abundance or decline and identify pest antagonists for potential biocontrol.

The annotation of plant species to Hemiptera, found as prey in wasp nests, revealed the indirect dependence of wasps on woody and herbaceous plants. Five forbs and 11 woody plant species served as hosts for herbivore-hunting wasp prey. Previous studies found an association of abundance and species richness of trap-nesting wasp species with forest habitat (Montagnana et al., 2021). Our results suggest that wasps are probably not directly associated with forests but indirectly with trees that serve as food resources for their prey.

CONCLUSIONS

The combination of trap nests as a standard monitoring tool and species level identification of all interaction partners using DNA barcoding allowed the assessment of spider and herbivore species used as food resources for larvae in wasp nests and identified their natural enemies. Thus, this approach allows the analysis of quantitative and qualitative multi-trophic interaction networks spanning primary producers, herbivores, predators, secondary predators, and natural enemies to hyper-parasitoids, and the construction of multiple multi-trophic interaction networks starting at different trophic positions. Moreover, unknown wasp-prey associations were found, revealing potential pest control species and ecosystem associations. The focus shift towards including food resources in trap nest research, together with current advances in network analysis, will increase our understanding of ecological mechanisms, responses to environmental change and quantify natural history observations.

AUTHOR CONTRIBUTIONS

Felix Fornoff: Conceptualization; methodology; data curation; investigation; formal analysis; funding acquisition; visualization; project administration; resources; writing – original draft; writing – review and editing. **Wenzel Halla:** Data curation; writing – review and editing; investigation. **Sarah Geiger:** Data curation; investigation; writing – review and editing. **Alexandra-Maria Klein:** Conceptualization; funding acquisition; resources. **Manuela Sann:** Conceptualization; data curation; formal analysis; methodology; validation; visualization; writing – original draft; writing – review and editing; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Data are deposited as supplementary files. All raw sequencing data are available for download from Mendeley repository: doi:10.17632/9sbx5rpmy6.1.

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SUPPORTING INFORMATION

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

Table S1. Taxon sampling and sample information. Provided are information on sampling date, nest opening date and nest material, nest parameters and the taxonomic level of wasps and prey species used for DNA barcoding. Detailed information on the ampling location can be found on the website: https://schulinsektenhaus.de.

Table S2. Primer combinations used for the DNA barcoding.

Table S3. DNA barcoding results. Provided are the results of the best hit for the studied wasps, prey, and parasite individuals obtained from the NCBI database search (species ID), results of the DNA extraction and primer combination to amplify the COI target gene for each individual, and the COI barcode sequence information that is sequence length before and after quality trimming with Geneious. Morphologically identified samples are indicated by an asterisk. NA = no individual sample available.

Table S4. Hemiptera species recorded in nests of cavity-nesting wasps (Table S3), their known host plants, and their known economic importance. If not otherwise stated, information was extracted from 'Leafminers and plant galls of Europe' by Dr. Willem N. Ellis <www.bladmineerders.nl>

Data S1. Supporting information.

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