Experimental loss of generalist plants reveals alterations in plant-pollinator interactions and a constrained flexibility of foraging

Paolo Biella *[1,2], Asma Akter [1,2], Jeff Ollerton [3], Sam Tarrant [3], Štěpán Janeček [4,5], Jana Jersáková [6], Jan Klečka [2]

[1] University of South Bohemia, Faculty of Science, Department of Zoology, České Budějovice, Czech Republic
[2] Czech Academy of Sciences, Biology Centre, Institute of Entomology, České Budějovice, Czech Republic
[3] Faculty of Arts, Science and Technology, University of Northampton, Northampton, UK
[4] Czech Academy of Sciences , Institute of Botany, Třeboň , Czech Republic.
[5] Department of Ecology, Faculty of Science, Charles University in Prague, Praha 2, Czech Republic
[6] University of South Bohemia, Faculty of Science, Department of Biology of ecosystems, České Budějovice, Czech Republic

*Author for correspondence:
Paolo Biella, email: paolo.biella@entu.cas.cz

Abstract

Species extinctions undermine ecosystem functioning but species do not disappear at random. Ecosystem dynamics are ruled by a subset of dominant species, but it is not clear how losing several of these key species would affect plant-pollinator interactions. We tested how the experimental loss of the most highly visited generalist plants would affect flower visitor abundances (visitation) and pollinator effectiveness (quantity of pollen-tubes within pistils) in several sites. Significant changes in the plant-pollinator communities were detected. Firstly, visitation decreased after removing 1-2 most visited plants, suggesting that these species mostly facilitate other plants by keeping high flower visitor abundances in the sites. Nevertheless, we recorded within-site variations of these trends, especially among sampled transects and among plant species, suggesting that complex facilitation-competition interactions occur among plants, but that these vary among transects within the same sites. Pollinator effectiveness fluctuated but not in a clear linear way and was not directly linked to changes of flower visitation. This suggests that fluctuations of pollinator effectiveness might be due to destabilization of the pollinators by the removal of key resources. In addition, we detected a constrained flexibility of the foraging of flower visitors because they did not switch among flower shapes and they favoured specific plant traits such as high sugar content, small inflorescences and taller plants. Indeed, these constraints would eventually limit utilisation of new resources after perturbations, possibly undermining the stability of the system. In conclusion, our work demonstrates that the loss of dominant, highly generalist plants alters plant-pollinator interactions with implications for pollination and insect foraging. Therefore, in order to preserve ecosystems, generalist plants should not get lost, because they sustain the complex pattern of interactions between plants and flower visitors.

Keywords: Pollination; Plant-pollinator network; Functional traits; Adaptive foraging; Community manipulation; Network conservation; Hub; Generalist core species; Grassland ecology; Dominant species; Community stability; Extinction;

Introduction

The rapid loss of biota brings consequences for ecosystem functioning (e.g. [1–3]). Furthermore, declines in several aspects of ecosystem functioning such as productivity, resistance to invasion, nutrient retention and animal pollination, among many others, have been shown to be consequences of species loss [4–6]. The consequences of these effects have been usually investigated by removing species at random in simulation models (e.g. [7]) and field experiments [8,9]. However, this is rarely the case in nature as species do not go extinct randomly, but rather non-random losses are the rule [10–14].

Most of the community-level dynamics and services to ecosystems are due to a small subset of a species pool [2,15]. For instance, few species may be responsible for the robustness of the entire system and their loss is expected to speed up the collapse of complex interaction relationships [3,16]. The loss of such a key set of species may cause additional species losses and even speed up the erosion of ecosystem functioning [5,17]. In multi-species assemblages, the key species facilitate others within the nested structure of their interactions [18], which contains a central core of generalist species that interact with both each other and with more specialised species [19].

Facilitative relationships are not uncommon in complex plant assemblages and have been shown to occur also in the case of the pollination niche of plants [20–22], even in the presence of competition [23,24]. In pollination, generalist plants provide floral resources (usually nectar or pollen) to a wide range of animal species visiting their flowers, and generalist pollinators transfer pollen to ensure sexual reproduction of many plant species. The importance of these generalists lies in their central, dominant ecological role within the community as they are often the most abundant species [25,26] and interact with a large subset of other species [27,28]. Thus, by providing a significant amount of floral resources and support for pollination services, the generalists may facilitate the ecological functioning of the entire community. Therefore, despite the focus of conservation biologists on rare species, conservation of abundant generalist plants may be an important focus for conservation purposes, because their persistence can sustain most of the complexity of interactions taking place in a community [17].

Despite their undoubted importance, relatively little is known about the effects of the local extinction of generalists on pollination. Simulation studies of plant or pollinator removals, and the consequent loss of other species in the community, have been undertaken [3,16,29]. However, it is only recently that experimental tests aimed at manipulating real plant-pollinator communities have been performed. Until now, only relatively small manipulations have been applied in the field, for instance by removing ether the one invasive plant species [30,31] or the one most visited (native) plant species [32]. Other studies have excluded one species or a target functional group of pollinators [33,34]. However, the lack of spatial replication of some of these experiments and the different criteria for choosing which species to remove, means that it is hard to find coherent conclusions among these studies. For instance, some studies point to small differences in the interaction networks after manipulation [33], while others find exactly the opposite [30]. In addition, it seems that only simulation-based works tested the effects of excluding several species sequentially [3,29], while the few field experiments removed one species only. Therefore, several questions remain open as to how multiple losses affect patterns of complex interactions such as pollination in natural ecosystems.

In this study we have addressed the question of what happens when the floral resources of the most generalist plant species are removed. The experiments comprised two parts. Firstly a pilot project in the United Kingdom experimentally manipulated a plant-pollinator community by removal of the most generalist plant (from here on called "pilot study"). This provided a proof-of-concept and suggested ways in which the pollinator assembly might react to such perturbation. Secondly, in the Czech Republic, we conducted a sequential removal experiment of the most highly visited species over a short time period to determine how the plant-pollinator community would respond to a more profound loss of resources (from here on called "sequential removal" experiment).

Specifically we asked whether the treatment produced effects on (1) the general overall visitation (that is the abundance of pollinators in the sites), (2) the visitation to each plant species, (3) the pollination effectiveness determined by the number of pollen tubes grown in the pistils after visitation, (4) the amount of used nectar resources ("standing crop"). Furthermore, we hypothesise that insects would perform a flexible foraging, which is the ability of using new resources after perturbations (Valdovinos et al. 2013). Therefore, we also aimed at (5) testing whether plants with traits different than the removed species would gather more visitor-abundances after removals; If so, the pollinator's foraging would show flexibility by visiting different plants after removal events.

The rationale behind this study is that it experimentally reproduces a situation in which a subset of floral resources, which are utilized by large proportion of a pollinating fauna, are removed over a short period of time. Examples of such situations include: a rapidly-spreading species-specific disease which affects flowers; natural burning or flooding of part of a site dominated by a single or few highly generalist plant species; or anthropogenic disturbance that selectively removes target plant species, such as conservation and agricultural practices involving eradications or suppressions.

Methods

Pilot study

The pilot study was conducted in the East Midlands of the UK, in Northampton, during summer 2008 on the 3 ha Quarry Field (52°16'12 N, 0°52'46W), part of the Bradlaugh Fields site, a network of parkland and Local Nature Reserves.

Surveys of insect visitation were undertaken at two stages: (i) before flower removal in order to determine the pollinator-plant interactions prior to the experiment; (ii) in the days following the removal of the plant with the highest visitation, i.e. the total abundance of flower visitors on the plant. During the same period the near Scrub Field was also surveyed two times using the same techniques, as a comparison control site to the Quarry Field. Insect flower visitation surveys were undertaken four times at each of the two stages between 1pm and 4pm on days which were warm and sunny with little or no wind. Surveys lasted 30 minutes and all flower visiting insects seen to be feeding within the flowers were captured from along a 2 metre wide belt and within 2 metres in front of the surveyor. The sampling followed a widening spiral from a randomly determined point at a standard pace of 10 m per minute (which makes for each survey an area of approximately 600 m^2). This method allowed for a large area to be surveyed owing to the relatively low pollinator density. Captured pollinators were placed into specimen jars. After

the survey was completed, those insects that could be identified in the field were recorded and released whilst the remaining individuals were taken for later identification.

Sequential removal

The sequential removal experiment was performed in the vicinity of Český Krumlov (South Bohemia, Czech Republic). Three experimental sites were chosen, all small meadows surrounded by large trees in a similar habitat (located at 48° 49' 26.8'' N, 14° 16' 26.2'' E Site 1; 48° 49' 51.63'' N 14° 17' 34.12'' E Site 2; 48° 49' 35.07'' N 14° 18' 8.2'' E Site 3). An untreated (control) site was located at 48° 49' 26.8'' N, 14° 16' 26.2'' E. The mean pairwise distance between sites was 2.01 ± 0.95 Km.

The sampling was based on walking six transects (10 m x 1 m) in a randomized order in each site between 9:00 and 17:00 hours. All transects were usually sampled twice during each day. Sampling was postponed in the case of rain or strong wind. All insects found while visiting flowers were sampled by netting or use of a mouth aspirator. The abundance of plants was recorded by counting the number of open flowers or compound inflorescences on each plant within each transect. Abundance was recorded during all stages of the experiment in order to capture variation of flower abundances across treatments.

In the experimental sites, all flower visitors feeding within flowers were sampled for two days (hereafter the "Before" period). At the end of the sampling days, the captured specimens were counted for each plant and thus we were able to determine the plant with the highest visitation, i.e. the total abundance of flower visitors on the plant. We call these highly visited plants "generalist" in accordance with the literature (see[32]). This species was removed from the site by cutting all inflorescences. We only removed flowers and left the stems otherwise intact so that the vegetation structure remained unaltered. Twenty four hours after the removal, the sites were sampled again for two sampling days ("Rem_1sp" = first species removed). After we had counted the abundances of the visitors for each plant, we determined the next plant species with the highest visitation, the inflorescences of which were removed ("Rem_2sp"). The flower visitors were sampled again after twenty four hours and again for two sampling days. The next plant species with the highest visitation was thus found in the same fashion as for the first two ones, and then removed ("Rem_3sp"). After twenty four hours, we again sampled flower visitors of the plant community for two sampling days, so that the next plant species with the highest visitation was found in the same way as for the others and later removed ("Rem_4sp"). After 24h, we again sampled the plant community. Site 1 and 2 were sampled from 25th June to 12th of July, Site 3 was sampled from 2nd to 17th of July; Control site was sampled at the same time as each of the experimental sites, but no treatment (removals) was applied to this one (data in S1 Table).

Pistil collection and pollen tubes

Pollinator effectiveness to the flowering plants at two experimental sites was tested by counting pollen tubes in the pistils. To do so, the pistils were collected after the insect sampling and preserved in formalin-acetic acid-alcohol (FAA) at room temperature. Flowers were collected outside (but nearby) the transects to avoid depletion of flower abundances in the transects. Later, in the laboratory, the flowers were dissected under the microscope and pistils were prepared for the softening and staining, following the technique of [35]. After softening the pistils in 4N NaOH,

they were stained with 0.1% aniline blue in 0.1M K_2HPO_4 for 12 hours. Then, the pistils were washed and mounted in a drop of 50% Glycerin on glass slides and covered with cover slips for observation under the fluorescence microscope. Pollen tubes were visible and counted for most of the species. However, in a few cases, pollen tubes were impossible to visualize properly. For these species the number of pollen grains on the stigmas were counted assuming that only germinated grains with tubes still attached would remain on the stigma after the preparation process. All processes were carried out at room temperature. After the observation, the edges of the cover slips were sealed with clear nail polish and stored at 4°C for future reference. Data were successfully obtained for 10 species because other plant species did not yield any countable pollen tubes (data in S2 Table).

Nectar content of flowers and other functional traits

We determined the daily production and the standing-crop of nectar in flowering plant species at two sites. The daily production was assessed by bagging flowers in anthesis for 24 hours. Flowers were bagged in the morning and sampled the next day in the morning. For the standing-crop of nectar, flowers were collected in each site after the pollination sampling on the same day (data in S3 Table). A 100µl Hamilton capillary syringe was used for the collection and for washing the nectar into distilled water. Flowers were selected randomly but outside the sampling transects in order to avoid impoverishment of flower resources in the transects; only flowers in full anthesis were sampled. Nectar samples were stored in a cool bag in the field and in a -20°C freezer in the laboratory. Sugar analysis of nectar was done using high performance anion exchange chromatography with pulsed amperometric detection using a Dionex ICS-3000 system and CarboPac PA1 analytical column. Nectar amount was expressed as milligrams of sugars per flower.

Other traits measured for each plant species in the two sites were: plant height (linear distance between the ground and the top of an inflorescence); inflorescence maximum size (the maximum dimension of the inflorescence); dominant colour of the corolla (categorical variable, e.g. “white”, “blue”, “pink”, “yellow”); flower shape coded according to [36] as follows: bell- or funnel-shaped, dish- or bowl-shaped flowers, flag-shaped flowers, gullet-shaped flowers, head- or brush-shaped, tube-shaped flowers; unclear cases were checked with [37].

Statistical analyses

All data were analysed by means of generalized linear mixed-effect models (GLMM) with library *lme4* [38] in the R environment [39]. Details of the models with the lowest AIC follow.

The overall visitation was derived by calculating the means of flower visitor abundances across plant species; mean values were calculated separately at each sampling event for each transect. Thus, the visiting insect abundance at transects was the response variable, treatment was a categorical predictor (“Before”, “Rem_1sp”, “Rem_2sp”, “Rem_3sp”, “Rem_4sp”). Transect identity was the random intercept. Poisson distribution was used. Data were also analysed after including an offset with the geometric means of visitation in the control site, in order to account for possible variations not due to treatment. Treated sites in the sequential removal experiment were analysed independently. In these experimental sites, a multiple comparison tests were performed using the package

multcomp with the function *glht* [40] to compare a given treatment level with the preceding treatment level (e.g. "Rem_1sp" vs "Before", "Rem_2sp" vs "Rem_1sp", and so on) in order to test for relative increases or decreases of pollinator abundances.

For the data from the pilot study, the visitation was analysed in a slightly different fashion because flower visitors were collected along a unique transect covering the entire surface of the site and insect species were taxonomically identified. Thus in a GLMM with Poisson distribution the abundance of visitation by flower visitor species was used as response, treatment was predictor ("before", "after"). Species identity was used as random factor on the intercept. Data were also analysed after including an offset with the visitation by insect species in the control site; the mean visitation on a given plant species was used for insects that were not present in the control.

To test the effect of treatment on each plant species at each site in the sequential removal experiment, plant-level visitation (visiting insect abundance for each plant species at each sampled transect, including unvisited plants) was the response variable and treatment was a predictor ("Before", "Rem_1sp", "Rem_2sp", "Rem_3sp", "Rem_4sp"). An offset of plant abundance was included, to account for possible variation of plant abundance during the experiment. Transect identity within plant species was included as random slope of treatment. Poisson distribution was used. The three treated sites were analysed separately.

To test whether the number of pollen tubes in each plant species changed in the sequential removal sites, the number of pollen tubes was the response variable, treatment ("Before", "Rem_1sp", "Rem_2sp", "Rem_3sp", "Rem_4sp") and the plant mean visitation across each transect were predictors. Random slopes of predictors were accounted with site identity within plant species as a random factor, and Poisson distribution was used.

To test the variation of standing crop of nectar, the amount of sugars per flower in each species was the response variable, treatment ("Before", "Rem_1sp", "Rem_2sp", "Rem_3sp", "Rem_4sp") and the plant mean visitation across transect were predictors. Random intercept was accounted with site identity within plant species as a random factor (no random slopes included due to lack of convergence of the model). Gamma distribution was used, link function was the natural logarithm.

To test for changes in foraging preferences of flower-visiting insects, we assessed how visitation related to differences between plant-species' traits and the plant removed (data in S4 Table). To do that, the log-ratio difference between a given continuous plant trait value and the trait value of the removed plant species was calculated for each treatment level and used as a predictor variable. For categorical variables, a binomial variable was used, i.e. "same" or "different" from the removed species. The variables included were flower colour, flower shape, inflorescence size, plant height and the daily production of sugars. All of these variables were included in the model in interaction with the treatment variable ("before", "after" removal to account for a simplified codification of the treatment levels in the statistical model). Visiting insect abundance for each plant species was used as a response variable. An offset of plant abundance was included, to account for possible variation of plant abundance across treatments. Random slopes of treatment was accounted with transect identity within plant species within site as a random factor, using Poisson distribution.

Results

In the pilot study, the surveys identified a total of 13 insect pollinated plant species in flower and 25 pollinating insect species in the treated site. The plant with the highest pollinator abundance was *Knautia arvensis* (Caprifoliaceae), with a total of 54 insects (of 11 species); the second generalist plant had 41 insects (of 11 species). After the removal of *K. arvensis*, *Centaurea nigra* (Asteraceae) was the plant with the highest pollinator abundance, having 86 insects (of 11 species). Other plants were visited by less than 20 insect specimens (and less than 8 species) in both experimental phases.

In the sequential removal experiment, the sites 1, 2 and 3 were surveyed for a total of 31, 26 and 23 insect pollinated plant species, respectively. The sequences of the removed plants specie were the following: Site 1, *Anthriscus sylvestris* (Apiaceae, 256 insect specimens in "Before"), *Rubus caesius* (Rosaceae, 315 insects in "Rem_1sp"), *Knautia arvensis* (Caprifoliaceae, 83 insects in "Rem_2sp"), *Galium mollugo* (Rubiaceae 39 insects in "Rem 3sp"); Site 2, *Veronica teucrium* (Plantaginaceae, 103 insect specimens in "Before"), *Agrimonia eupatoria* (Rosaceae, 96 insects in "Rem_1sp"), *Centaurea scabiosa* (Asteraceae, 96 insects in "Rem_2sp"), *Securigera varia* (Fabaceae, 26 insects in "Rem 3sp"); Site 3, *Aegopodium podagraria* (Apiaceae, 141 insect specimens in "Before"), *Veronica teucrium* (Plantaginaceae, 26 insects in "Rem_1sp"), *Knautia arvensis* (Caprifoliaceae, 45 insects in "Rem_2sp"), *Origanum vulgare* (Lamiaceae, 55 insects in "Rem_3sp").

Results from the pilot study

In the pilot study, visitation to plants slightly increased after removing one species (Fig. 1). The treatment was a significant predictor of visitation: χ^2=8.49, df=1, p < 0.01. However, in the model including an offset of the control's visitation, the treatment was not significant. No significant change was detected in the control site during the period.

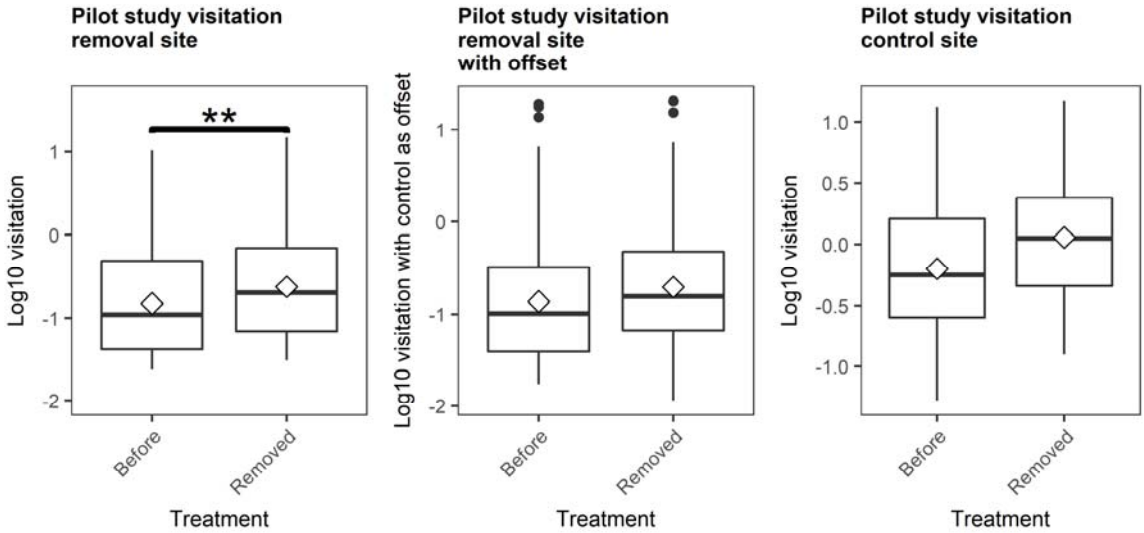

*Figure 1 – Pilot study's flower-visitor species abundances for each plant ("visitation") on a logarithmic scale. The left panel shows the trend at the treated site, the middle panel shows the trend when including an offset of control site's visitation, and the right panel shows the visitation at the control site. Diamonds indicate means, significance codes are the following: *** p<0.001, ** p<0.01, * p<0.05.*

General pattern of visitation

In the sequential removal sites, visitation per transect decreased sharply after the removal of plants and treatment was a significant predictor (Site1: χ^2= 1197.2, df=6, p < 0.001; Site 2: χ^2= 168.15, df=6, p < 0.001; Site3: χ^2= 785.92, df=6, p < 0.001). Treatment was also significant when visitation in the control site was used as an offset in the models. Multiple-comparison test of treatment-levels gave significant results in most cases, especially when variation of visitation in the control was accounted in the model (Table 1). Namely, the removal of the first plant caused only minor changes in overall flower visitor abundances in two sites, while it decreased sharply afterwards (Fig. 2); in one site, however, flower visitors abundances decreased after the first species was removed.

Table 1 – Multiple comparison statistics on the effect of treatment on general visitation, i.e. the total abundance of flower-visiting insects per transect, in the sequential removal experiment for the three experimental sites. Statistical models either not including visitation in control site ("Unstandardized") or accounting for it by an offset ("Standardized") are presented. Treatment levels are coded as "Before" for no species removed, and Rem_Nsp with N referring to either the first (1), second (2), third (3), or fourth (4) plant species removed.

Unstandardized	Hypothesis	Estimate	Std. Error	Significance
Site 1	Rem_1sp - Before == 0	0.141	0.048	0.013
	Rem_2sp - Rem_1sp == 0	-0.861	0.066	<0.001
	Rem_3sp - Rem_2sp == 0	-0.930	0.093	<0.001
	Rem_4sp - Rem_3sp == 0	-0.021	0.109	0.999
Site 2	Rem_1sp - Before == 0	-0.063	0.080	0.855
	Rem_2sp - Rem_1sp == 0	-0.478	0.097	<0.001
	Rem_3sp - Rem_2sp == 0	-0.407	0.120	0.003
	Rem_4sp - Rem_3sp == 0	0.016	0.133	0.999
Site 3	Rem_1sp - Before == 0	-0.941	0.116	<0.001
	Rem_2sp - Rem_1sp == 0	0.156	0.127	0.567
	Rem_3sp - Rem_2sp == 0	0.042	0.111	0.987
	Rem_4sp - Rem_3sp == 0	-0.682	0.144	<0.001
Standardized				
Site 1	Rem_1sp - Before == 0	0.278	0.048	<0.001
	Rem_2sp - Rem_1sp == 0	-1.232	0.066	<0.001
	Rem_3sp - Rem_2sp == 0	-0.959	0.093	<0.001
	Rem_4sp - Rem_3sp == 0	-0.508	0.109	<0.001
Site 2	Rem_1sp - Before == 0	0.098	0.080	0.573
	Rem_2sp - Rem_1sp == 0	-0.852	0.097	<0.001
	Rem_3sp - Rem_2sp == 0	-0.422	0.120	0.002
	Rem_4sp - Rem_3sp == 0	-0.476	0.133	0.001
Site 3	Rem_1sp - Before == 0	-0.916	0.116	<0.001
	Rem_2sp - Rem_1sp == 0	-0.346	0.127	0.024
	Rem_3sp - Rem_2sp == 0	-0.061	0.111	0.951
	Rem_4sp - Rem_3sp == 0	-0.377	0.144	0.032

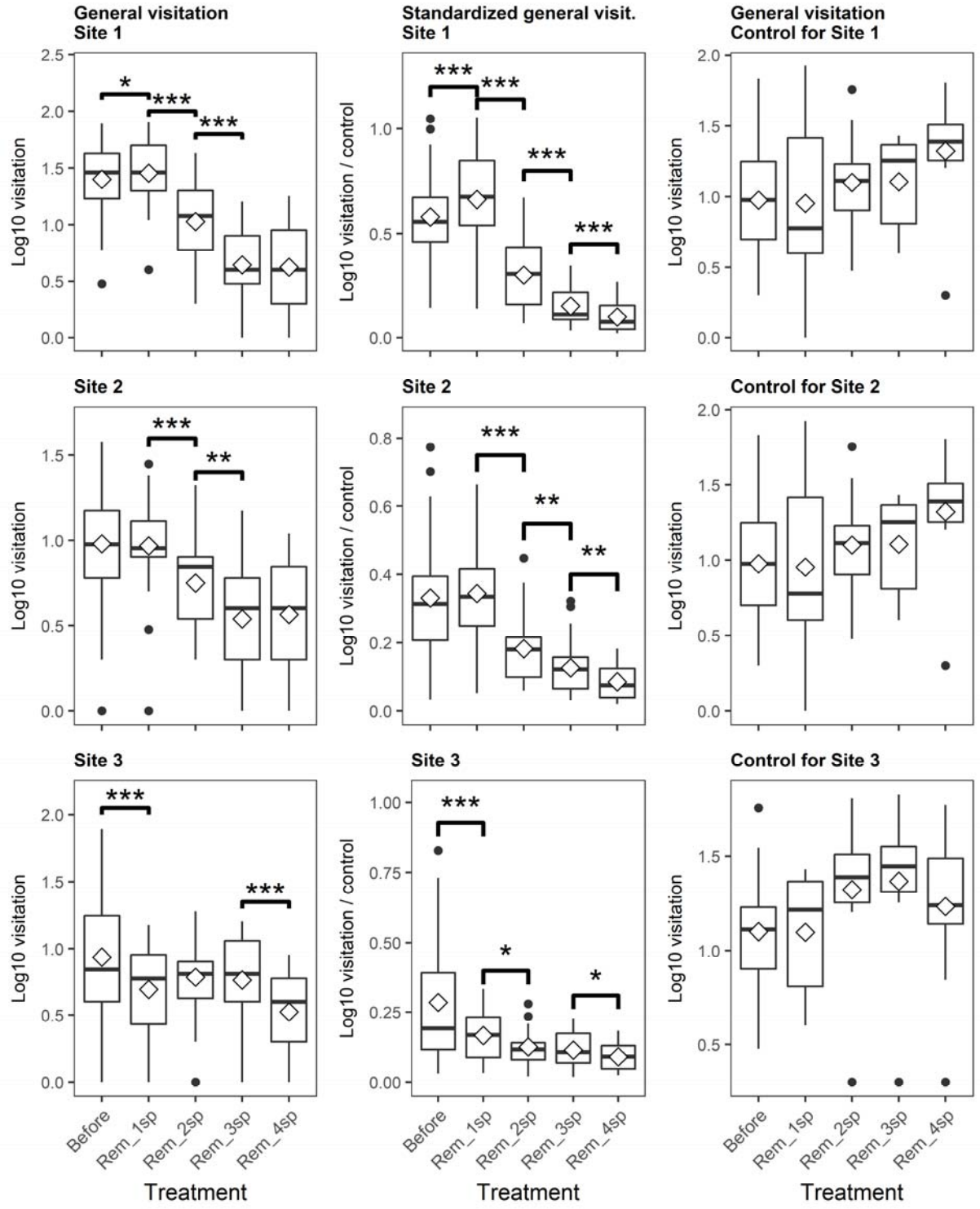

*Figure 2 – General trends of flower visitor abundances ("visitation") in the sequential removal experiment. Boxplots indicate visitation as means across plant species of the same sampling event (i.e. a transect walk) on a logarithmic scale. The left column shows the trend at the treated site, the middle column shows the trend when including an offset of control site's visitation, and the right column shows the visitation at the control site. Each treated site is shown in a separate row. Diamonds indicate means, significance codes of multiple comparison tests are the following: *** p<0.001, ** p<0.01, * p<0.05.*

Visitation to each plant species

The community averaged values for visitation to single plant species showed non-linear trends in flower visitor abundances (Fig. 3) across treatment levels. In site 1 and 2 there was a sharp decrease of visitation only after removal of the second species. Before the second species was removed, the trend was nearly monotonic in site 1, while it increased in site 2. In site 3 there was an important decrease of visitation after the first species had been removed but at the following removals the trend was nearly monotonic. The trends were significantly predicted by treatment in two sites, but not in the third one (Site1: χ^2= 14.195, df=20, p < 0.01; Site 2: χ^2=11.887, df=20, p <0.05; Site3: χ^2=6.3598, df=20, p =0.17). Plants showed high variation in visitation both within sites (among transects) and between sites (coloured lines and confidence intervals in Fig. 3). Nevertheless, the trend was not uniform across plant species, as idiosyncratic responses took place: visitation to some plants increased while in others it decreased during the same treatment level.

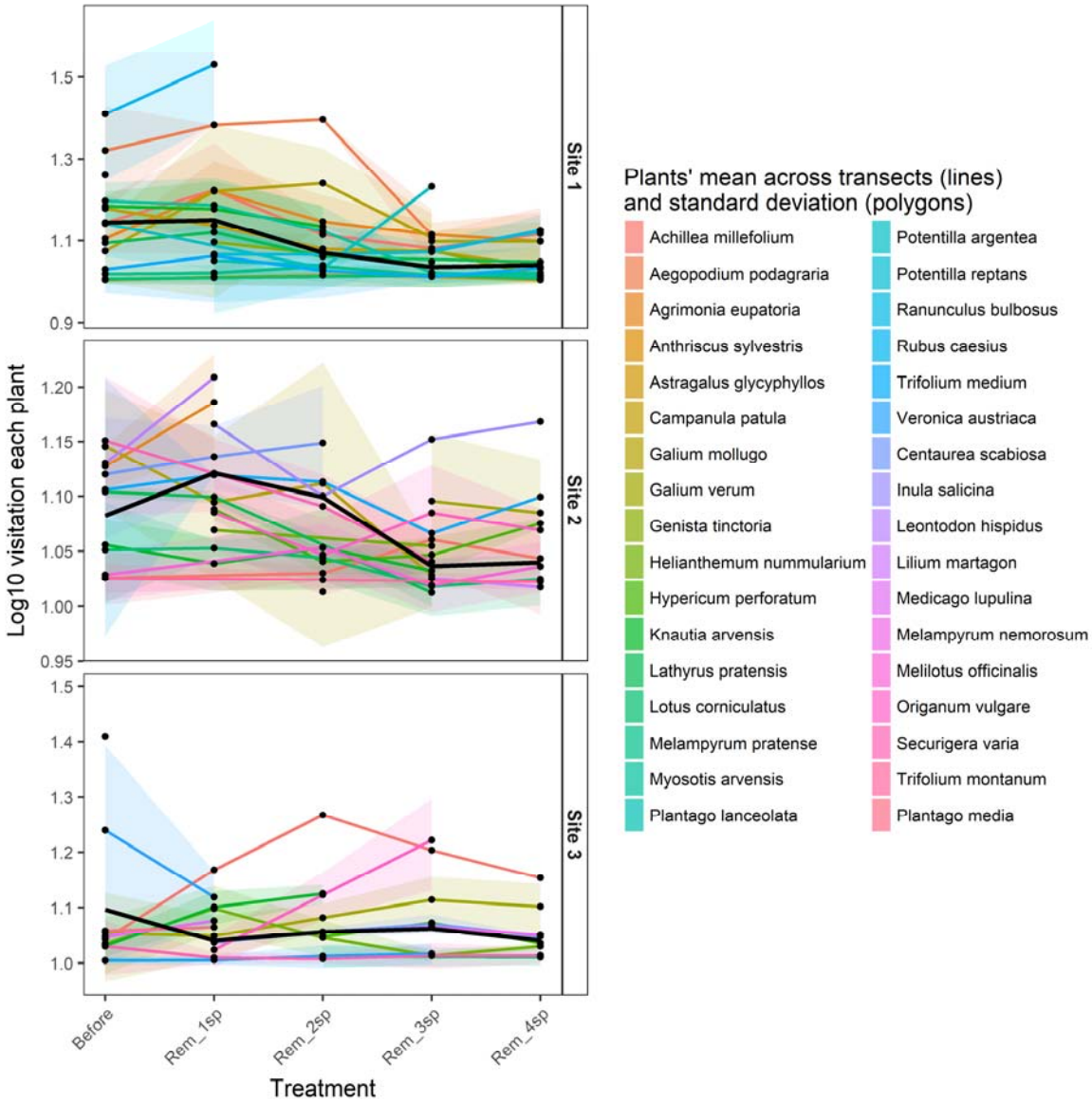

Figure 3 – Trends of flower visitor abundances ("visitation") on individual plant species in the sequential removal experiment. Visitation is shown on a logarithmic scale, coloured lines are means across transects of a given plant species, coloured polygons are confidence intervals around the means, black line is the plant community average trend.

Pollination effectiveness

The number of pollen tubes per stigma fluctuated significantly during the experiment, but there was no consistent trend (Fig. 4). Treatment was a significant predictor of pollen tube number (χ^2= 19.9, df=20, p < 0.001), but visitation was not (χ^2= 0.62, df=20, p = 0.43).

The average trend of pollen tube number was not linear but fluctuating (Fig. 4), because it decreased after the first species had been removed but it slightly increased during the following removals, then decreased again after the last species had been removed. However, the trend was not uniform across plant species, as idiosyncratic responses took place (coloured lines in Fig. 4).

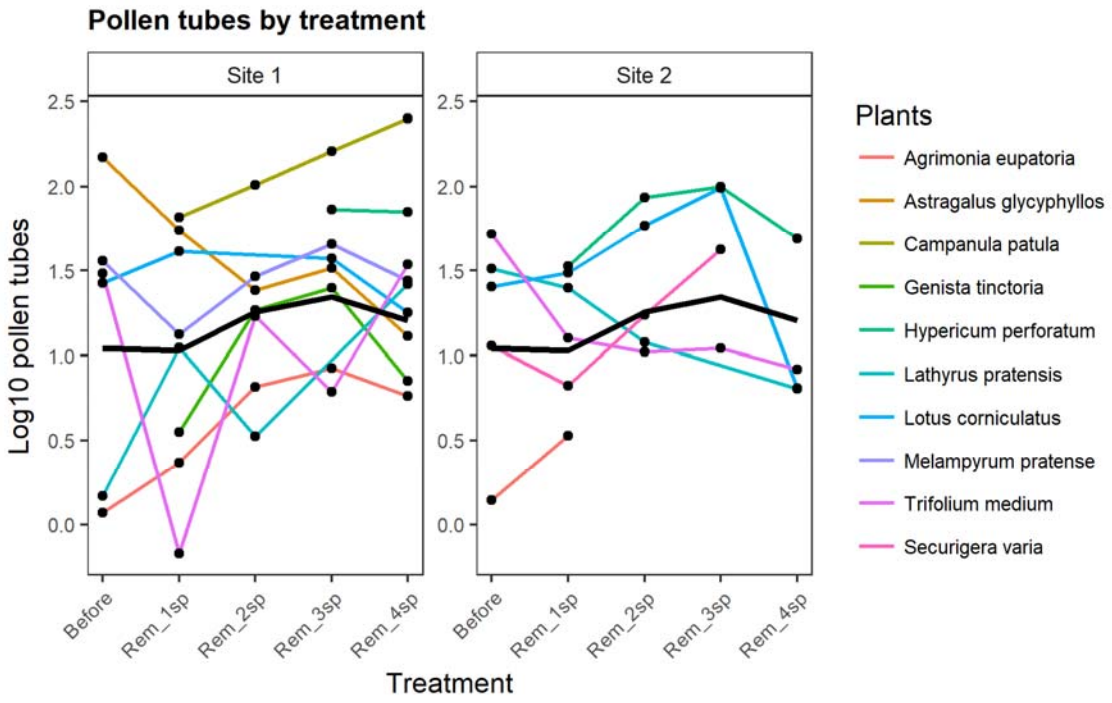

Figure 4 – Trends of the number of pollen tubes in two of the sites in the sequential removal experiment. The number of pollen tubes is shown on a logarithmic scale, coloured lines are the means across transects of a given plant species, the black line is the plant community average trend.

Standing crop of nectar

The standing crop of nectar did not change significantly as plants were removed (Fig. 5) (χ^2= 2.14, df=4, p = 0.71) and visitation had no significant effect (χ^2= 0.12, df=1, p = 0.71).

Plant traits

All plant-traits included in the models except colour were significant predictors of insect visitation (Table 2). After removals, visitation increased in plants with the same flower shape as the removed plant, with smaller inflorescences, slightly taller heights and with a higher sugar content than the removed plant (Fig. 6).

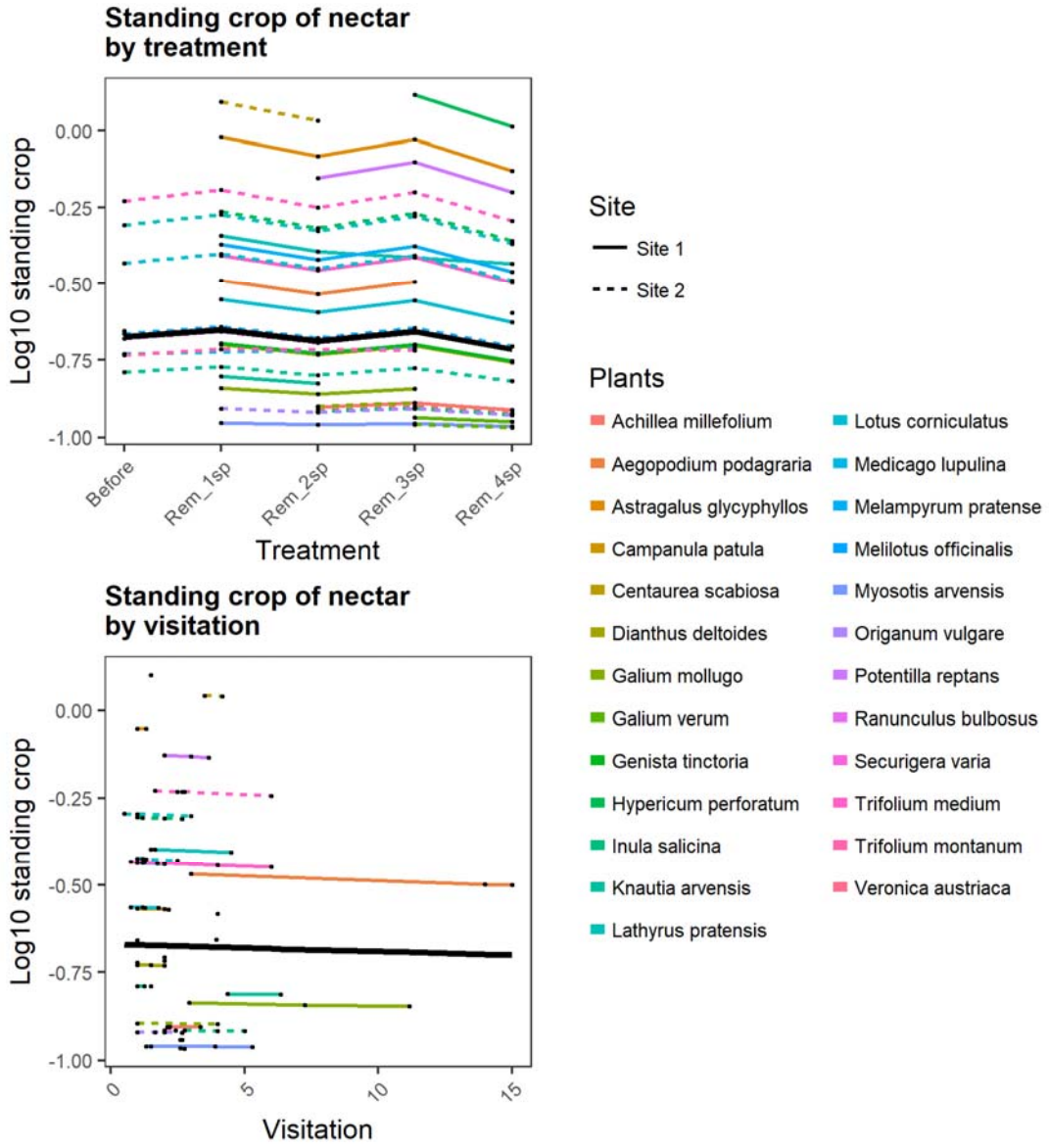

Figure 5 – Standing crop in both sites in logarithmic scale vs. treatment and flower visitor abundances ("visitation"), coloured lines are means across transects of a given plant species, black line is the community-average trend.

Table 2 – The effect of plant traits on changes of visitation after plant removal evaluated by single-term deletion of interaction terms from a GLMM model. Colons are used for interactions between treatment ("Treatment" referring to before-after removal of one plant species) and difference of trait values of individual plants from the removed species. Trait difference for colour and flower shape was classified as same or different. Log-ratio difference was used for plant height ("Height"), inflorescence size ("Size") and amount of sugars in nectar ("Sugars").

	AIC	Pr(Chi)	Significance
null model	6781.7		
Treatment:Color	6780	0.628	0.628
Treatment:Shape	6820	<0.001	<0.001
Treatment:Size	6806.1	<0.001	<0.001
Treatment:Height	6788.2	<0.01	0.004
Treatment:Sugars	6825.7	<0.001	<0.001

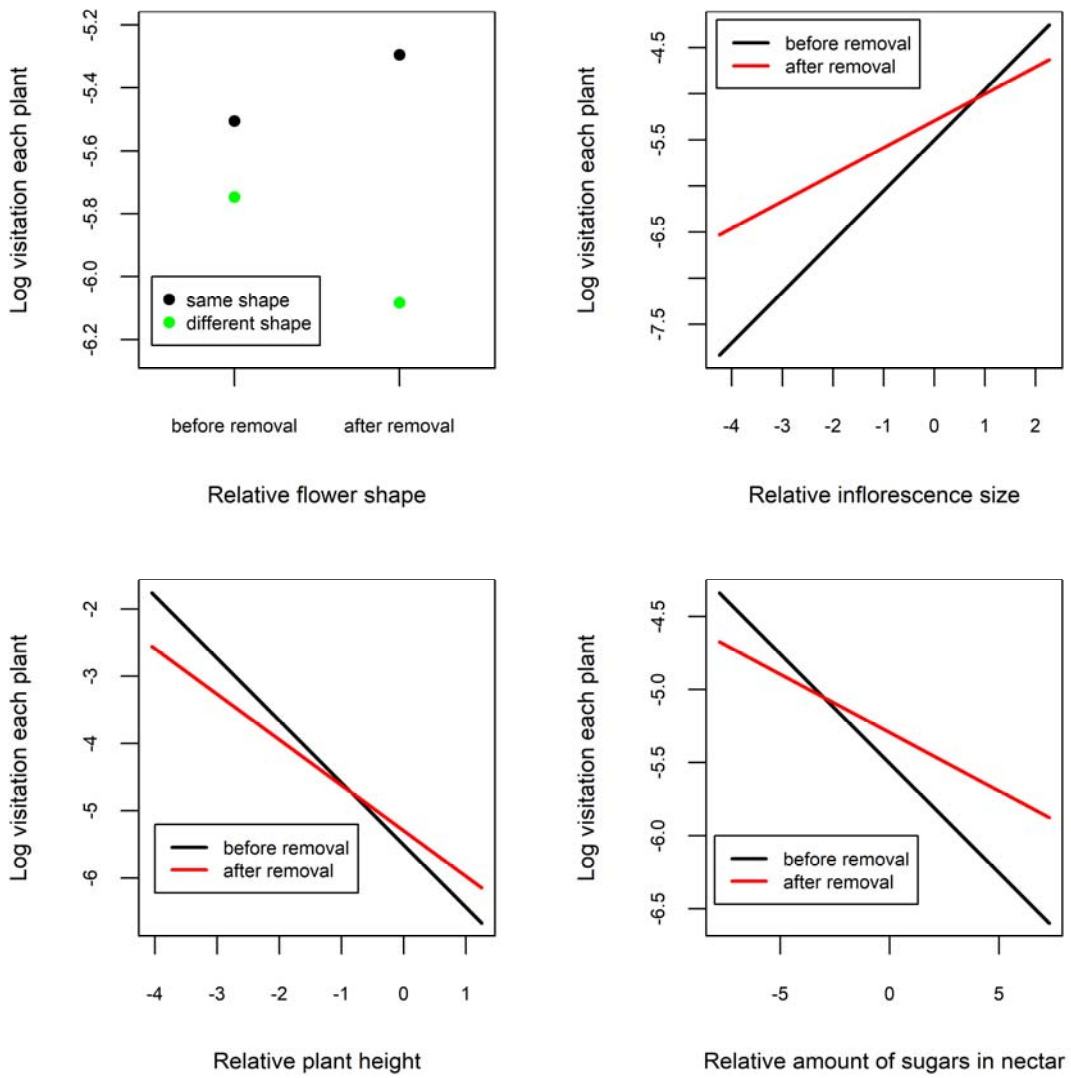

Figure 6 – Sequential removal experiment's flower visitors abundances as response to plant functional traits expressed as the difference between a given plant trait value and the trait value of the removed plant species across treatment.

Discussion

The pilot study showed that it was feasible to remove flowers from the dominant, most visited plant ("generalist") within a community and that the pollinators would respond by increasing the abundance to the next most visited plant. Moreover, this pilot study showed that these plants with the highest visitation are also true generalist species, because the plants with the highest insect abundances were also the plants with highest pollinator species richness (generalist *sensu* [41]). These results gave us some understanding of what we might expect to happen in a more extensive test using replicated sites and sequential removal of species.

In the sequential removal experiment, four generalist plant species were sequentially removed from 3 sites and changes in the visitation, pollination effectiveness and nectar standing crop were tested across the experiment. Also, each plant species' functional traits were recorded in order to evaluate exploitation of new resources by insects after the loss of highly visited plants. Overall, we observed decreases of overall flower-visiting insect abundance and

visitation of remaining plant species after the removal of 1 or 2 most generalist plants, some variation in pollination effectiveness unrelated to changes in visitation, and hints of rewiring of interactions by insects in relation to specific plant traits.

Responses of visitation

In complex communities, a few dominant, generalist species support the overall interactions structure by facilitating many other species, as outlined by [18]. This scenario likely occurred also in the treated sites of our experiments, as the presence of a small number of most generalist plants supported high visitation in the plant communities. When a few of these generalists were removed, the overall abundance of flower-visiting insects in the transects and the visitation of the remaining plants decreased. We interpret this as a case of facilitation among plants because when the most generalists were present, the visitation was high to the overall plant assemblage. This reaction of plant-pollinator assemblages reinforces the idea that community-level complex interactions are based on a core of few important species, without which the system appears as altered and impoverished [17].

Visitation to plants decreased especially after the second most generalist plant was removed. In the pilot study, removing the most generalist plant did not induce decreases in the overall visitation and actually [32] showed that this is because a new generalist plant takes the role of the removed one. However, sharp decreases took place in the sequential removal experiment after the second most generalist was removed. Therefore, there was only a few species of these generalists that maintained a high visitation in the sites, namely two species in sites 1 and 2, and only one species in site 3; after these species were removed, visitation started to decrease sharply. Therefore, the hierarchy of the important species is based on few plant species only [27]. This pattern have important implications for both conservation of community-level interactions and functionality of these systems, as discussed below. Nevertheless, the removal of key resources could have forced insects to explore and utilize other flowers, if their foraging were flexible [42].

The constrained flexibility of foraging

In the sequential removal experiment, we tested for the hypothesis that insects would perform a flexible foraging by recording changes in insect abundances in relation to several plant functional traits after the removal events. Foraging by insects was altered by our experiment, because after the removal events the changes of visitation of remaining plants depended on how their traits related to the removed plants. However, flower visitors showed only a limited degree of flexibility while foraging and looking for new resources.

Although previous simulations of consequences of species extinctions in plant-pollinator networks did not account for the redistribution of pollinators following the removal of key resources [29,43], our study has shown that static responses in foragers are not the rule. Instead, these assemblages are dynamic and there is considerable flexibility in foraging and new interactions are established after perturbations [3,42]. Foraging flexibility by consumers within a food web has been suggested to be important for the stability of complex communities [44]. This is because the system could buffer alterations by switches in interactions [42]. However, in our results, flexible foraging assessed at the level of the entire flower visitor community was not random but it was constrained by particular plant traits.

Specifically, the flower visitors did not swap between flower shapes across treatments, thus the level of foraging flexibility of insects was constrained. The sugar content of nectar also affected the rewiring process. As flower visitors were attracted by more rewarding flowers once the plant community was impoverished, the energetic intake constrained the rewiring. Furthermore, other plant traits also played a role in mediating the exploitation of new resources by flower visitors. Taller plants gained more visitation, possibly because they produce more effective displays and thus appear more attractive. However, smaller inflorescences also became more important after each removal. Although preference of smaller inflorescences seems to be contradictory as they have a smaller floral display, smaller inflorescences might provide more resources per flower, and thus be more rewarding to flower visitors on a per-visit basis [45]. Therefore, our results support the hypothesis that flexibility of foraging and exploitation of new resources is constrained by morphological (e.g. flower shape) or physiological aspects (e.g. sugar production) and that it can take place only within a specific plant-trait space [46]. Indeed, these constraints would eventually limit the accessibility of some new resources after perturbations, thus possibly undermining the stability of the system.

Responses in pollination effectiveness

The average pattern of pollination effectiveness (as measured by the number of pollen tubes grown in the pistils) did not follow the average trend of visitation, and visitation did not have a statistically significant effect on predicting pollination. This shows that it is not always possible to use flower visitors as an effective proxy for pollinators [47], although it is commonly done that way (though see Ballantyne et al. 2017). To address pollination, the amount of carried pollen might be obtained from DNA barcoding [48,49] or morphological identification of the pollen [50], while pollination service or effectiveness could be addressed by counting the number of pollen tubes growing within the pistils [51]. Although these and other similar approaches might be time- or resource- consuming when they have to be applied to species-rich communities, they provide better insights into the arguments about mutualisms and about ecosystem functions.

Growth of pollen tubes provides information about the effectiveness of the pollination service because they link pollen deposition and seed production [52]. In our sites, pollinator service varied across the treatment in a non-linear way. This might be due to destabilizations in the pollinators by the removal of key resources, which thus reacted in a fluctuating way. In fact, exclusion experiments highlighted that in the absence of a dominant pollinator, other pollinators can compensate by becoming themselves more effective pollen vectors [34]. However, other work has shown that once the abundant pollinator is excluded, plant fitness and fidelity to flowers can decrease [33]. Although these two studies contradict each other, in fact our results showed both responses: increasing or decreasing pollination effectiveness. This resulted from species-specific responses as some plants benefited by receiving more pollen, while other species received less, after removing key plant species. Thus, an idiosyncratic, fluctuating trend of pollination was the outcome at increasing impoverishment of the plant assemblage.

Sources of within-site variation in visitation

This community-level experimental approach allowed us to gather information even on within-site variation and species-specific responses. Specifically, we detected two types of variation. Firstly, some plant species had different

trends compared to the average community trend after treatment (Fig. 3). Such plants could be highly competitive taxa, able to gain pollinator visitation above average while other plants were losing visitors. This allows us to indirectly determine another feature of the most generalist species. Namely, it seems that they do not equally facilitate the visitation to all plants, but rather that some plants are facilitated by the most generalist species while others are limited by them. Such species-specific facilitation of pollination has been observed also in the case of invasive species (e.g. [31]).

The second case of variation is that of plant species which fluctuated in visitation responses among sampling units (within the same site at a given treatment level: see the wide confidence intervals around plant means in Fig. 3). It has been already demonstrated that visitation by pollinators often varies both spatially and in time (see [53,54]). However, most of these studies addressed variations over large areas such as regions or between years. In our work we have found variation in flower visitor abundances even among patches within the same site. That is, a given plant had high visitation in one sampling transect and low in another transect, even on the same day. Such patchy responses could be due to either a local heterogeneity of abiotic factors [55] or a changing set of neighbouring plant species and their relative abundances as local plant abundances often define visitation rates [23,54,56]. In other words, in one patch the facilitation might predominate, while competition might outweigh facilitation in another patch, thus resulting in very complex overall patterns [57]. A similar conclusion is pointed out in [53], in which the fluctuations of generalization levels are interpreted as variations of competition for pollinators between plants in a community. Therefore, given the heterogeneity in distribution of plants and the complexity of facilitation-competition relationships within a site, community ecologists should assess the interactions while accounting for nested sampling designs (sampling units within sites) and not only as cumulative (summing up all interactions on a plant species across sampling units). This would probably increase the amount of within-site variation in interaction patterns, as we also observed, but it would depict the detailed complexity of interactions and reveal the consistency of patterns and potentially their drivers [58].

Conclusions and implications for conservation

The species loss performed in our experiments led to important decreases of visitation and significant fluctuations of pollination effectiveness. We also showed that some of the responses were highly variable and idiosyncratic. Furthermore, the insects changed their foraging behaviours after the perturbation but their foraging flexibility was mediated by physiological and morphological traits of plants, which likely limited the utilization of new resources after plant removal. Thus, it seems clear that the stability of this system depends on a small subset of important species whose loss has severe consequences for the entire community of plants and flower-visiting insects. Generalist plants visited by a wide range of insects thus play a key role in plant-flower visitor complex interactions, and they could serve as hubs in the interaction networks [27,32,59]. They were also proposed to be important for the conservation of species-rich assemblages [60,61]. Therefore, in order to preserve ecosystems, high generalists should be preserved first, because they sustain the complex pattern of interactions in the community [17,27].

Supporting information

S1 Table. Data on the pollinator abundances per plant species from sequential removal experiment CSV

S2 Table. Data on the pollen tubes numbers per plant species CSV

S3 Table. Data on the standing crop of nectar per plant species CSV

S4 Table. Data on the difference between the removed plant species' and the other plants' functional traits CSV

Acknowledgements

PB, JK and AA would like to thank Dagmar Hucková, Tomáš Gregor, Michal Rindoš, Michal Bartoš, and Zuzana Chlumská for their help during field work in CZ. In the UK, JO & ST wish to thank The Wildlife Trust for Bedfordshire, Cambridgeshire, Northamptonshire and Peterborough for permission to work at Bradlaugh Fields, and the field assistants Joanna Tarrant, Lee Muttock, Claire Hepinstall Md Lutfor Rahman, and Christine Berrill.

References

1. Chapin FSI, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM, Reynolds HL, et al. Consequences of changing biodiversity. Nature. 2000;405: 234–242.

2. Smith MD, Knapp AK. Dominant species maintain ecosystem function with non-random species loss. Ecol Lett. 2003;6: 509–517. doi:10.1046/j.1461-0248.2003.00454.x

3. Kaiser-Bunbury CN, Muff S, Memmott J, Müller CB, Caflisch A. The robustness of pollination networks to the loss of species and interactions: a quantitative approach incorporating pollinator behaviour. Ecol Lett. 2010;13: 442–452. doi:10.1111/j.1461-0248.2009.01437.x

4. Cardinale BJ, Srivastava DS, Duffy JE, Wright JP, Downing AL, Sankaran M, et al. Effects of biodiversity on the functioning of trophic groups and ecosystems. Nature. 2006;443: 989. doi:10.1038/nature05202

5. Hegland SJ, Nielsen A, Lázaro A, Bjerknes A-L, Totland Ø. How does climate warming affect plant-pollinator interactions? Ecol Lett. 2009;12: 184–195.

6. Schmid B, Balvanera P, Cardinale BJ, Godbold J, Pfisterer AB, Raffaelli D, et al. Consequences of species loss for ecosystem functioning: meta-analyses of data from biodiversity experiments. Biodivers Ecosyst Funct Hum Wellbeing Ecol Econ Perspect. 2009; 14–29.

7. Dunne JA, Williams RJ, Martinez ND. Network structure and biodiversity loss in food webs: robustness increases with connectance. Ecol Lett. 2002;5: 558–567. doi:10.1046/j.1461-0248.2002.00354.x

8. Hooper DU, Vitousek PM. The effects of plant composition and diversity on ecosystem processes. Science. 1997;277: 1302–1305.

9. Tilman D, Reich PB, Knops J, Wedin D, Mielke T, Lehman C. Diversity and productivity in a long-term grassland experiment. Science. 2001;294: 843–845.

10. Seabloom EW, Dobson AP, Stoms DM. Extinction rates under nonrandom patterns of habitat loss. Proc Natl Acad Sci. 2002;99: 11229–11234.

11. Gross K, Cardinale BJ. The functional consequences of random vs. ordered species extinctions. Ecol Lett. 2005;8: 409–418. doi:10.1111/j.1461-0248.2005.00733.x

12. Rezende EL, Lavabre JE, Guimarães PR, Jordano P, Bascompte J. Non-random coextinctions in phylogenetically structured mutualistic networks. Nature. 2007;448: 925–928.

13. Smith KG, Lips KR, Chase JM. Selecting for extinction: nonrandom disease-associated extinction homogenizes amphibian biotas. Ecol Lett. 2009;12: 1069–1078.

14. Ollerton J, Erenler H, Edwards M, Crockett R. Extinctions of aculeate pollinators in Britain and the role of large-scale agricultural changes. Science. 2014;346: 1360–1362. doi:10.1126/science.1257259

15. Grime JP. Declining plant diversity: empty niches or functional shifts? J Veg Sci. 2002;13: 457–460.

16. Santamaría S, Galeano J, Pastor JM, Méndez M. Removing interactions, rather than species, casts doubt on the high robustness of pollination networks. Oikos. 2016;125: 526–534. doi:10.1111/oik.02921

17. Biella P, Ollerton J, Barcella M, Assini S. Network analysis of phenological units to detect important species in plant-pollinator assemblages: can it inform conservation strategies? Community Ecol. 2017;18: 1–10. doi:10.1556/168.2017.18.1.1

18. Verdú M, Valiente-Banuet A. The Nested Assembly of Plant Facilitation Networks Prevents Species Extinctions. Am Nat. 2008;172: 751–760. doi:10.1086/593003

19. Jordano P, Bascompte J, Olesen JM. The ecological consequences of complex topology and nested structure in pollination webs. In: Waser NM, Ollerton J, editors. Plant-pollinator interactions: from specialization to generalization. Chicago: University of Chicago Press; 2006. pp. 173–199.

20. Johnson SD, Peter CI, Nilsson LA, Ågren J. Pollination Success in a Deceptive Orchid Is Enhanced by Co-Occurring Rewarding Magnet Plants. Ecology. 2003;84: 2919–2927. doi:10.1890/02-0471

21. Feldman TS, F. Morris W, G. Wilson W. When can two plant species facilitate each other's pollination? Oikos. 2004;105: 197–207. doi:10.1111/j.0030-1299.2004.12845.x

22. Tur C, Sáez A, Traveset A, Aizen MA. Evaluating the effects of pollinator-mediated interactions using pollen transfer networks: evidence of widespread facilitation in south Andean plant communities. Ecol Lett. 2016;19: 576–586. doi:10.1111/ele.12594

23. Ye Z-M, Dai W-K, Jin X-F, Gituru RW, Wang Q-F, Yang C-F. Competition and facilitation among plants for pollination: can pollinator abundance shift the plant–plant interactions? Plant Ecol. 2014;215: 3–13. doi:10.1007/s11258-013-0274-y

24. Albrecht M, Ramis MR, Traveset A. Pollinator-mediated impacts of alien invasive plants on the pollination of native plants: the role of spatial scale and distinct behaviour among pollinator guilds. Biol Invasions. 2016;18: 1801–1812. doi:10.1007/s10530-016-1121-6

25. Dupont YL, Hansen DM, Olesen JM. Structure of a plant–flower-visitor network in the high-altitude sub-alpine desert of Tenerife, Canary Islands. Ecography. 2003;26: 301–310. doi:10.1034/j.1600-0587.2003.03443.x

26. Ollerton J, Johnson SD, Cranmer L, Kellie S. The Pollination Ecology of an Assemblage of Grassland Asclepiads in South Africa. Ann Bot. 2003;92: 807–834. doi:10.1093/aob/mcg206

27. Martín González AM, Dalsgaard B, Olesen JM. Centrality measures and the importance of generalist species in pollination networks. Ecol Complex. 2010;7: 36–43. doi:10.1016/j.ecocom.2009.03.008

28. Watts S, Dormann CF, González AAM, Ollerton J. The influence of floral traits on specialisation and modularity of plant-pollinator networks in a biodiversity hotspot in the Peruvian Andes Watts S, Dormann CF, González AMM, Ollerton J. Ann Bot. 2016;in press.

29. Memmott J, Waser NM, Price MV. Tolerance of pollination networks to species extinctions. Proc R Soc Lond B Biol Sci. 2004;271: 2605–2611.

30. Lopezaraiza–Mikel ME, Hayes RB, Whalley MR, Memmott J. The impact of an alien plant on a native plant–pollinator network: an experimental approach. Ecol Lett. 2007;10: 539–550.

31. Ferrero V, Castro S, Costa J, Acuna P, Navarro L, Loureiro J. Effect of invader removal: pollinators stay but some native plants miss their new friend. Biol Invasions. 2013;15: 2347–2358.

32. Goldstein J, Zych M. What if we lose a hub? Experimental testing of pollination network resilience to removal of keystone floral resources. Arthropod-Plant Interact. 2016;10: 263–271. doi:10.1007/s11829-016-9431-2

33. Brosi BJ, Briggs HM. Single pollinator species losses reduce floral fidelity and plant reproductive function. Proc Natl Acad Sci. 2013;110: 13044–13048.

34. Hallett AC, Mitchell RJ, Chamberlain ER, Karron JD. Pollination success following loss of a frequent pollinator: the role of compensatory visitation by other effective pollinators. AoB PLANTS. 2017;9. doi:10.1093/aobpla/plx020

35. Martin FW. Staining and observing pollen tubes in the style by means of fluorescence. Stain Technol. 1959;34: 125–128.

36. Faegri K, van der Pijl L. The principles of pollination ecology. Pergamon Press; 1979.

37. Kugler H. Blütenökologie. G. Fischer; 1970.

38. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J Stat Softw. 2015;67: 1–48. doi:10.18637/jss.v067.i01

39. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria; 2017. Available: https://www.R-project.org/

40. Hothorn T, Bretz F, Westfall P. Simultaneous inference in general parametric models. Biom J. 2008;50: 346–363.

41. Waser NM, Chittka L, Price MV, Williams NM, Ollerton J. Generalization in Pollination Systems, and Why it Matters. Ecology. 1996;77: 1043–1060. doi:10.2307/2265575

42. Valdovinos FS, Moisset de Espanés P, Flores JD, Ramos-Jiliberto R. Adaptive foraging allows the maintenance of biodiversity of pollination networks. Oikos. 2013;122: 907–917.

43. Saavedra S, Stouffer DB, Uzzi B, Bascompte J. Strong contributors to network persistence are the most vulnerable to extinction. Nature. 2011;478: 233–235.

44. Kondoh M. Foraging adaptation and the relationship between food-web complexity and stability. Science. 2003;299: 1388–1391.

45. Akter A, Biella P, Klecka J. Effects of small-scale clustering of flowers on pollinator foraging behaviour and flower visitation rate. PLOS ONE. 2017;12: e0187976. doi:10.1371/journal.pone.0187976

46. Vizentin-Bugoni J, Maruyama PK, Sazima M. Processes entangling interactions in communities: forbidden links are more important than abundance in a hummingbird–plant network. Proc R Soc Lond B Biol Sci. 2014;281: 20132397. doi:10.1098/rspb.2013.2397

47. King C, Ballantyne G, Willmer PG. Why flower visitation is a poor proxy for pollination: measuring single-visit pollen deposition, with implications for pollination networks and conservation. Methods Ecol Evol. 2013;4: 811–818.

48. Galimberti A, De Mattia F, Bruni I, Scaccabarozzi D, Sandionigi A, Barbuto M, et al. A DNA barcoding approach to characterize pollen collected by honeybees. PLoS One. 2014;9: e109363.

49. Bruni I, Galimberti A, Caridi L, Scaccabarozzi D, De Mattia F, Casiraghi M, et al. A DNA barcoding approach to identify plant species in multiflower honey. Food Chem. 2015;170: 308–315.

50. Fisogni A, Quaranta M, Grillenzoni F-V, Corvucci F, de Manincor N, Bogo G, et al. Pollen load diversity and foraging niche overlap in a pollinator community of the rare Dictamnus albus L. Arthropod-Plant Interact. 2017; 1–10.

51. Aizen MA. Influences of animal pollination and seed dispersal on winter flowering in a temperate mistletoe. Ecology. 2003;84: 2613–2627.

52. Alonso C, Herrera CM, Ashman T-L. A piece of the puzzle: a method for comparing pollination quality and quantity across multiple species and reproductive events. New Phytol. 2012;193: 532–542.

53. Lázaro A, Nielsen A, Totland Ø. Factors related to the inter-annual variation in plants' pollination generalization levels within a community. Oikos. 2010;119: 825–834. doi:10.1111/j.1600-0706.2009.18017.x

54. Carstensen DW, Sabatino M, Trøjelsgaard K, Morellato LPC. Beta Diversity of Plant-Pollinator Networks and the Spatial Turnover of Pairwise Interactions. PLOS ONE. 2014;9: e112903. doi:10.1371/journal.pone.0112903

55. Janovský Z, Mikát M, Hadrava J, Horčičková E, Kmecová K, Požárová D, et al. Conspecific and Heterospecific Plant Densities at Small-Scale Can Drive Plant-Pollinator Interactions. PLOS ONE. 2013;8: e77361. doi:10.1371/journal.pone.0077361

56. Vázquez DP, Aizen MA. Community-wide patterns of specialization in plant–pollinator interactions revealed by null models. Plant–pollinator Interact Spec Gen. 2006; 200–219.

57. Gross K. Positive interactions among competitors can produce species-rich communities. Ecol Lett. 2008;11: 929–936.

58. Trøjelsgaard K, Olesen JM. Ecological networks in motion: micro- and macroscopic variability across scales. Funct Ecol. 2016;30: 1926–1935. doi:10.1111/1365-2435.12710

59. Peters VE, Carlo TA, Mello MA, Rice RA, Tallamy DW, Caudill SA, et al. Using plant–animal interactions to inform tree selection in tree-based agroecosystems for enhanced biodiversity. BioScience. 2016;66: 1046–1056.

60. Menz MH, Phillips RD, Winfree R, Kremen C, Aizen MA, Johnson SD, et al. Reconnecting plants and pollinators: challenges in the restoration of pollination mutualisms. Trends Plant Sci. 2011;16: 4–12.

61. Montoya D, Rogers L, Memmott J. Emerging perspectives in the restoration of biodiversity-based ecosystem services. Trends Ecol Evol. 2012;27: 666–672.