



Bumblebee footprints on bird's-foot trefoil uncover increasing flower visitation with land-use intensity



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ABSTRACT

Wild pollinators are declining in abundance, diversity and richness and this puts the ecosystem function pollination at risk. Here, we investigated how land-use intensity and the three main components of land use used on the study sites (mowing, grazing, fertilisation) affect bumblebee visitation to bird's-foot trefoil, *Lotus corniculatus*, using a novel chemistry-based approach that overcomes the limitations of classical visitation monitoring. This approach uses the hydrocarbon footprints left behind by bumblebees that accumulate within the epicuticular wax of flowers. Along with land-use intensity, we investigated whether the small-scale (patch) population density and local abundance of *L. corniculatus* affected visitation. We found that the amount of footprints, and thus visitation, of bumblebees to *L. corniculatus* increased with land-use and grazing intensity while traditionally recorded visitation rates did not show such an effect. We further found that the patch density and local abundance of *L. corniculatus* had no effect on visitation. We conclude that bumblebee visitation to *L. corniculatus* increased with land use using the novel approach but emphasise that foraging decisions by bumblebees can be affected by many different parameters unrelated to the land-use intensity of a specific site.

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1. Introduction

Pollination is an ecosystem function provided by many wild pollinator species, particularly insects, birds and bats. Without pollination by animals, many plant species that rely on pollinators for the movement of their pollen would go extinct (De Groot et al., 2002). Relative to other ecosystem functions, pollination is highly beneficial to human welfare.

However, intensification of land use has caused habitat loss, modification and fragmentation, resulting in declines in biodiversity (Pimm and Raven, 2000). Likewise, the abundance and species richness of pollinators have declined with disturbances, agricultural intensification and habitat loss. Furthermore, these changes in pollinator community may cause a reduction of pollination provided to many plant populations (Kremen et al., 2002; Natural Research Council, 2006; Ricketts et al., 2008; Winfree et al., 2009; Potts et al., 2010). Recently, Clough et al. (2014) found a decrease of insect-pollinated plants with land-use intensification. Accordingly, a parallel decline of pollinators and insect-pollinated plant species has been reported (Biesmeijer et al., 2006). Consequences of

pollinator loss are likely particularly severe for wild plant populations because ~80% depend on insect pollination and for most of these plant species pollen limitation has been observed (Burd, 1994; Ashman et al., 2004; Becker et al., 2011).

Pollen limitation can have effects on plant fecundity and ultimately population viability (Bond, 1994; Ishii and Masahiko, 2001; Lennartsson, 2002). It has been suggested that pollinators preferably visit abundant plant species so that they can maximise their rate of reward (Goulson, 2000), while pollinators are less likely to visit small populations, which as a consequence are more likely to suffer from pollen limitation (Lennartsson, 2002; Aizen and Harder, 2007; Kolb, 2008; Dauber et al., 2010). Likewise, the density (per unit area) at which plants occur might lead to differences in visitation and degree of pollen limitation. Grindeland et al. (2005) and Ye et al. (2014) found that the visitation rate of bumblebees increased with plant density.

In this study, we investigated how land-use intensity and its three main components, grazing, mowing and fertilisation, as well as small-scale patch density of *Lotus corniculatus* affected bumblebee visitation, and potentially pollination. We further investigated whether local abundance of *L. corniculatus* affected bumblebee visitation. Grassland study sites represented a land-use

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gradient and were mown, grazed or both with variable intensity, and fertilised to different degrees.

Traditionally, pollinator visitation rates have been recorded by direct observations or sometimes camera traps. The enormous amount of time needed for these methods often causes researchers to limit the number of replicate plants/sites that are investigated in their study designs as well as the choice of plant species, because for sufficient measuring accuracy a relatively high visitation rate per observed unit is required. Indeed, many studies that measured flower visitation rates in the field focused on shrubs with multiple flowers that can be observed synchronously or on other highly visited plant species (Cunningham, 2000; Kay and Schemske, 2003; Vázquez and Aizen, 2003; Greenleaf and Kremen, 2006; Winfree et al., 2008; Dauber et al., 2010; Kaiser-Bunbury et al., 2010). Here, we used a chemistry-based approach that aims at overcoming such limitations. During flower visits, bumblebees involuntarily deposit traces of cuticular hydrocarbons (CHCs). Bumblebees leave substantial amounts of CHCs wherever they walk (Wilms and Eltz, 2008) whereas non-bumblebee visitors appear to leave no or comparatively insignificant amounts of hydrocarbon footprints. Bumblebee 'footprints' accumulate within the epicuticular wax of the corolla, which consequently holds information about past bumblebee visitation (Eltz, 2006). Due to the species specificity of footprint chemical composition (Schmitt et al., 1991; Goulson et al., 2000; Saleh et al., 2007; Martin et al., 2010) deposits have also been used to assess species composition of visiting bumblebees (Witjes et al., 2011). CHCs cover insect surfaces and have the primary function to seal the cuticle against the environment, e.g. for preventing water loss (Howard and Blomquist, 2005). A secondary function is the improvement of tarsal adhesion on smooth surfaces (Lockey, 1988; Jiao et al., 2000; Drechsler and Federle, 2006). While some plant species produce alkanes in their epicuticular layer, unsaturated hydrocarbons (UHCs) are rarely found in plants but occur commonly in bumblebee footprints (Griffiths et al., 1999; Griffiths et al., 2000; Goodwin et al., 2003; Zeisler and Schreiber, 2016). Consequently, cumulative bumblebee visitation to natural flowers can be inferred from the UHCs in solvent extracts of inflorescences using Gas Chromatography/Mass Spectrometry (GC/MS) (Eltz, 2006; Witjes and Eltz, 2009).

Using this novel method, we estimated bumblebee visitation, i.e. the quantity of bumblebee footprints, on *L. corniculatus* populations in grassland sites in Germany. We chose *L. corniculatus* because it is pollinated by bumblebees (Rasmussen and Brødsgaard, 1992; Pellissier et al., 2012), is declining with land use (Weiner et al., 2014; Kämper et al., 2016) and, due to its relatively low nectar secretion rates (Stout and Goulson, 2002), has low visitation making it difficult to measure visitation rates using conventional methods. To illustrate the problems with conventional records of visitation rates, which are often dominated by many zeros and skewed by single or very few observations, we compare data collected with this new method with observations of *L. corniculatus* patches recorded on the same grassland sites.

2. Methods

Our study was conducted within the framework of the Biodiversity Exploratories (www.biodiversity-exploratories.de). For details on study regions and site selection see Fischer et al. (2010).

2.1. Data collection – classical visitor observations

To estimate visitation rates conventionally, we used previously collected data from the same region (Martina Tospaun, unpublished data). In total, 17 grassland sites were visited in the

'Schwäbische Alb' from 5-Aug-2010 to 2-Sep-2010 and 16 grassland sites in the 'National Park Hainich' from 28-Jun-2011 to 25-Aug-2011. Each grassland site was visited once, with observations conducted between 9:29 and 18:10. On 8 subplots (50 × 50 cm) per grassland site all visits to *L. corniculatus* were recorded for 15 min each. We also counted all open flowers on each subplot at the time of observation. For each subplot, we calculated the number of visits per flower per hour. We then used the average per grassland site for subsequent analyses.

2.2. Data collection – footprint-derived visitation

To assess footprint-derived UHCs of bumblebees on *Lotus corniculatus*, we visited 35 grassland sites from 04-Jun-2014 to 04-Jul-2014 and collected flowers between 15:15 and 21:15. Thirteen sites were located in the UNESCO Biosphere Reserve 'Schwäbische Alb', 15 in the National Park 'Hainich', and seven in the UNESCO Biosphere Reserve 'Schorfheide-Chorin', Germany. During each visit, we assessed the local abundance of *L. corniculatus* by counting open flowers along a 100 × 6 m transect or, if highly abundant, estimated local abundance by extrapolation from a small area.

To assess bumblebee visitation and the effect of patch density, we collected two pooled samples containing 15 *L. corniculatus* flowers on each site, respectively. For one sample, flowers were collected from *L. corniculatus* plants in low-density patches with on average 1.34 ± 0.67 *L. corniculatus* flowers per 1 m². For the second sample, flowers were collected in high-density patches with on average 28.41 ± 17.04 *L. corniculatus* flowers per 1 m². For comparison, because some plant species produce alkenes in their epicuticular wax layer (Cseke et al., 2006), we also sampled unvisited flowers. For this we covered a patch of *L. corniculatus* with mosquito mesh, removed open flowers, waited for fresh flowers to open, and took two samples of 15 flowers, both from the 'Schwäbische Alb'.

We extracted each pooled flower sample in 2 ml of n-hexane (Roth, Karlsruhe, Germany) with 10 µl of 2-undecanone (MERCK-Schuchardt, Hohenbrunn, Germany) as an internal standard for a minimum of 30 s. 30 s are sufficient to dissolve the majority of petal surface hydrocarbons along with the footprint-derived UHCs of bumblebees (T. Eltz, unpublished data). Afterwards, the inflorescences were removed and discarded. Extracts were stored at -18 °C prior to chemical analysis in Bochum, Germany, with coupled gas chromatography and mass spectrometry (GC-MS). To increase sample concentration, each extract was transferred to a 2 ml vial (Agilent, Ratingen, Germany), evaporated to dryness under a stream of laboratory air, and then filled up with 50 µl n-hexane. 1 µl of the more concentrated sample was injected splitless on a DB-5 MS column (30 m, 0.25 µm film thickness, 0.25 mm diameter). The oven of the GC (HP5890 II) was heated from 60 to 300 °C at 10° per min. A mass spectrometer (HP5972) served as detector.

Compounds were identified by comparison of mass spectra and retention times with those of reference samples (series of linear alkanes) in combination with spectral interpretation using Agilent ChemStation software (Agilent Technologies, Germany). For the quantification of hydrocarbons, we manually integrated all compounds that were potentially derived from bumblebees (based on our own data as well as Goulson et al. (2000), Martin et al. (2010) and Witjes et al. (2011)), i.e. odd-numbered alkanes, alkenes and alkadienes of a chain length of 21–31 C-atoms. The separation of the different isomers of alkenes and alkadienes, which for a given chain length have very similar spectra and retention times, was based on our own inventories of all isomers found in leg extracts of 13 bumblebee species. Alkene and alkadiene isomers of a given chain length were numbered according to their

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