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

Short title: Removing generalist plants alters plant-pollinator interactions

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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].

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

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

99 Specifically we asked whether the treatment produced effects on (1) the general overall visitation
100 (that is the abundance of pollinators in the sites), (2) the visitation to each plant species, (3) the pollination
101 effectiveness determined by the number of pollen tubes grown in the pistils after visitation, (4) the amount
102 of used nectar resources ("standing crop"). Furthermore, we hypothesise that insects would perform a
103 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

130 pollinator density. Captured pollinators were placed into specimen jars. After the survey was completed,
131 those insects that could be identified in the field were recorded and released whilst the remaining
132 individuals were taken for later identification.
133
134 ***Sequential removal***
135 The sequential removal experiment was performed in the vicinity of Český Krumlov (South Bohemia, Czech
136 Republic). Three experimental sites were chosen, all small meadows surrounded by large trees in a similar
137 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'
138 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.
139 The mean pairwise distance between sites was 2.01 ± 0.95 Km.
140 The sampling was based on walking six transects (10 m x 1 m) in a randomized order in each site between
141 9:00 and 17:00 hours. All transects were usually sampled twice during each day. Sampling was postponed in
142 the case of rain or strong wind. All insects found while visiting flowers were sampled by netting or use of a
143 mouth aspirator. The abundance of plants was recorded by counting the number of open flowers or
144 compound inflorescences on each plant within each transect. Abundance was recorded during all stages of
145 the experiment in order to capture variation of flower abundances across treatments.
146 In the experimental sites, all flower visitors feeding within flowers were sampled for two days (hereafter
147 the "Before" period). At the end of the sampling days, the captured specimens were counted for each plant
148 and thus we were able to determine the plant with the highest visitation, i.e. the total abundance of flower
149 visitors on the plant. We call these highly visited plants "generalist" in accordance with the literature
150 (see[32]). This species was removed from the site by cutting all inflorescences. We only removed flowers
151 and left the stems otherwise intact so that the vegetation structure remained unaltered. Twenty four hours
152 after the removal, the sites were sampled again for two sampling days ("Rem_1sp" = first species
153 removed). After we had counted the abundances of the visitors for each plant, we determined the next
154 plant species with the highest visitation, the inflorescences of which were removed ("Rem_2sp"). The
155 flower visitors were sampled again after twenty four hours and again for two sampling days. The next plant
156 species with the highest visitation was thus found in the same fashion as for the first two ones, and then

157 removed ("Rem_3sp"). After twenty four hours, we again sampled flower visitors of the plant community
158 for two sampling days, so that the next plant species with the highest visitation was found in the same way
159 as for the others and later removed ("Rem_4sp"). After 24h, we again sampled the plant community. Site 1
160 and 2 were sampled from 25th June to 12th of July, Site 3 was sampled from 2nd to 17th of July; Control site
161 was sampled at the same time as each of the experimental sites, but no treatment (removals) was applied
162 to this one (data in S1 Table).
163
164 ***Pistil collection and pollen tubes***
165 Pollinator effectiveness to the flowering plants at two experimental sites was tested by counting pollen
166 tubes in the pistils. To do so, the pistils were collected after the insect sampling and preserved in formalin-
167 acetic acid-alcohol (FAA) at room temperature. Flowers were collected outside (but nearby) the transects
168 to avoid depletion of flower abundances in the transects. Later, in the laboratory, the flowers were
169 dissected under the microscope and pistils were prepared for the softening and staining, following the
170 technique of [35]. After softening the pistils in 4N NaOH, they were stained with 0.1% aniline blue in 0.1M
171 K_2HPO_4 for 12 hours. Then, the pistils were washed and mounted in a drop of 50% Glycerin on glass slides
172 and covered with cover slips for observation under the fluorescence microscope. Pollen tubes were visible
173 and counted for most of the species. However, in a few cases, pollen tubes were impossible to visualize
174 properly. For these species the number of pollen grains on the stigmas were counted assuming that only
175 germinated grains with tubes still attached would remain on the stigma after the preparation process. All
176 processes were carried out at room temperature. After the observation, the edges of the cover slips were
177 sealed with clear nail polish and stored at 4°C for future reference. Data were successfully obtained for 10
178 species because other plant species did not yield any countable pollen tubes (data in S2 Table).
179
180 ***Nectar content of flowers and other functional traits***
181 We determined the daily production and the standing-crop of nectar in flowering plant species at two sites.
182 The daily production was assessed by bagging flowers in anthesis for 24 hours. Flowers were bagged in the
183 morning and sampled the next day in the morning. For the standing-crop of nectar, flowers were collected

184 in each site after the pollination sampling on the same day (data in S3 Table). A 100µl Hamilton capillary
185 syringe was used for the collection and for washing the nectar into distilled water. Flowers were selected
186 randomly but outside the sampling transects in order to avoid impoverishment of flower resources in the
187 transects; only flowers in full anthesis were sampled. Nectar samples were stored in a cool bag in the field
188 and in a -20°C freezer in the laboratory. Sugar analysis of nectar was done using high performance anion
189 exchange chromatography with pulsed amperometric detection using a Dionex ICS-3000 system and
190 CarboPac PA1 analytical column. Nectar amount was expressed as milligrams of sugars per flower.
191 Other traits measured for each plant species in the two sites were: plant height (linear distance between
192 the ground and the top of an inflorescence); inflorescence maximum size (the maximum dimension of the
193 inflorescence); dominant colour of the corolla (categorical variable, e.g. "white", "blue", "pink", "yellow");
194 flower shape coded according to [36] as follows: bell- or funnel-shaped, dish- or bowl-shaped flowers, flag-
195 shaped flowers, gullet-shaped flowers, head- or brush-shaped, tube-shaped flowers; unclear cases were
196 checked with [37].
197
198 ***Statistical analyses***
199 All data were analysed by means of generalized linear mixed-effect models (GLMM) with library *lme4* [38]
200 in the R environment [39]. Details of the models with the lowest AIC follow.
201 The overall visitation was derived by calculating the means of flower visitor abundances across plant
202 species; mean values were calculated separately at each sampling event for each transect. Thus, the visiting
203 insect abundance at transects was the response variable, treatment was a categorical predictor ("Before",
204 "Rem_1sp", "Rem_2sp", "Rem_3sp", "Rem_4sp"). Transect identity was the random intercept. Poisson
205 distribution was used. Data were also analysed after including an offset with the geometric means of
206 visitation in the control site, in order to account for possible variations not due to treatment. Treated sites
207 in the sequential removal experiment were analysed independently. In these experimental sites, a multiple
208 comparison tests were performed using the package *multcomp* with the function *glht* [40] to compare a
209 given treatment level with the preceding treatment level (e.g. "Rem_1sp" vs "Before", "Rem_2sp" vs
210 "Rem_1sp", and so on) in order to test for relative increases or decreases of pollinator abundances.

211 For the data from the pilot study, the visitation was analysed in a slightly different fashion because flower
212 visitors were collected along a unique transect covering the entire surface of the site and insect species
213 were taxonomically identified. Thus in a GLMM with Poisson distribution the abundance of visitation by
214 flower visitor species was used as response, treatment was predictor ("before", "after"). Species identity
215 was used as random factor on the intercept. Data were also analysed after including an offset with the
216 visitation by insect species in the control site; the mean visitation on a given plant species was used for
217 insects that were not present in the control.
218 To test the effect of treatment on each plant species at each site in the sequential removal experiment,
219 plant-level visitation (visiting insect abundance for each plant species at each sampled transect, including
220 unvisited plants) was the response variable and treatment was a predictor ("Before", "Rem_1sp",
221 "Rem_2sp", "Rem_3sp", "Rem_4sp"). An offset of plant abundance was included, to account for possible
222 variation of plant abundance during the experiment. Transect identity within plant species was included as
223 random slope of treatment. Poisson distribution was used. The three treated sites were analysed
224 separately.
225 To test whether the number of pollen tubes in each plant species changed in the sequential removal sites,
226 the number of pollen tubes was the response variable, treatment ("Before", "Rem_1sp", "Rem_2sp",
227 "Rem_3sp", "Rem_4sp") and the plant mean visitation across each transect were predictors. Random
228 slopes of predictors were accounted with site identity within plant species as a random factor, and Poisson
229 distribution was used.
230 To test the variation of standing crop of nectar, the amount of sugars per flower in each species was the
231 response variable, treatment ("Before", "Rem_1sp", "Rem_2sp", "Rem_3sp", "Rem_4sp") and the plant
232 mean visitation across transect were predictors. Random intercept was accounted with site identity within
233 plant species as a random factor (no random slopes included due to lack of convergence of the model).
234 Gamma distribution was used, link function was the natural logarithm.
235 To test for changes in foraging preferences of flower-visiting insects, we assessed how visitation related to
236 differences between plant-species' traits and the plant removed (data in S4 Table). To do that, the log-ratio
237 difference between a given continuous plant trait value and the trait value of the removed plant species

238 was calculated for each treatment level and used as a predictor variable. For categorical variables, a
239 binomial variable was used, i.e. “same” or “different” from the removed species. The variables included
240 were flower colour, flower shape, inflorescence size, plant height and the daily production of sugars. All of
241 these variables were included in the model in interaction with the treatment variable (“before”, “after”
242 removal to account for a simplified codification of the treatment levels in the statistical model). Visiting
243 insect abundance for each plant species was used as a response variable. An offset of plant abundance was
244 included, to account for possible variation of plant abundance across treatments. Random slopes of
245 treatment was accounted with transect identity within plant species within site as a random factor, using
246 Poisson distribution.
247

248 **Results**

249 In the pilot study, the surveys identified a total of 13 insect pollinated plant species in flower and 25
250 pollinating insect species in the treated site. The plant with the highest pollinator abundance was *Knautia*
251 *arvensis* (Caprifoliaceae), with a total of 54 insects (of 11 species); the second generalist plant had 41
252 insects (of 11 species). After the removal of *K. arvensis*, *Centaurea nigra* (Asteraceae) was the plant with
253 the highest pollinator abundance, having 86 insects (of 11 species). Other plants were visited by less than
254 20 insect specimens (and less than 8 species) in both experimental phases.
255 In the sequential removal experiment, the sites 1, 2 and 3 were surveyed for a total of 31, 26 and 23 insect
256 pollinated plant species, respectively. The sequences of the removed plants specie were the following: Site
257 1, *Anthriscus sylvestris* (Apiaceae, 256 insect specimens in “Before”), *Rubus caesius* (Rosaceae, 315 insects
258 in “Rem_1sp”), *Knautia arvensis* (Caprifoliaceae, 83 insects in “Rem_2sp”), *Galium mollugo* (Rubiaceae 39
259 insects in “Rem 3sp”); Site 2, *Veronica teucrium* (Plantaginaceae, 103 insect specimens in “Before”),
260 *Agrimonia eupatoria* (Rosaceae, 96 insects in “Rem_1sp”), *Centaurea scabiosa* (Asteraceae, 96 insects in
261 “Rem_2sp”), *Securigera varia* (Fabaceae, 26 insects in “Rem 3sp”); Site 3, *Aegopodium podagraria*
262 (Apiaceae, 141 insect specimens in “Before”), *Veronica teucrium* (Plantaginaceae, 26 insects in “Rem_1sp”),

263 *Knautia arvensis* (Caprifoliaceae, 45 insects in "Rem_2sp"), *Origanum vulgare* (Lamiaceae, 55 insects in
264 "Rem_3sp").
265
266 ***Results from the pilot study***
267 In the pilot study, visitation to plants slightly increased after removing one species (Fig. 1). The treatment
268 was a significant predictor of visitation: χ^2=8.49, df=1, p < 0.01. However, in the model including an offset of
269 the control's visitation, the treatment was not significant. No significant change was detected in the control
270 site during the period.
271
272 Figure 1 – Pilot study's flower-visitor species abundances for each plant ("visitation") on a logarithmic scale.
273 The left panel shows the trend at the treated site, the middle panel shows the trend when including an
274 offset of control site's visitation, and the right panel shows the visitation at the control site. Diamonds
275 indicate means, significance codes are the following: *** p<0.001, ** p<0.01, * p<0.05.
276
277
278 ***General pattern of visitation***
279 In the sequential removal sites, visitation per transect decreased sharply after the removal of plants and
280 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;
281 Site3: χ^2= 785.92, df=6, p < 0.001). Treatment was also significant when visitation in the control site was
282 used as an offset in the models. Multiple-comparison test of treatment-levels gave significant results in
283 most cases, especially when variation of visitation in the control was accounted in the model (Table 1).
284 Namely, the removal of the first plant caused only minor changes in overall flower visitor abundances in
285 two sites, while it decreased sharply afterwards (Fig. 2); in one site, however, flower visitors abundances
286 decreased after the first species was removed.
287
288 Table 1 – Multiple comparison statistics on the effect of treatment on general visitation, i.e. the total
289 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 ("Standarized") 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).

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.

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.

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, $p = 0.43$).

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.

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).

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, $p = 0.71$).

339

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

343

344 ***Plant traits***

345 All plant-traits included in the models except colour were significant predictors of insect visitation (Table 2).
346 After removals, visitation increased in plants with the same flower shape as the removed plant, with
347 smaller inflorescences, slightly taller heights and with a higher sugar content than the removed plant (Fig.
348 6).

349

350 Table 2 – The effect of plant traits on changes of visitation after plant removal evaluated by single-term
351 deletion of interaction terms from a GLMM model. Colons are used for interactions between treatment
352 ("Treatment" referring to before-after removal of one plant species) and difference of trait values of
353 individual plants from the removed species. Trait difference for colour and flower shape was classified as
354 same or different. Log-ratio difference was used for plant height ("Height"), inflorescence size ("Size") and
355 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

356

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

383 case of facilitation among plants because when the most generalists were present, the visitation was high
384 to the overall plant assemblage. This reaction of plant-pollinator assemblages reinforces the idea that
385 community-level complex interactions are based on a core of few important species, without which the
386 system appears as altered and impoverished [17].
387 Visitation to plants decreased especially after the second most generalist plant was removed. In the pilot
388 study, removing the most generalist plant did not induce decreases in the overall visitation and actually
389 [32] showed that this is because a new generalist plant takes the role of the removed one. However, sharp
390 decreases took place in the sequential removal experiment after the second most generalist was removed.
391 Therefore, there was only a few species of these generalists that maintained a high visitation in the sites,
392 namely two species in sites 1 and 2, and only one species in site 3; after these species were removed,
393 visitation started to decrease sharply. Therefore, the hierarchy of the important species is based on few
394 plant species only [27]. This pattern have important implications for both conservation of community-level
395 interactions and functionality of these systems, as discussed below. Nevertheless, the removal of key
396 resources could have forced insects to explore and utilize other flowers, if their foraging were flexible [42].
397
398 ***The constrained flexibility of foraging***
399 In the sequential removal experiment, we tested for the hypothesis that insects would perform a flexible
400 foraging by recording changes in insect abundances in relation to several plant functional traits after the
401 removal events. Foraging by insects was altered by our experiment, because after the removal events the
402 changes of visitation of remaining plants depended on how their traits related to the removed plants.
403 However, flower visitors showed only a limited degree of flexibility while foraging and looking for new
404 resources.
405 Although previous simulations of consequences of species extinctions in plant-pollinator networks did not
406 account for the redistribution of pollinators following the removal of key resources [29,43], our study has
407 shown that static responses in foragers are not the rule. Instead, these assemblages are dynamic and there
408 is considerable flexibility in foraging and new interactions are established after perturbations [3,42].
409 Foraging flexibility by consumers within a food web has been suggested to be important for the stability of

410 complex communities [44]. This is because the system could buffer alterations by switches in interactions
411 [42]. However, in our results, flexible foraging assessed at the level of the entire flower visitor community
412 was not random but it was constrained by particular plant traits. Specifically, the flower visitors did not
413 swap between flower shapes across treatments, thus the level of foraging flexibility of insects was
414 constrained. The sugar content of nectar also affected the rewiring process. As flower visitors were
415 attracted by more rewarding flowers once the plant community was impoverished, the energetic intake
416 constrained the rewiring. Furthermore, other plant traits also played a role in mediating the exploitation of
417 new resources by flower visitors. Taller plants gained more visitation, possibly because they produce more
418 effective displays and thus appear more attractive. However, smaller inflorescences also became more
419 important after each removal. Although preference of smaller inflorescences seems to be contradictory as
420 they have a smaller floral display, smaller inflorescences might provide more resources per flower, and thus
421 be more rewarding to flower visitors on a per-visit basis [45]. Therefore, our results support the hypothesis
422 that flexibility of foraging and exploitation of new resources is constrained by morphological (e.g. flower
423 shape) or physiological aspects (e.g. sugar production) and that it can take place only within a specific
424 plant-trait space [46]. Indeed, these constraints would eventually limit the accessibility of some new
425 resources after perturbations, thus possibly undermining the stability of the system.
426
427 ***Responses in pollination effectiveness***
428 The average pattern of pollination effectiveness (as measured by the number of pollen tubes grown in the
429 pistils) did not follow the average trend of visitation, and visitation did not have a statistically significant
430 effect on predicting pollination. This shows that it is not always possible to use flower visitors as an
431 effective proxy for pollinators [47], although it is commonly done that way (though see Ballantyne et al.
432 2017). To address pollination, the amount of carried pollen might be obtained from DNA barcoding [48,49]
433 or morphological identification of the pollen [50], while pollination service or effectiveness could be
434 addressed by counting the number of pollen tubes growing within the pistils [51]. Although these and other
435 similar approaches might be time- or resource- consuming when they have to be applied to species-rich

436 communities, they provide better insights into the arguments about mutualisms and about ecosystem
437 functions.
438 Growth of pollen tubes provides information about the effectiveness of the pollination service because
439 they link pollen deposition and seed production [52]. In our sites, pollinator service varied across the
440 treatment in a non-linear way. This might be due to destabilizations in the pollinators by the removal of key
441 resources, which thus reacted in a fluctuating way. In fact, exclusion experiments highlighted that in the
442 absence of a dominant pollinator, other pollinators can compensate by becoming themselves more
443 effective pollen vectors [34]. However, other work has shown that once the abundant pollinator is
444 excluded, plant fitness and fidelity to flowers can decrease [33]. Although these two studies contradict each
445 other, in fact our results showed both responses: increasing or decreasing pollination effectiveness. This
446 resulted from species-specific responses as some plants benefited by receiving more pollen, while other
447 species received less, after removing key plant species. Thus, an idiosyncratic, fluctuating trend of
448 pollination was the outcome at increasing impoverishment of the plant assemblage.
449
450 ***Sources of within-site variation in visitation***
451 This community-level experimental approach allowed us to gather information even on within-site
452 variation and species-specific responses. Specifically, we detected two types of variation. Firstly, some plant
453 species had different trends compared to the average community trend after treatment (Fig. 3). Such plants
454 could be highly competitive taxa, able to gain pollinator visitation above average while other plants were
455 losing visitors. This allows us to indirectly determine another feature of the most generalist species.
456 Namely, it seems that they do not equally facilitate the visitation to all plants, but rather that some plants
457 are facilitated by the most generalist species while others are limited by them. Such species-specific
458 facilitation of pollination has been observed also in the case of invasive species (e.g. [31]).
459 The second case of variation is that of plant species which fluctuated in visitation responses among
460 sampling units (within the same site at a given treatment level: see the wide confidence intervals around
461 plant means in Fig. 3). It has been already demonstrated that visitation by pollinators often varies both
462 spatially and in time (see [53,54]). However, most of these studies addressed variations over large areas

463 such as regions or between years. In our work we have found variation in flower visitor abundances even
464 among patches within the same site. That is, a given plant had high visitation in one sampling transect and
465 low in another transect, even on the same day. Such patchy responses could be due to either a local
466 heterogeneity of abiotic factors [55] or a changing set of neighbouring plant species and their relative
467 abundances as local plant abundances often define visitation rates [23,54,56]. In other words, in one patch
468 the facilitation might predominate, while competition might outweigh facilitation in another patch, thus
469 resulting in very complex overall patterns [57]. A similar conclusion is pointed out in [53], in which the
470 fluctuations of generalization levels are interpreted as variations of competition for pollinators between
471 plants in a community. Therefore, given the heterogeneity in distribution of plants and the complexity of
472 facilitation-competition relationships within a site, community ecologists should assess the interactions
473 while accounting for nested sampling designs (sampling units within sites) and not only as cumulative
474 (summing up all interactions on a plant species across sampling units). This would probably increase the
475 amount of within-site variation in interaction patterns, as we also observed, but it would depict the
476 detailed complexity of interactions and reveal the consistency of patterns and potentially their drivers [58].
477

478 ***Conclusions and implications for conservation***

479 The species loss performed in our experiments led to important decreases of visitation and significant
480 fluctuations of pollination effectiveness. We also showed that some of the responses were highly variable
481 and idiosyncratic. Furthermore, the insects changed their foraging behaviours after the perturbation but
482 their foraging flexibility was mediated by physiological and morphological traits of plants, which likely
483 limited the utilization of new resources after plant removal. Thus, it seems clear that the stability of this
484 system depends on a small subset of important species whose loss has severe consequences for the entire
485 community of plants and flower-visiting insects. Generalist plants visited by a wide range of insects thus
486 play a key role in plant-flower visitor complex interactions, and they could serve as hubs in the interaction
487 networks [27,32,59]. They were also proposed to be important for the conservation of species-rich
488 assemblages [60,61]. Therefore, in order to preserve ecosystems, high generalists should be preserved first,
489 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

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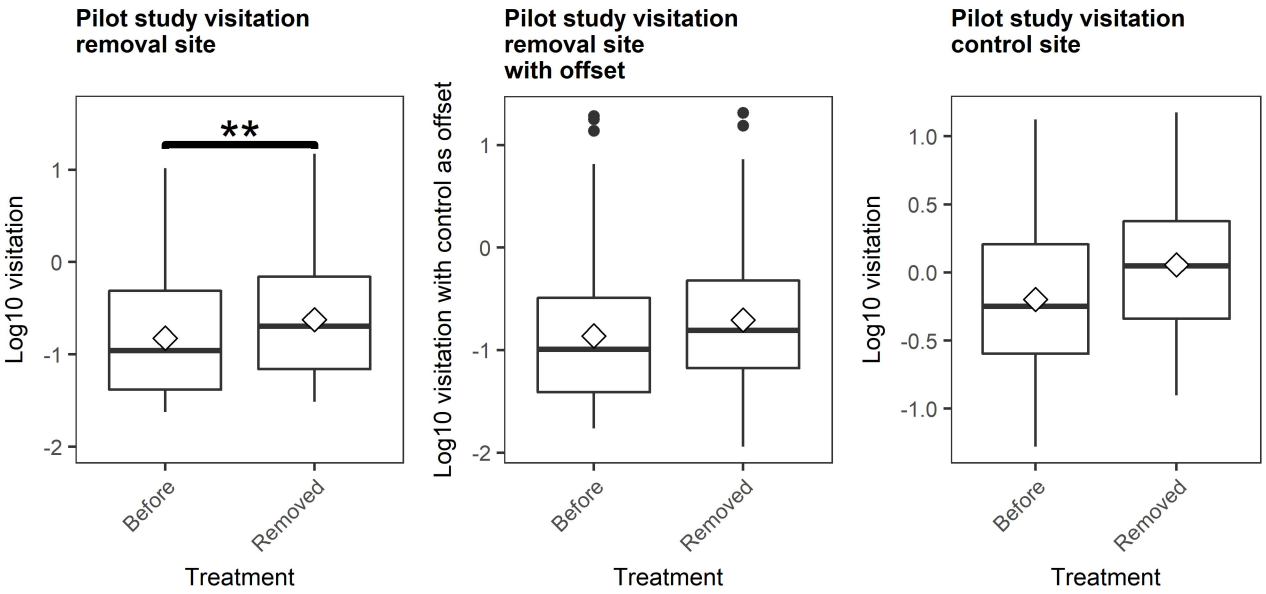
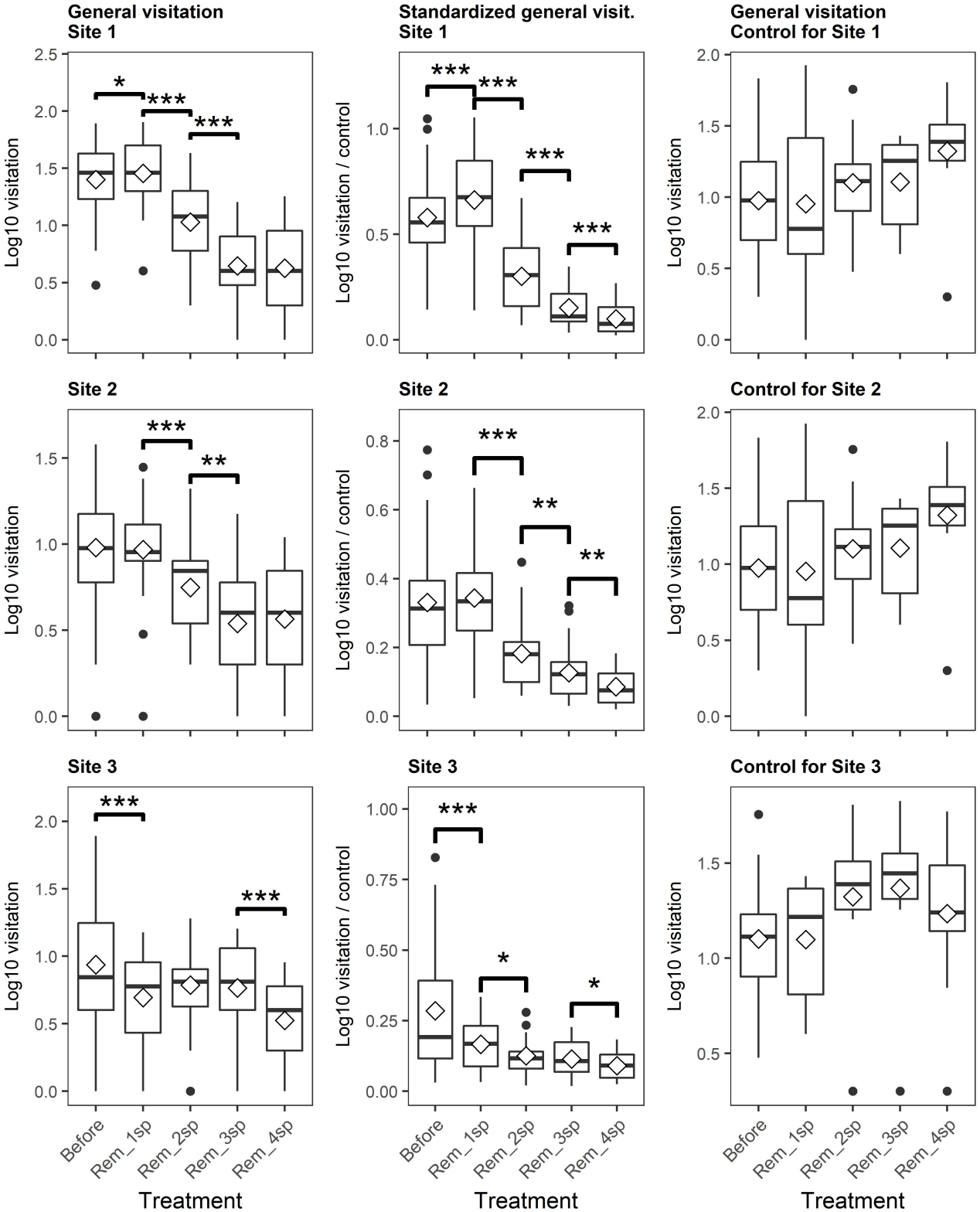
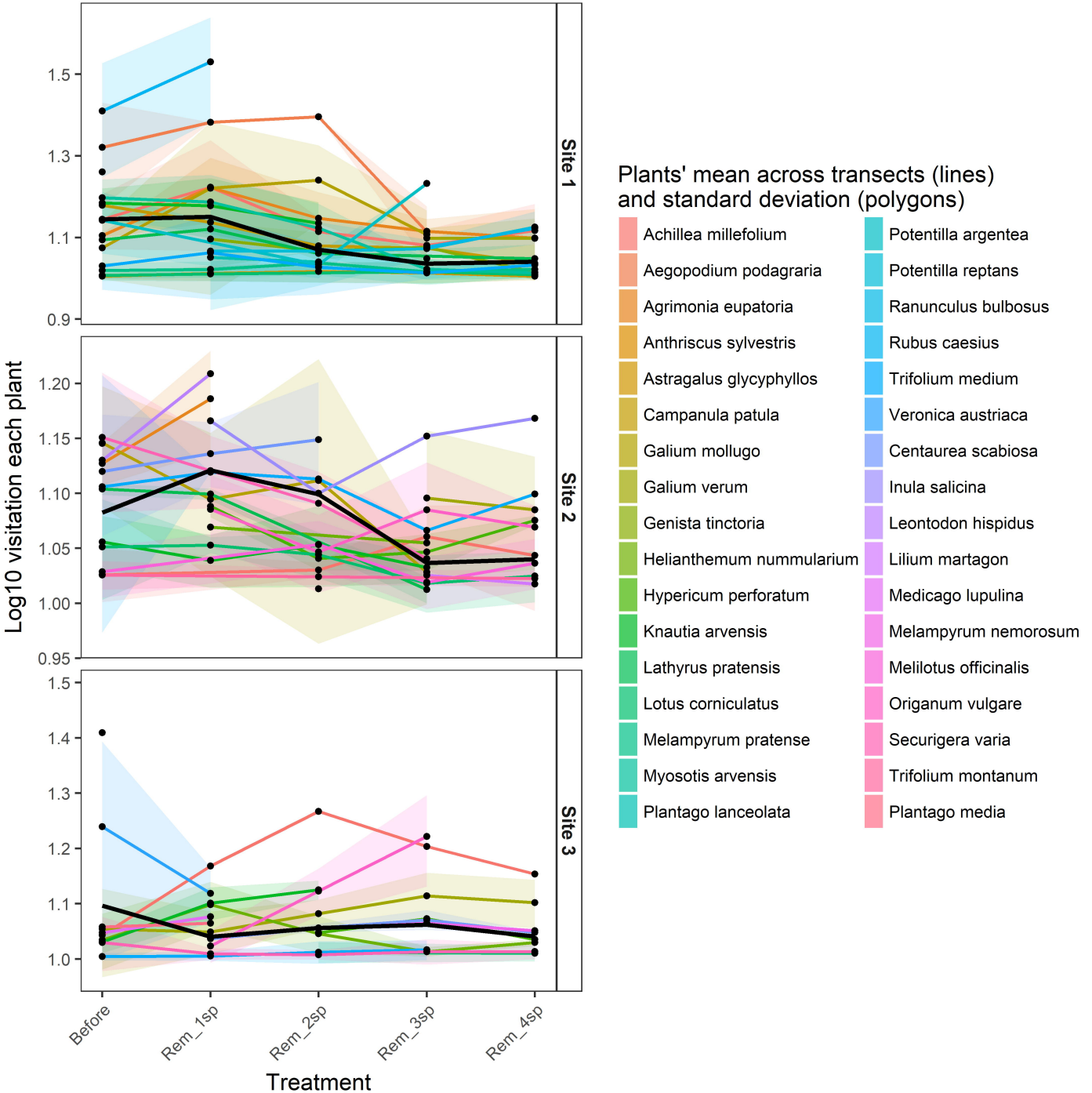
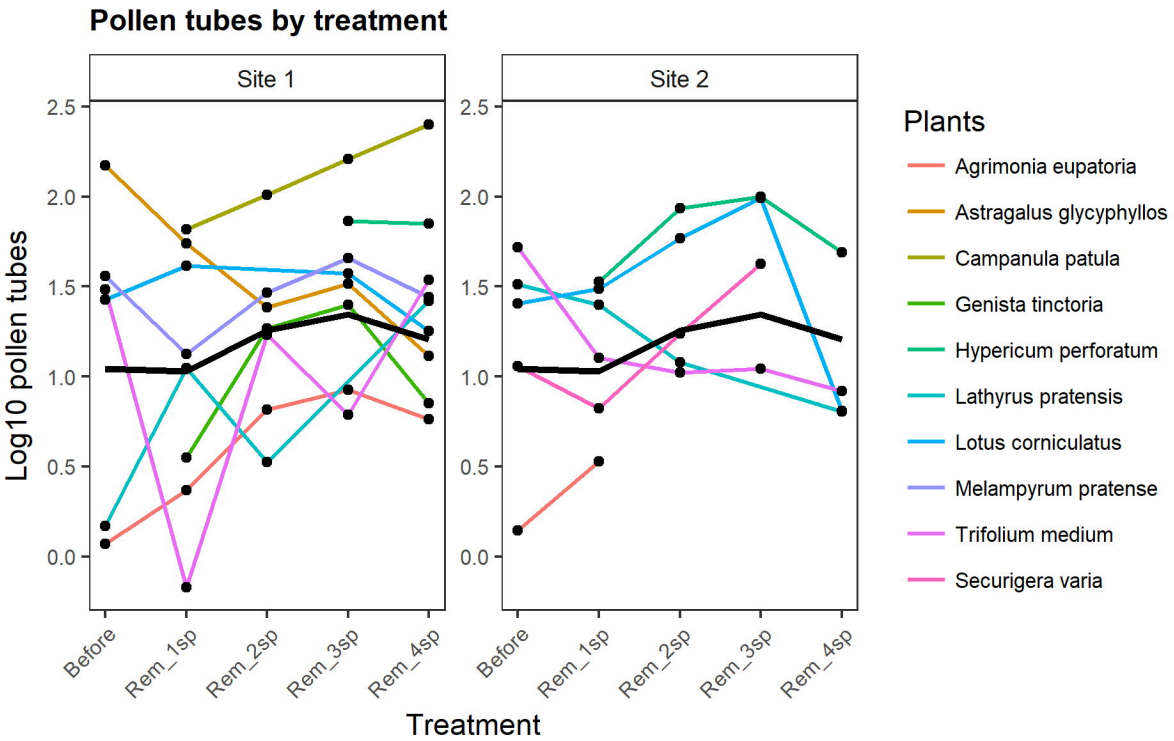
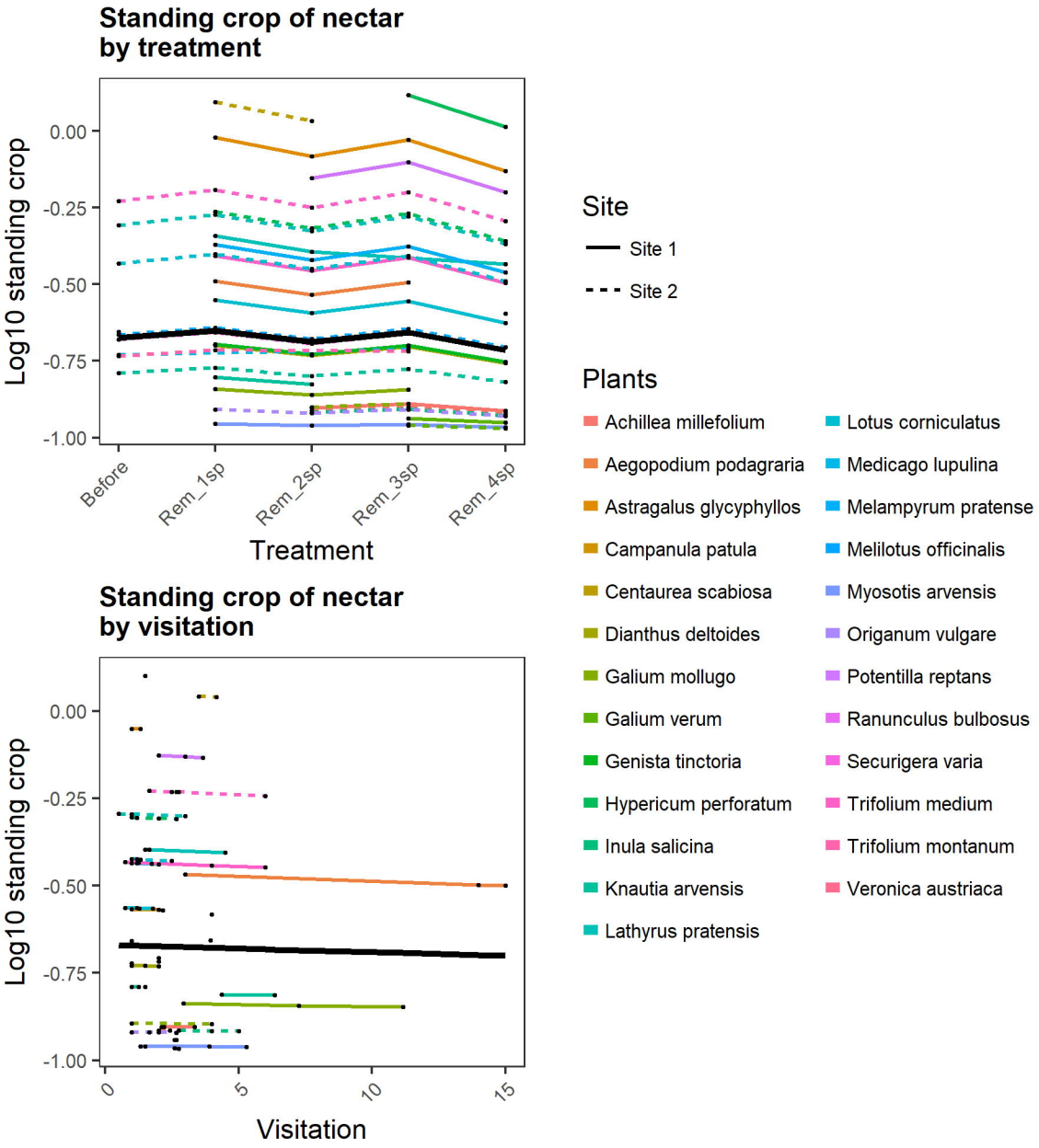
644

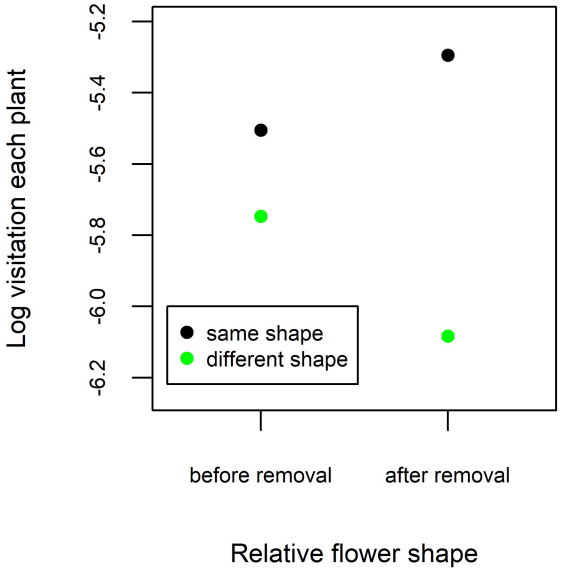

Pilot study visitation
removal site
**
Log10 visitation
1
0
-1
-2
Before
Removed
Treatment
Pilot study visitation
removal site
with offset
Log10 visitation with control as offset
1
0
-1
-2
Before
Removed
Treatment
Pilot study visitation
control site
Log10 visitation
1.0
0.5
0.0
-0.5
-1.0
Before
Removed
Treatment

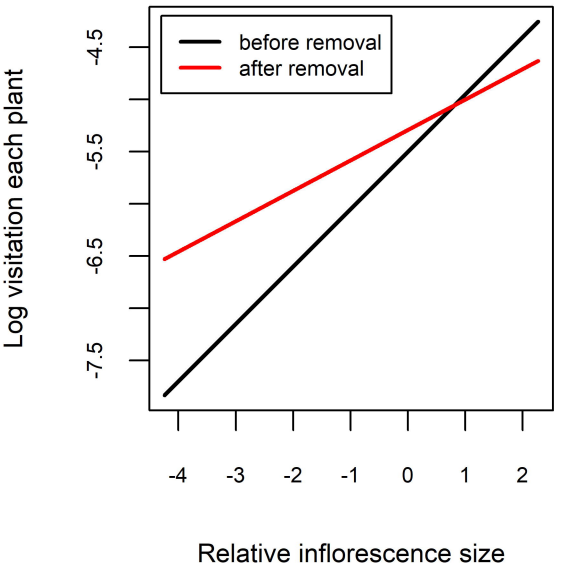

General visitation
Site 1
Standardized general visit.
Site 1
General visitation
Control for Site 1
Site 2
Site 2
Control for Site 2
Site 3
Site 3
Control for Site 3
Log10 visitation
Log10 visitation / control
Before
Rem_1sp
Rem_2sp
Rem_3sp
Rem_4sp
Treatment


Site 1
Site 2
Site 3
Log10 visitation each plant
Treatment
Before
Rem_1sp
Rem_2sp
Rem_3sp
Rem_4sp
Plants' mean across transects (lines) and standard deviation (polygons)
Achillea millefolium
Aegopodium podagraria
Agrimonia eupatoria
Anthriscus sylvestris
Astragalus glycyphyllos
Campanula patula
Galium mollugo
Galium verum
Genista tinctoria
Helianthemum nummularium
Hypericum perforatum
Knautia arvensis
Lathyrus pratensis
Lotus corniculatus
Melampyrum pratense
Myosotis arvensis
Plantago lanceolata
Potentilla argentea
Potentilla reptans
Ranunculus bulbosus
Rubus caesius
Trifolium medium
Veronica austriaca
Centaurea scabiosa
Inula salicina
Leontodon hispidus
Lilium martagon
Medicago lupulina
Melampyrum nemorosum
Melilotus officinalis
Origanum vulgare
Securigera varia
Trifolium montanum
Plantago media

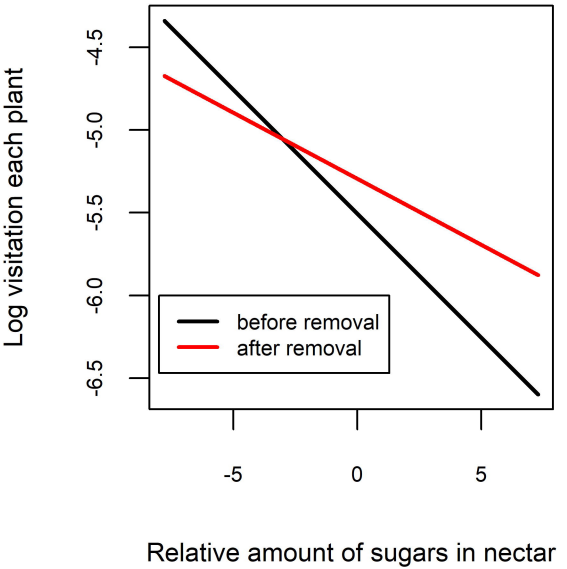

Pollen tubes by treatment
Site 1
Site 2
Log10 pollen tubes
2.5
2.0
1.5
1.0
0.5
0.0
Before
Rem_1sp
Rem_2sp
Rem_3sp
Rem_4sp
Treatment
Plants
Agrimonia eupatoria
Astragalus glycyphyllos
Campanula patula
Genista tinctoria
Hypericum perforatum
Lathyrus pratensis
Lotus corniculatus
Melampyrum pratense
Trifolium medium
Securigera varia


Standing crop of nectar
by treatment
Log10 standing crop
0.00
-0.25
-0.50
-0.75
-1.00
Before
Rem_1sp
Rem_2sp
Rem_3sp
Rem_4sp
Treatment
Standing crop of nectar
by visitation
Log10 standing crop
0.00
-0.25
-0.50
-0.75
-1.00
0
5
10
15
Visitation
Site
Site 1
Site 2
Plants
Achillea millefolium
Aegopodium podagraria
Astragalus glycyphyllos
Campanula patula
Centaurea scabiosa
Dianthus deltoides
Galium mollugo
Galium verum
Genista tinctoria
Hypericum perforatum
Inula salicina
Knautia arvensis
Lathyrus pratensis
Lotus corniculatus
Medicago lupulina
Melampyrum pratense
Melilotus officinalis
Myosotis arvensis
Origanum vulgare
Potentilla reptans
Ranunculus bulbosus
Securigera varia
Trifolium medium
Trifolium montanum
Veronica austriaca


Log visitation each plant
-5.2
-5.4
-5.6
-5.8
-6.0
-6.2
same shape
different shape
before removal
after removal
Relative flower shape


Log visitation each plant
-4.5
-5.5
-6.5
-7.5
before removal
after removal
-4
-3
-2
-1
0
1
2
Relative inflorescence size

Log visitation each plant
-2
-3
-4
-5
-6
before removal
after removal
-4
-3
-2
-1
0
1
Relative plant height


Log visitation each plant
-4.5
-5.0
-5.5
-6.0
-6.5
before removal
after removal
-5
0
5
Relative amount of sugars in nectar