Functional Ecology

An empirical attack tolerance test alters the structure and species richness of plant-pollinator networks

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Abstract

1. Ecological network theory hypothesizes that the structuring of species interactions can convey stability to the system. Investigating how these structures react to species loss is fundamental for understanding network disassembly or their robustness. However, this topic has mainly been studied *in-silico* so far.
2. Here, in an experimental manipulation, we sequentially removed four generalist plants from real plant-pollinator networks. We explored the effects on, and drivers of, species and interaction disappearance, network structure and interaction rewiring. Firstly, we compared both the local extinctions of species and interactions and the observed network indices with those expected from three co-extinction models. Secondly, we investigated the trends in network indices and rewiring rate after plant removal and the pollinator tendency at establishing novel links in relation to their proportional visitation to the removed plants. Furthermore, we explored the underlying drivers of network assembly with probability matrices based on ecological traits.
3. Our results indicate that the cumulative local extinctions of species and interactions increased faster with generalist plant loss than what was expected by co-extinction models, which predicted the survival or disappearance of many species incorrectly, and the observed network indices were lowly correlated to those predicted by co-extinction models. Furthermore, the real networks reacted in complex ways to plant removal. Firstly, network *nestedness* decreased and *modularity* increased. Secondly, although species abundance was a main assembly rule, opportunistic random interactions and structural unpredictability emerged as plants were removed. Both these reactions could indicate network instability and fragility. Other results showed network reorganization, as rewiring rate was high and asymmetries between network levels emerged as plants increased their centrality. Moreover, the generalist pollinators that had frequently visited both the plants targeted of removal and the non-target plants tended to establish novel links more than who either had only visited the removal plants or avoided to do so.
4. With the experimental manipulation of real networks, our study shows that despite their reorganizational ability, plant-pollinator networks changed towards a more fragile state when generalist plants are lost.

Keywords (8): adaptive foraging; assembly and disassembly of network; community stability; ecosystem services; network reorganization; pollination; restoration; species coextinction.

Introduction

Interactions are organized in complex networks, and the way these structures react to disturbance is crucial for understanding network functioning, their ability to buffer negative impacts and also for their conservation (Albert, Jeong, & Barabasi, 2000; Brodie, Redford, & Doak, 2018; Reis et al., 2014; Strogatz, 2001). This is usually verified with “attack tolerance tests” that assess the functionality of a system after knocking out its important components (Burgos et al., 2007). In ecology, such tests usually consist of removing all species in one trophic level and then in assessing how many species in another level lost all interactions (Dunne, Williams, & Martinez, 2002; Memmott, Waser, & Price, 2004). So far, in pollination networks, this has been addressed mainly theoretically with numerical simulations that show a higher rate of pollinator extinction when highly linked plants are removed (Fortuna, Krishna, & Bascompte, 2013; Kaiser-Bunbury et al., 2017; Memmott et al., 2004; Vieira & Almeida-Neto, 2015). However, these theoretical predictions were not compared to empirical data from similar manipulations, which is urgently needed to assess their reliability (Curtsdotter et al., 2011).

Manipulative experiments of plant-pollinator networks can illuminate the factors maintaining network stability and the processes of network re-organization (e.g., Brosi & Briggs, 2013). For instance, previous experiments removing only one generalist plant (Ferrero et al., 2013; Goldstein & Zych, 2016) showed that networks are quite stable to this loss, and that other species occupy the role in the network of the removed species. Conversely, when multiple invasive plants are removed, network interaction diversity and generalisation are impacted (Kaiser-Bunbury et al., 2017), indicating that losing multiple species can strongly affect real networks. Moreover, after disturbance, network stability could depend on the amount of interaction rewiring (Kondoh, 2003), i.e. foragers’ ability to use alternative resources after depletion or disappearance of those previously used (Biella, Tommasi, et al., 2019; CaraDonna et al., 2017; Valdovinos, Moisset de Espanés, Flores, & Ramos-Jiliberto, 2013). Rewiring and the establishment of interactions between plants and pollinators may be regulated by several ecological drivers, such as species trait matching (Stang, Klinkhamer, Waser, Stang, & Meijden, 2009; Watts, Dormann, Martín González, & Ollerton, 2016), flower's rewards (Junker et al., 2013; Klumpers, Stang, & Klinkhamer, 2019) or species abundances (Ollerton, Johnson, Cranmer, & Kellie, 2003; Vázquez, Chacoff, & Cagnolo, 2009). Similarly, it was shown that, after altering the plant community, the redistribution of pollinators is constrained by plant traits (Biella, Akter, et al., 2019). Nevertheless, opportunism can prevail over strict interaction rules if foragers, to avoid competition, exploit less rewarding resources (Ponisio, Gaiarsa, & Kremen, 2017). Still, it is unknown how the above-mentioned or similar ecological drivers would rule a perturbed plant-pollinator network.

In this study, we conducted a field experiment in which we sequentially removed several generalist plant species from real networks and investigated the impact on pollinators, their interactions and network structure. We present two alternative expectations that link pollinator foraging strategy and network structure. After plant removal, if foragers will predominantly increase their use of alternative resources (i.e., high rewiring), then network compartmentalization (modularity) will likely decrease, because new interactions might happen with different kinds of resources (i.e., across different compartments)(Thébault & Fontaine, 2010). The other expectation is based on the central position that generalist plants cover in the networks, i.e. hubs (Biella, Ollerton, Barcella, & Assini, 2017). The loss of central nodes, that maintain network cohesiveness and links different modules would break a network down to isolated subnetworks or compartments following generalist plant removal (Reis et al., 2014).

Here, we investigated (a) if the rate of species and interactions disappearance from our systems and the network indices are similar to those simulated by established co-extinction models; (b) alterations in the structure of plant-pollinator networks and the rate of interaction rewiring that emerges during the plant removal; and (c) what ecological factors mediate these changes.

Materials and methods

The study included three treatment sites and one control site, located at a mean distance of 2.01±0.95 km from each other, near Český Krumlov, in the Czech Republic (treatments: Site 1 ca 1500 m^2 in size, 48°49'26.8"N-14°16’26.2"E; Site 2, ca 1800 m^2, 48°49'51.63"N-14°17'34.12"E; Site 3, ca 1600 m^2, 48°49'35.07"N-14°18'8.2"E; untreated control: 48°49'26.8"N-14°16'26.2"E). Each site was a small grassland with a barrier of trees to likely limit pollinator movements to the surrounding landscape. Due to the high mobility of pollinators, we deemed that an experimental design based on small within-site treatment plots would not be appropriate as plots would not be independent (e.g. McKinlay, 1953). The experiment consisted of sequentially removing, by clipping, all inflorescences of the most generalist plant species from the entire surface of the treatment sites, one species at a time until four species were removed, while the untreated control site was sampled synchronously to the sampling of each treated site (see Supporting information Table S1 for a list of the removed species and their relative floral abundance). Before and after each species was removed, we sampled flower-visiting insects in six 10m x 1m transects per site during two days for each experimental phase (but the sampling was postponed in the case of rain or strong wind; in total, the experiment took about two weeks); the transects were walked very slowly in a randomized order between 9:00 and 17:00 hours and usually twice a day and the sampling effort in term of number of transect walks and time spent on each transect was even during the experiment (see Appendix S1 for sampling completeness estimation). While walking the transects, all insects visiting flowers were sampled by a hand net or a mouth aspirator. After each “before” phase, flower-visitors were counted and this was used as a proxy of generalization to determine which plant species should be removed next; this proxy was reliable and in fact we later verified that these plants were visited by the most diverse set of pollinators, similarly to (Biella, Akter, et al., 2019; Goldstein & Zych, 2016). We identified all insects to species where possible, otherwise morpho-species were used when necessary (after pre-sorting into families and genera). In addition, we counted the number of flowers or inflorescences of all plant species within transects over the sampling period.

Species co-extinctions

We compared the number of pollinator and of interaction disappearances from the sampled sites after the removal of each generalist plant to what was expected from two co-extinction models without network rewiring and a co-extinction model allowing species rewiring: these were the Topological co-extinction model (“TCM”, (Memmott et al., 2004), the Stochastic co-extinction model (“SCM”, (Vieira & Almeida-Neto, 2015) and a model allowing rewiring by (Vizentin-Bugoni, Debastiani, Bastazini, Maruyama, & Sperry, 2019) that we name “REW” here for practical reasons. The TCM model assumes that a species is extinct when all its interacting partners disappear from the network, and this model is based on the presence-absence of links between species but it does not consider the interaction strength between them. The SCM model is not based on the assumption that coextinctions require the loss of all partners, but species disappearance from the network is derived from an extinction probability considering interaction strength between partners and a parameter of interaction dependency (R), and it allows cascading extinction chains. The REW model with rewiring estimates species extinctions while allowing them to create new links after the disappearance of a partner, in where the rewiring probability depends on several possible factors, for example species abundances, matching of morphological traits, or phenological overlap. In our study, separately for each plant removal stage of the treatment sites, these co-extinction models were triggered by removing the same generalist plant species as the field manipulations, and the number of species without interactions left were counted as local extinctions. In the SCMs, we ran 10^3 simulations, and, following (Dalsgaard et al., 2018), we assigned random values to the parameter R of plants and pollinators as we did not have information on the real values of species interaction dependency. In the REW rewiring model we ran 10^3 simulations, we assigned random probability values of receiving new links in order to avoid adding additional assumptions and allowed the option of one rewiring attempt with a single partner as in (Vizentin-Bugoni et al., 2019).

We counted local extinctions as the number of pollinators or of interactions recorded before a plant removal that were lost after a plant removal, for both the observed networks and the model predictions. To avoid overestimations, in the observed networks we considered (i) as disappeared species, the pollinators interacting with the plant targeted by removal that were not recorded afterwards, and (ii) as lost interactions, the difference in the amount of interactions after excluding the species unique to the after phases. In addition, all singletons (i.e. species with interaction abundance of 1) were removed from the observed networks and also from the simulations, to avoid overestimations due to species with extremely small populations and sampling stochasticity (Kantsa, Raguso, Lekkas, Kalantzi, & Petanidou, 2019). We tested the trends in the cumulative extinctions of species or of interactions during the sequential removal as proportions of the total pollinator richness or of the total interaction quantity with generalized mixed models in the *glmmTMB* package (Bates, Mächler, Bolker, & Walker, 2015) in R (R Core Team, 2017). The number of pollinator extinctions (or of interactions) was the response variable, the number of removed plant species was included as a numerical predictor and the observed/TCM/SCM/REW was a categorical one, the total number of pollinators (or of interactions) was an Offset term as indicated by (Reitan & Nielsen, 2016); site identity was used as a random intercept.

In addition, we also recorded the amount of species "extinctions" predicted by the models that were true positives (predicted extinctions which happened in the observed networks), false positives (predicted extinctions which did not happened), true negatives (extinctions not predicted which did not happen in the observed networks) or false negatives (extinctions not predicted which did happen in reality) with both TCM and each SCM simulation at each plant removal stage.

Networks indices and rewiring

We assembled interaction matrices for each stage of the experiment in all sites and calculated several network-level indices that describe different aspects of species interactions (Bennett, Evans, & Powell, 2019): the binary *connectance*, indicates the proportion of realised links in relation to all possible links (range 0-1); the weighted *nestedness NODF* (Nestedness based on Overlap and Decreasing Fill) quantifies the tendency of generalist species to interact with both other generalists and with specialists and ranges 0-100 (the maximum is for fully nested networks); the weighted *modularity* measures the interactions partitioning into groups, it was computed by the algorithm DIRTLPAwb+ and ranges 0-1 (the maximum is for full compartmentalization); the weighted H_2' measures specialization considering the diversity of interactions based on Shannon entropy and ranges 0-1 (the maximum is for perfect specialisation). The following species-level indices were also calculated: the weighted *connectivity* and *participation*, which express the ability of a species to connect partners of different modules (*connectivity*) or to interact with species of the same module (*participation*). All these indices were calculated with the *rnetcarto* and *bipartite* packages for R (Dormann, Gruber, & Fründ, 2008; Doulcier & Stouffer, 2015). In addition, an index of network robustness that we name *Stochastic robustness* was calculated as the area under a curve drawn from the rate of pollinators surviving a sequential removal of all plants from the most generalist to the most specialist as simulated by 10^3 SCM; this was drawn as a mean number across simulations of pollinator considered as disappeared and was calculated separately for each experimental plant removal phase using the *robustness* function of the *bipartite* R package.

We have also compared the observed network indices and the network indices calculated from the networks simulated by each of the co-extinction models detailed above (i.e., TCM, SCM, REW co-extinction models). For each network index, a correlation test was used to verify the similarity between the real and the predicted networks (using Kendall correlation tests).

We quantified the turnover of interactions across the removal stages using the approach developed in Poisot, Canard, Mouquet, & Hochberg (2012). This method quantifies the total interaction turnover as $\beta_{WN} = \beta_{ST} + \beta_{OS}$ and partitions it into species turnover (i.e., β_{ST}, the interaction diversity in the pool of species that are not shared between two networks) and interactions rewiring (i.e. β_{OS}, switching of interacting partners in species occurring in both networks). These were calculated for all sites and consecutive stages of the experimental removal (before - 1 sp. removed, 1 sp. removed – 2 spp. removed, and so forth) with Whittaker's beta-diversity index and its components extracted with the package *betalink* (Poisot, 2016). Values for these indices range from 0 to 1; higher values indicate higher turnover or rewiring. Two types of interaction matrix were used for the turnover analyses; one uses binary matrices and focuses on the number of interaction links per species. In addition, to account for the frequency of interactions, we also employed a quantitative version of beta-diversity that is calculated as above but in which the sum of interaction frequency per species is used instead of the number of links.

The effects of plant species removal on network indices and on beta-diversity components were tested with generalized linear mixed models (GLMM) with the *glmmTMB* package in the R environment (Bates et al., 2015); a given index was the response, the site identity was a random intercept, and Beta or Gaussian distributions were used depending on the response variable. For the beta-diversity components, pairs of successive removal stages were used as categorical predictor variables. For the network indices, the number of removed plants was used as numerical predictor. As in (Olesen & Jordano, 2002), network size (the number of animal species * the number of plant species in the matrix) and the number of network interactions (the quantitative matrix sum) were included in the models in order to account for their effects on index variation over the experiment. We favoured this approach rather than the delta- or z-transformations because those can cause biases (Chagnon, 2015) and they are more useful for testing departures from a random expectation (Biella et al., 2017), while we aimed at testing the effect of a treatment in causing specific trends (i.e. increase or decrease of an index). To compare the trend of a given index with that of the control, the values from the control site during the experiment were included as an Offset term in the GLMM. For *connectivity* and *participation* indices, plants and pollinators were analysed separately in GLMMs with a given index as a response variable, the number of removed plant species as numerical predictor and species identity within site as the random intercept. Here, it was not possible to include the control site for direct comparison because not all species were shared with the removal sites.

Furthermore, we described pollinators tendency to establish new links after plant removal in relation to the visitation to the plant species to be removed, as follows. Firstly, we considered network pairs of before-after each plant removal, thus likewise the rewiring analysis above. Secondly, we obtained the tendency at visiting alternative resources after a perturbation, and we calculated the proportion of new links being established by pollinators as the number of plant species that were visited only in after removal and divided it by the total number of plants being visited during the given before-after phases. Thirdly, we calculated how pollinator interaction strength is distributed among plants, and for each pollinator species we obtained the proportional visitation to the plant targeted of removal as the number of pollinator specimens recorded visiting flowers of the plant to be removed (i.e. “visitation”), divided by the total visitation recorded on the entire plant assemblage during a given before-after phase. The relationship between the two variables was analysed with generalized additive mixed model to account for nonlinear patterns, with the proportion of new links as a response variable, the proportional visitation as a predictor variable, site and removed plant identities nested within site as random slopes, and quasibinomial distribution accounting for proportional data, with the *mgcv* package for R (Wood, 2004).

Drivers of interactions

For each site and for each plant removal stage, several simulation models were constructed from different probability matrices to explore the factors driving the observed interactions and indices. The following factors were chosen based on previous evidences of their importance in influencing plant-pollinator interactions (Olito & Fox, 2015; Vázquez et al., 2009; Vizentin-Bugoni, Maruyama, & Sazima, 2014). The matrices used for the models were: “NULL” explores the possible effect of randomness and all species have the same probability of interactions (=1); “ABUNDANCES” investigates the role of species abundances of either or both plants and pollinators in determining interaction establishment and the matrix is filled with either the number of flowers of a plant (“PLANTS”), or the abundance of the pollinator species calculated as total amount of flower visitors of a given pollinator species over the entire study period (“POLLINATORS”), or the element-wise multiplication of these two (“ABUNDANCES”); (3) “MORPHOLOGY” assumes that interactions happen when traits match and the matrix is filled with 1 only when a morphological match between the length of insect mouthparts and a flower’s nectar allocation depth occurs (Stang et al., 2009). Firstly, as in (Olito & Fox, 2015), insect mouthparts were measured with a calliper and then categorized as having a long tongue (>9 mm), intermediate tongue (4-9 mm) or short (<4 mm), and plants were categorized based on flower structure, such as for having nectar hidden in flower structures (e.g. larger Fabaceae and flowers with tubular corolla), semi hidden nectaries (more open tubes, smaller Fabaceae) and accessible nectaries (very short tubes or open flowers). After this, the matching was determined between the analogous categories, such as an insect’s “long-mouthparts” with a flower’s “hidden-nectaries”, “intermediate mouthparts” with “semi-hidden nectaries” and “short-mouthparts” with “accessible nectaries”. (4) “SUGAR” assumes that the probability of interaction is proportional to amount of sugar per flower in the nectar and the matrix is filled with the amount of sugar/flowers per plant species (Junker et al., 2013); these data were obtained from (Biella, Akter, et al., 2019) that includes values of nectar sugars from flowers bagged for 24h, using a100 µl Hamilton capillary syringe for washing the nectar into distilled water and a high performance anion exchange chromatography for sugars quantification; The total amount of sugar was afterwards divided by the number of washed flowers per each species (an average of 45 flowers per plant species).

For each matrix, probabilities were obtained by dividing the cells of the matrices by the matrix sum. In addition, while the matrices above describe the contribution of single factors, the interactions of these drivers were included by building models based on multiplying two or three of the matrices described above, as in (Vázquez et al., 2009; Vizentin-Bugoni et al., 2014), specifically: *ABUNDANCES x MORPHOLOGY*, *ABUNDANCES x SUGAR*, *MORPHOLOGY x SUGAR*, and *ABUNDANCES x MORPHOLOGY x SUGAR*. We ran 10^3 simulated networks with the *mgen* function of the *bipartite* R package that distributes the interaction quantities of the real networks according to the probabilities of the model matrix, that means we kept both the network size and interaction strength as those of each real network in the simulations. For each simulated network, network indices and beta-diversity components were calculated as for the real networks (see above). A given driver is considered as consistent with the empirical observations when its 95% confidence interval includes the real network index (Vázquez et al., 2009).

To investigate which of the above drivers provided the best fit in terms of predicting the occurrence and frequency of the species pairwise interactions in the observed networks, we used a likelihood approach. Following (Vázquez et al., 2009), a multinomial distribution was calculated from the interaction frequencies of the observed network and from a given probability matrix. Then, the delta of the Akaike information criteria (ΔAIC) was used to evaluate the ability of each probability model to predict the likelihood of pairwise interactions. As in (Vizentin-Bugoni et al., 2014), in the AIC calculation, the number of parameters was set as the number of species in each probability matrix multiplied by the number of matrices used in order to weight each model's complexity.

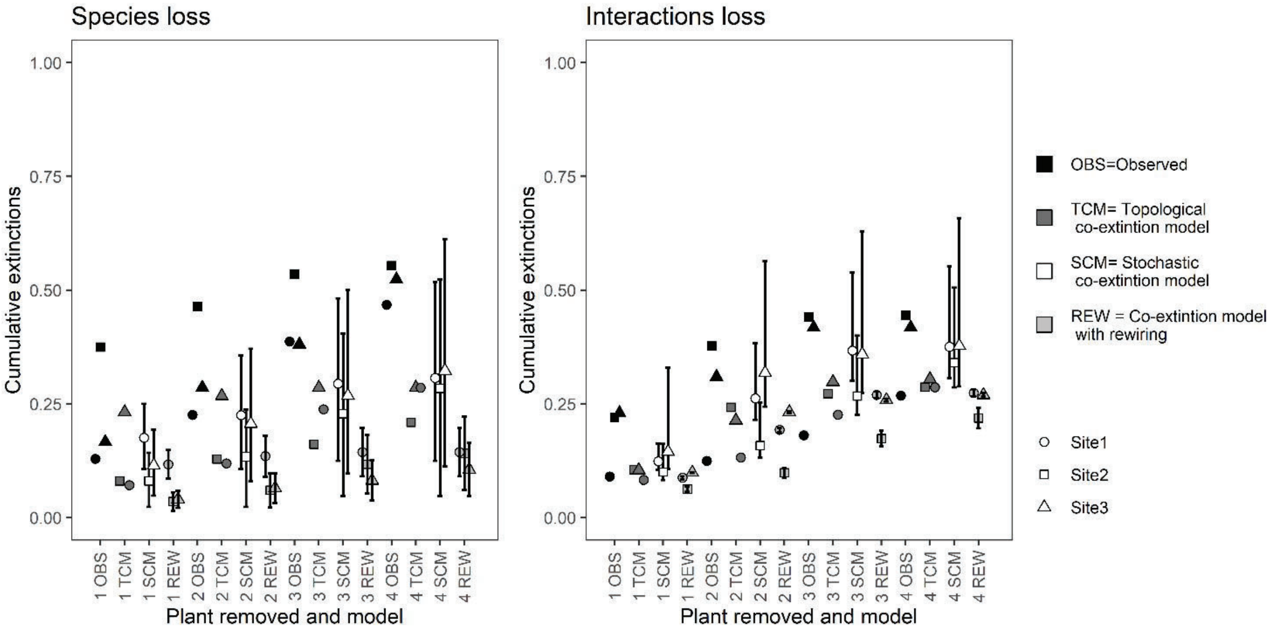
Results

The plant-flower visitor networks of the experimental sites were similar in species richness (plants 28, pollinators 157 in Site1; plants=24, pollinators=171 in Site2; plants=20, pollinators=106 in Site3).

Species co-extinctions

The cumulative proportion of observed and predicted local extinctions increased linearly with the number of removed plants for both species and interactions ($\beta_{species}$= 0.158, likelihood ratio test $\chi^2_{plant\ removal}$= 176.356, df=1, $p<0.001$; $\beta_{interactions}$= 0.178, likelihood ratio test $\chi^2_{plant\ removal}$= 3838.7, df=1, $p<0.001$, Fig. 1). The observed networks (OBS) registered more species extinctions than the TCM, the SCM and REW models ($\beta_{OBS\text{-}TCM}$=1.03, $\beta_{OBS\text{-}SCM}$=0.958, $\beta_{OBS\text{-}REW}$=1.278, likelihood ratio test $\chi^2_{OBS/TCM/SCM/REW}$=110.14, df=3, $p<0.001$). Similarly, the observed networks lost more interactions than what was predicted by the two models ($\beta_{OBS\text{-}TCM}$= 0.906, $\beta_{OBS\text{-}SCM}$= 0.713, $\beta_{OBS\text{-}REW}$=0.956, likelihood ratio test $\chi^2_{observed/TCM/SCM}$= 612.7, df=3, $p<0.001$).

Fig. 1. Cumulative proportion of extinctions of species and of interactions over the sequential plant removal as observed in the real networks ("OBS") and as predicted by TCM, SCM and REW co-extinction models for each site. In SCM and REW, the symbols and lines indicate the mean and 5%-95% quantiles of 10^3 simulations. Statistical tests are presented in the Results.



Among pollinator species which went extinct in the field experiment, on average 85.33% (range across sites and plant removal stages: 33 – 100%) were species which were predicted to go extinct by the TCM (true positives), while the rest were species which the model incorrectly predicted to survive (false positives). SCM provided worse predictions of extinctions of individual species with the mean of 26.62 % true positives (range: 10 – 37.7 %). On the other hand, among species which survived in the field experiment, on average 33.66% were species predicted to survive based on TCM (range 15 – 50 %) and 41.29 % based on SCM (range 10.88 – 85.53 %) (true negatives), the rest were extinctions observed in the field, but not predicted by the models (false negatives).

Network structure and rewiring during plant removal

Network *modularity* and *specialization* significantly increased with the number of removed plants, while *nestedness* decreased significantly, in the treated sites (Table 1, Fig. 2 and Fig. S1). When the values from the control site were used as an offset, the statistical significance of the increase in *modularity* and decrease in *nestedness* was confirmed, while, however, the significance of *specialization* was not confirmed (Table 1). The trends of other network indices were not significant during the sequential plant removal. In the species-level indices, plants and pollinators responded differently (Table 1, Fig. 2 and Fig. S1). Only the plant *connectivity* increased significantly, while plant *participation* and the pollinator indices were nearly constant during the sequential plant removal. The interaction turnover was high in both quantitative and binary versions (Fig. 3), with a larger proportion attributable to rewiring than to species turnover; however, no statistically significant trend was found in these indices in response to the treatment (Table 1).

The proportion of new established links by pollinators after plant removal varied in a significant and nonlinear way in relation to the proportion of visitation on the plant to be removed (F= 4.46, $p<0.05$, Fig. 4). In particular, the lowest proportion of new links being established tended to occur in pollinators that either had visited exclusively the plant species to be removed or those pollinators who had avoided visiting the plant to be removed (that are the highest and the lowest values of proportional visitation to the plant to remove, respectively); conversely, the highest amount of new links being established after plant removal tended to occur in the set of pollinators with intermediate levels of proportional visitation to the plant removed.

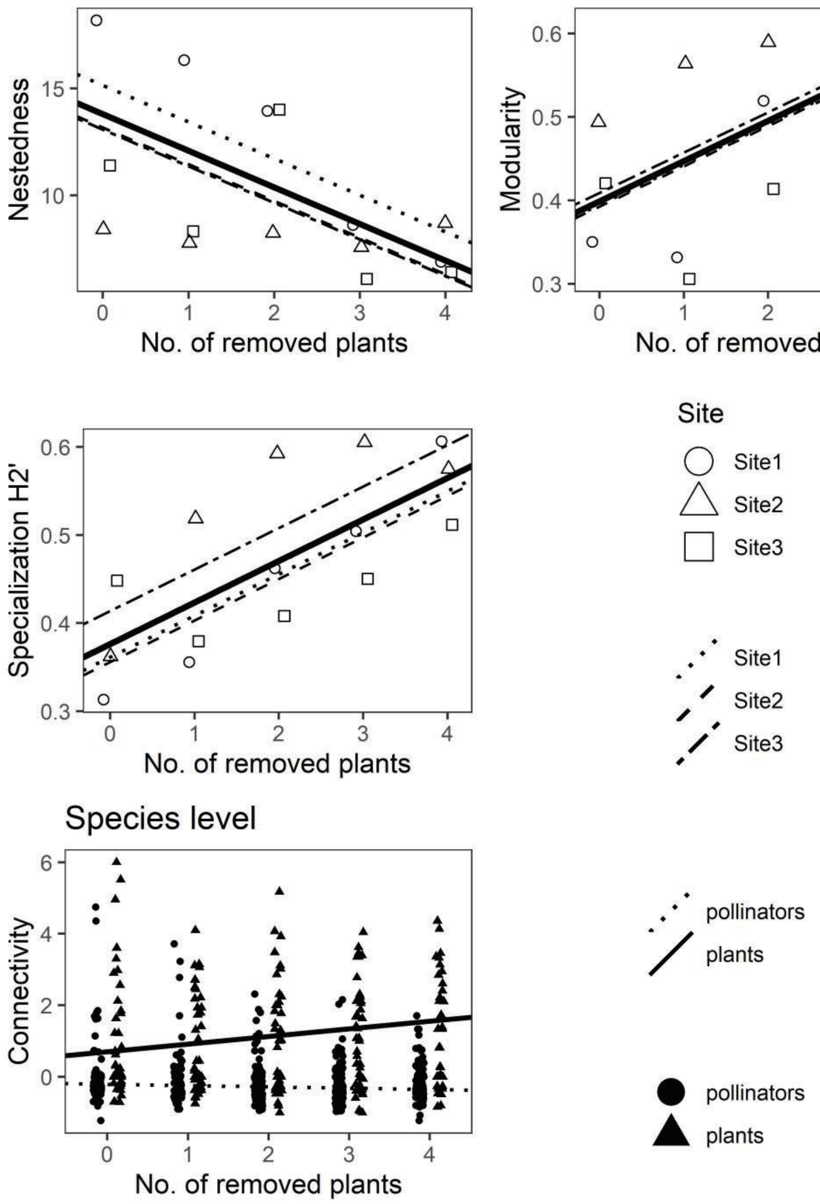
The network indices predicted by the TCM co-extinction model were usually lowly although significantly correlated with the observed network indices, while the indices obtained from SCM and REW co-extinction models were not significantly correlated (see Fig. S2). In detail, for *connectance* $\tau_{\mathrm{OBS,TCM}} = 0.17$ p= 0.45, $\tau_{\mathrm{OBS,SCM}} = 0.18$ p= 0.45, $\tau_{\mathrm{OBS,REW}} = 0.18$ p= 0.45; For *nestedness* $\tau_{\mathrm{OBS,TCM}} = 0.45$ p= 0.04, $\tau_{\mathrm{OBS,SCM}} = 0.24$ p= 0.31, $\tau_{\mathrm{OBS,REW}} = 0.27$ p= 0.25; For *modularity* $\tau_{\mathrm{OBS,TCM}} = 0.75$ p< 0.001, $\tau_{\mathrm{OBS,SCM}} = 0.36$ p= 0.11, $\tau_{\mathrm{OBS,REW}} = 0.33$ p= 0.15; For specialization *H2'* $\tau_{\mathrm{OBS,TCM}} = 0.51$ p= 0.02, $\tau_{\mathrm{OBS,SCM}} = 0.36$ p= 0.11, $\tau_{\mathrm{OBS,REW}} = 0.39$ p= 0.08.

Table 1. - Statistics of changes in the network indices and *Beta* diversity components in response to plant removal. Each row is a separate generalized mixed effect model (see Material and Methods for further details). *ΔAIC* is calculated as $AIC_i - AIC_{min}$. Statistically significant predictors (P<0.05) are highlighted in bold. Significance of the models including the values of the indices in the control site as an offset are also given.

	Df	ΔAIC	χ^2	P	P with control offset
Connectance	1	1.274	0.726	0.394	0.719
Nestedness	1	6.032	8.032	**0.005**	**0.001**
Modularity	1	7.246	9.246	**0.002**	**0.007**
Specialization H2’	1	11.076	13.076	**<0.001**	0.073
Stochastic robustness	1	1.819	3.819	0.051	0.350
Connectivity plants	1	10.439	12.439	**<0.001**	NA
Participation plants	1	1.857	0.143	0.705	NA
Connectivity pollinators	1	5.285	7.285	**0.007**	NA
Participation pollinators	1	1.509	0.491	0.484	NA
β diversity (binary)	3	0.604	5.396	0.145	0.110
rewiring (binary)	3	3.793	2.207	0.531	0.525

turnover (binary)	3	2.262	3.738	0.291	0.323
β diversity (quantitative)	3	4.170	1.830	0.608	0.688
rewiring (quantitative)	3	2.581	3.419	0.331	0.698
turnover (quantitative)	3	1.400	4.600	0.204	0.890

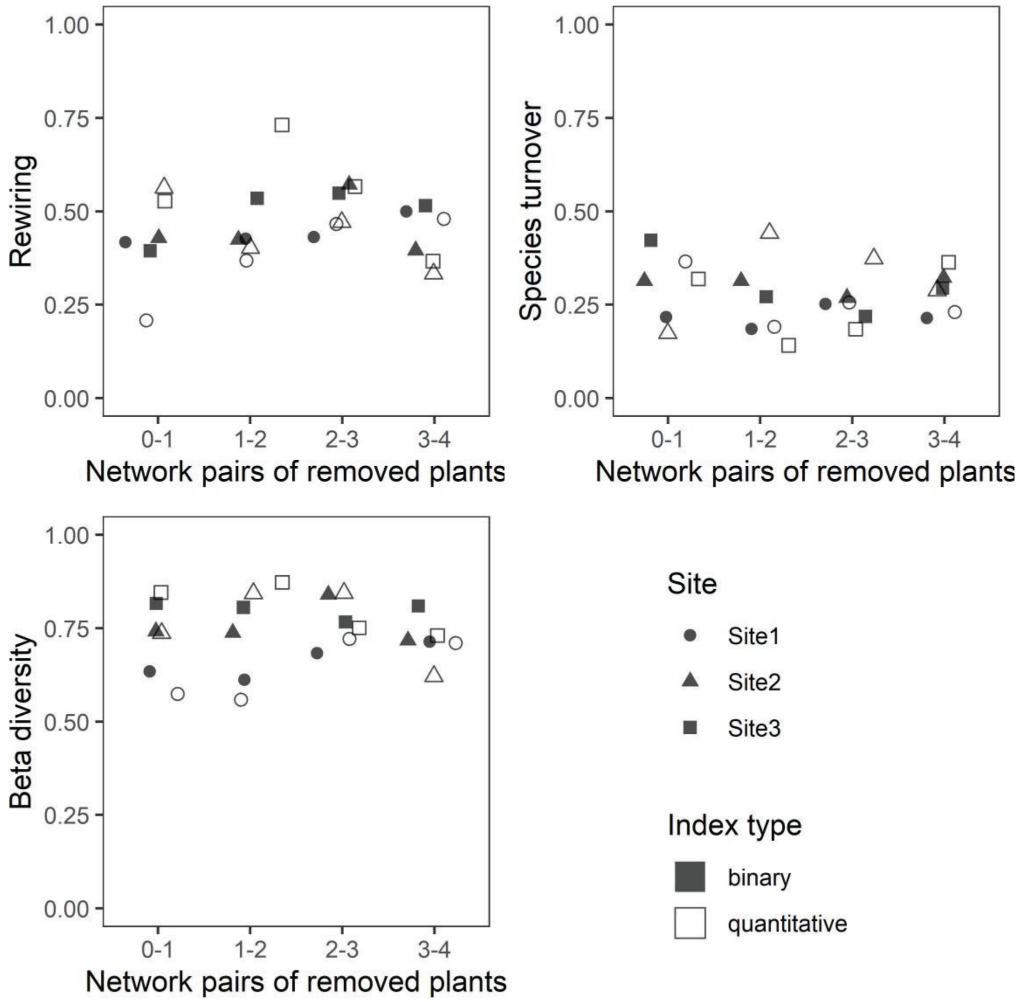
Fig. 2. Responses of network- and species- level indices to the removal of generalist plants. The significances of predictors are expressed in Table 1. The solid line is the average trend significantly predicted by the models. See Fig. S1 in Supporting Information for other indices used in this study that resulted as not significant (i.e. *connectance*, *stochastic robustness*, *participation*).



Drivers of network structure and rewiring

In the likelihood analysis (Table 2), the models based on species abundance usually provided the best fit to the observed species interactions, especially in the case of the pollinator abundances model; the model assuming equal probability of all interactions (NULL) predicted the observed interactions as the plant removal progressed; the model based on the amount of sugars in nectar also contributed to describing the interactions (i.e. it had low ΔAIC values).

Fig. 3. Fig. 3. Total Beta diversity and its components of species rewiring and turnover between network pairs after each stage of plant removal. Both the binary indices and their quantitative counterparts are plotted. Significances of predictors are included in Table 1.



In the networks and *Beta* diversity components none of the models generated confidence intervals including every observed index (Fig. 5, and Fig. S3, S4, S5, S6). The *connectance* and *specialization H2'* were particularly poorly predicted. Remarkably, the model based on plant abundances, the one based on pollinator abundances and the multiplication of the models based on abundances with other models were explaining the observed indices in several cases (e.g. *nestedness, modularity, rewiring* and *species turnover*). Remarkably, the model based on morphological matching and sugar resources predicted *nestedness* in most cases. In addition, the predictors usually changed as the removal of plants progressed, such as the model based on morphological matching and sugar resources that predicted both *rewiring* and *species turnover* only after the first removal events, while only before plant removal the model based on morphological matching predicted *modularity* and the model based on morphological matching and abundances predicted *specialization H2'*. In some cases, the complexity of the models (i.e. from the multiplication of several probability matrices) increased the predicting power (*nestedness, modularity* and *rewiring*) as the removal progressed.

Table 2 – Models' likelihood of pairwise species interactions drivers (*ΔAIC*). In bold, the probability models that best predicted the interactions are highlighted; the second important probability models are underlined. The models (in columns) are described in the Material and Methods.

Site	Species removed	NULL	PLANT ABUNDANCES	POLLINATOR ABUNDANCES	ABUNDANCES (plant and pollinators)	MORPHOLOGY	SUGAR	ABUNDANCE and SUGAR	ABUNDANCE and MORPHOLOGY	SUGAR and MORPHOLOGY	ABUNDANCE and MORPHOLOGY and SUGAR
Site1	0 spp.	4812.74	5218.36	**0.00**	5580.98	6752.64	707.81	7539.28	184.16	3004.32	2281.85
Site2	0 spp.	1539.41	1664.42	**0.00**	3079.29	2698.48	322.79	4176.45	1214.89	3052.62	3906.86
Site3	0 spp.	62.43	35.79	**0.00**	83.98	307.85	91.98	300.99	263.12	292.37	450.94
Site1	1 spp.	2304.65	2376.03	**0.00**	2564.01	3131.56	253.93	3597.58	188.34	1196.39	1072.54
Site2	1 spp.	817.87	1108.17	**0.00**	1525.95	1833.28	416.13	2614.27	1427.68	1349.81	2280.14
Site3	1 spp.	57.07	127.65	**0.00**	250.72	357.14	156.32	537.18	373.98	455.47	664.68
Site1	2 spp.	346.69	305.53	**0.00**	1017.21	2078.38	95.36	2709.90	1094.55	1165.87	1823.93
Site2	2 spp.	326.20	361.91	**0.00**	1341.76	422.59	167.22	1578.92	223.22	1616.49	1704.16
Site3	2 spp.	61.12	92.82	**0.00**	562.00	505.00	147.55	1036.19	491.58	774.95	1027.15
Site1	3 spp.	**0.00**	230.70	219.08	143.55	935.75	532.68	1187.56	1068.96	825.74	1062.64
Site2	3 spp.	**0.00**	163.23	123.26	387.34	526.63	388.07	900.07	751.48	693.46	972.02
Site3	3 spp.	97.72	**0.00**	74.24	520.31	701.97	88.27	1095.48	520.76	664.28	935.12
Site1	4 spp.	**0.00**	124.06	162.01	28.97	760.64	408.50	991.81	1023.36	650.01	973.84
Site2	4 spp.	**0.00**	155.87	127.40	330.28	902.66	447.01	1298.55	944.37	795.11	1023.23
Site3	4 spp.	2.81	**0.00**	11.59	114.62	230.76	64.75	317.72	249.87	241.70	311.29

Fig. 4. Pollinator tendency of establishing new links after plant removal in relation to the strength of the pollinator visitation to the plant being removed. Both axes are calculated as proportions (see Material and Methods), the solid and dashed lines indicate the estimated nonlinear relationship and the confidence intervals respectively, while single points and darker areas represents the raw data (notice that they have been jittered to decrease overlaps).

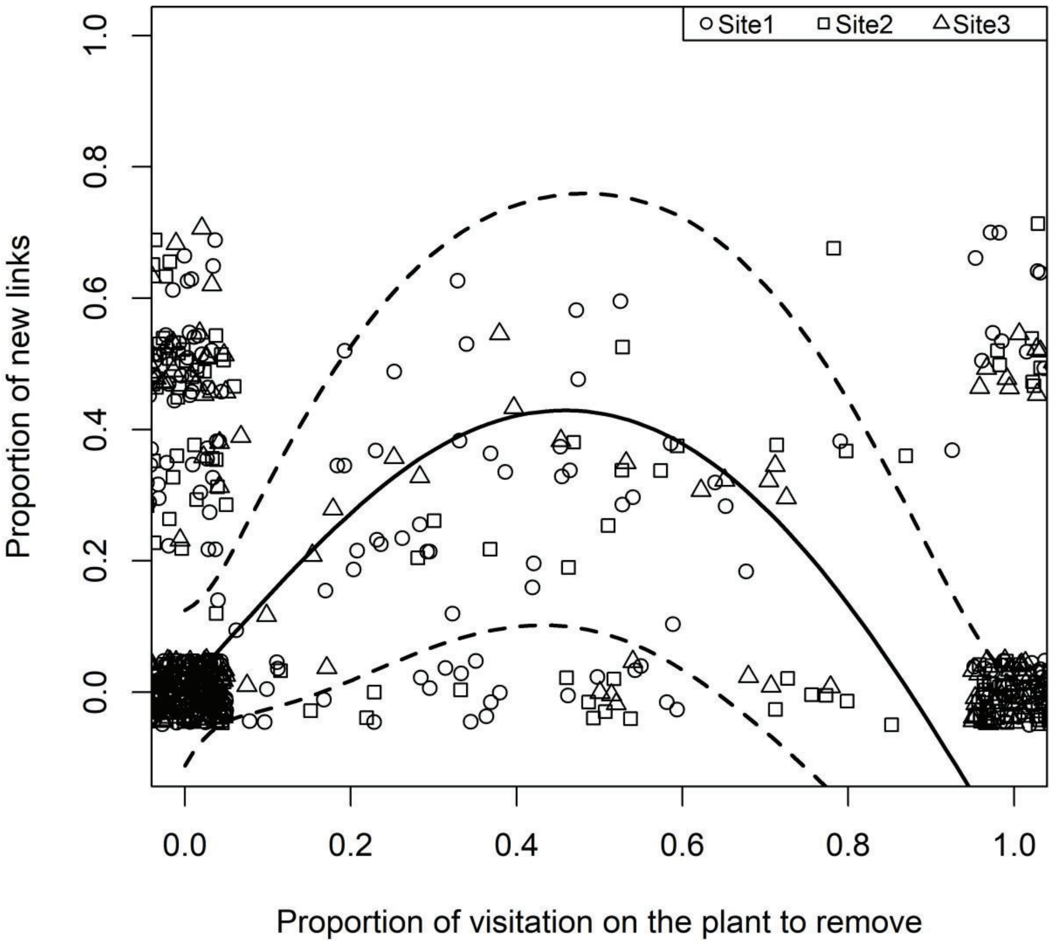


Fig. 5. Heatmap indicating the overlap between the observed main network or *Beta*-diversity indices and the confidence intervals of 10^3 simulations generated from probability matrices (specified in columns in the figure and described in the Material and Methods). Colours symbolize the number of sites being correctly predicted, as shown in the legend. See Fig. S3-S6 in Supporting Information for all indices used and site-specific predictions.

Index	Phases	NULL	PLANT ABUNDANCES	POLLINATOR ABUNDANCES	ABUNDANCES (PLANTS and POLLINATORS)	ABUNDANCES and SUGAR	ABUNDANCES and MORPHOLOGY	ABUNDANCES and SUGAR and MORPHOLOGY	SUGAR and MORPHOLOGY	MORPHOLOGY	SUGAR
Nestedness	removal 0		🟧	🟧					🟧	▨	▨
	removal 1		🟧	▨				▨	🟧	🟧	▨
	removal 2		🟥	🟥			🟧	🟧			▨
	removal 3		🟥	▨			🟧	🟧	🟧		
	removal 4		▨	🟧	▨		▨	🟧	🟧	▨	🟧
Modularity	removal 0						▨	▨	▨	🟧	
	removal 1								▨		
	removal 2							🟧		▨	
	removal 3							🟧			
	removal 4	▨						🟧	▨	▨	
Specialization H2'	removal 0		▨		▨	▨	🟧	▨		▨	▨
	removal 1							▨			
	removal 2						▨	▨	▨		
	removal 3						▨	▨			
	removal 4		▨		▨		▨				
Rewiring	pair 1-2		▨	🟥	🟧	🟧	▨	▨	▨	▨	▨
	pair 2-3		🟥	🟥	🟥	🟥		🟧	▨	🟥	🟧
	pair 3-4		🟧	🟥	▨	▨			🟧	🟧	🟥
	pair 4-5			🟧	🟧	🟥	🟧	🟧	▨	▨	🟧
Species Turnover	pair 1-2		▨	🟥	🟥	🟧	🟧	▨		▨	▨
	pair 2-3	🟧	▨	🟧	🟧	🟥	▨	▨		▨	🟧
	pair 3-4		🟧	🟥	🟥	🟧	▨	▨	▨	🟥	🟧
	pair 4-5	▨	▨	🟧	🟧	🟥	▨	▨	▨	🟥	🟥
Rewiring (Quantitative)	pair 1-2		🟧	🟧	🟧	🟥	🟥	🟥	▨	▨	▨
	pair 2-3	▨	▨	▨	🟧	🟧	🟧	🟧	▨	🟥	▨
	pair 3-4		🟧	🟥	🟧	🟧	🟧	▨	🟧	🟧	🟧
	pair 4-5		▨	🟧	🟥	🟥	🟥	🟧	▨	▨	🟧
Species Turnover (Quantitative)	pair 1-2	🟧	▨	🟧	🟥	🟧	🟧	🟥		▨	🟧
	pair 2-3	🟧	▨	▨	🟧	🟧	🟧	🟧	▨	▨	
	pair 3-4	▨	🟧	🟧	🟥	🟥	🟥	🟧	▨	🟥	🟧
	pair 4-5		🟥	🟧	🟥	🟥	🟥	🟧	▨	🟥	🟥

Legend 🟥 Predicted in 3 sites ▨ Predicted in 1 site 🟧 Predicted in 2 sites ⬜ No site predicted

Discussion

In this study, we conducted an experiment based on manipulating real plant communities and tested the effects on the pollinator assemblages and on the interactions between plants and pollinators. In practice, we sequentially removed several, highly visited, plant species, and investigated the effects on pollinator and interaction disappearance rate, on plant-pollinator network structures, on interaction rewiring, and explored what ecological traits contributed at network assembly during the experiment. Experiments of this kind can indicate what departures exist between the observed local extinctions and network indices and what inferred from theoretical models, and the changing structure of a plant-pollinator network after a disturbance can provide information on its functionality thanks to the ecological interpretation of network indices (Jordano, Bascompte, & Olesen, 2006; Kaiser-Bunbury & Blüthgen, 2015). Furthermore, looking at the ecological factors that describe linkage rules between interacting species could reveal the hidden mechanisms that built plant-pollinator networks during the experimental manipulation (Vázquez et al., 2009).

In our study, after removing generalist plants from real plant pollinator networks, the cumulative number of disappeared species and interactions increased more than expected from co-extinction models. Previous studies have only used *in-silico* estimation of extinctions (Bane, Pocock, & James, 2018; Evans, Pocock, & Memmott, 2013; Kaiser-Bunbury, Muff, Memmott, Müller, & Caflisch, 2010; Vanbergen, Woodcock, Heard, & Chapman, 2017), but our experiment clarifies that TCM, SCM and REW models underestimated species extinction rates, and the rate of false positives and false negatives was high in relation to the identity of the species that were lost. Furthermore, these models underestimated the rate of interaction loss, an issue that has been already pointed out (Santamaría, Galeano, Pastor, & Méndez, 2016). In addition to these discrepancies, our data showed that the observed network indices were also poorly correlated to the indices predicted by the co-extinction models. Altogether, field experiments such as the one we performed have a big potential for validating, rejecting or refining the theoretical insights gained by simulation models, and could trigger further development of more accurate models on network functioning, stability and co-extinction rates. We speculate that the differences between predicted and observed extinctions of this study could be due to ecological factors not accounted for by the coextinction models. The two simplest possible explanations might be that, firstly, the treated sites became progressively less attractive to foraging pollinators which emigrated even when resources they were using were still available, because pollinators are usually attracted by total flowering plant richness and abundance (e.g. Baldock et al., 2019). This is confirmed in our study systems in some cases, that occurred when the removed species were also highly abundant (see Table S1). However, another possible explanation of the observed higher disappearance rate comes from graph theory, as in fact we removed the plants that had a central position in the network, and thus the interacting species would be less anchored to others in the interaction web and therefore more exposed to extinction (Dunne & Williams, 2009; Eklöf & Ebenman, 2006).

In addition to causing local extinctions, the removal of generalist plants clearly impacted network structure. The loss of generalist plants triggered a decrease of *nestedness* and an increase of *modularity*. The observed increase in *modularity* indicates the emergence of a progressively more compartmentalised structure, which is some studies was used as a sign of potential network breakdown into separated and poorly-connected units (Reis et al., 2014). Although compartmentalization of predator-prey food-webs is considered beneficial as it buffers against alterations spreading throughout the entire web (Stouffer & Bascompte, 2011), in mutualistic networks a very high modularity actually prevents the access to alternative resources and it have been associated to a decreased network stability (Thébault & Fontaine, 2010). Therefore, the observed increase of *modularity* in our study could hint that, after removing key elements, the network shifts towards a less cohesive structure that is fragmented in compartments, that could even decrease species persistence after a perturbation (Thébault & Fontaine, 2010).

The observed decrease of *nestedness* could be linked to theoretical studies showing that a network with low *nestedness* is also fragile (Burgos et al., 2007). This is based on the fact that nested networks contain a central core of generalist species that interact with each other and with more specialised species (Biella et al., 2017; Nielsen & Bascompte, 2007). In so doing, these core generalists support the specialised species by either providing them with their sole source of floral resources (plants) or their sole pollen vector (pollinators). Thus, a decrease of *nestedness* hints for a scenario where specialist species are less connected to the generalist network core and thus more prone to disappear from the network (Jordano et al., 2006; Thébault & Fontaine, 2010). The trends in *nestedness* and *modularity*, that are usually negatively correlated, could be related to each other and could be based on the fact that specialisation increased during the successive plant removal events, possibly as a result of the concurrent decrease in pollinator abundances (Biella, Akter, et al., 2019), i.e. reductions in the number of interactions triggers changes in network structure (Bomfim, Guimarães, Peres, Carvalho, & Cazetta, 2018; Moreira, Boscolo, & Viana, 2015; Vanbergen et al., 2017).

The changes in *nestedness* and *modularity* did not translate into a lower *stochastic robustness* index, possibly due to the dynamic yet asymmetric re-organization of species interactions along the sequence of plant removal. In other words, our results showed that the remaining plant species became increasingly centralized in the network, but there was no trend in the average centralization of pollinator species, hence indicating that network re-organization was based on the asymmetric responses between mutualistic network levels. In addition, the recorded high rewiring rate shows dynamism in establishing new interactions after network disturbance, and it played a larger role than species turnover in determining the total *Beta* diversity during the experiment, as in (CaraDonna et al., 2017), although without a clear trend during the experiment. Additional evidence of rewiring emerges when we explored which pollinators visited novel plants after the network perturbation. Our data showed that only a subset of the pollinator guild visited new plants after the removal events. In particular, the specialist pollinators visiting the removal plants or those ones which did not visit those tended to avoid creating new links once the target plant had been removed. On the other hand, the generalist pollinators that visited both the plant targeted of removal and other plants were more prone at establishing new links. However, a previous study within the same system of this study showed that pollinator redistribution to the rest of the plant assemblage is constrained within particular species-trait spaces, which either favoured or prevented using alternative flower resources (Biella, Akter, et al., 2019). In particular, in that study, the pollinators visited new plants according to the sugar content of the nectar, the plant tallness and inflorescence size, while they did not swap between flower shapes. These results are relevant because they show constraints that could impede accessing to some new resources after perturbations, with possible negative effect on the persistence of mutualsitic interactions, while the results of the present study further clarifies that establishing new links happens more often if the pollinators are foraging as generalists.

The ecological mechanisms linked to the above-mentioned network indices and rewiring could additionally show how species reorganized their interactions and what linkage rules are relevant before and after network perturbation. When several plants were removed, pairwise interaction were explained by the null model assuming equal probability of interactions, which suggests an emergence of randomness in species interactions of disturbed communities. That randomness rules pairwise interactions of disturbed networks is particularly alarming, because it would indicate the disruption of established interaction assembly mechanisms, and may also be linked to opportunism in interactions and high rewiring (Ponisio et al., 2017). In addition to this, at earlier stages of the experiment, individual pairwise interactions were explained best by the model using pollinator abundances, reflecting the relationship between abundance and generalization of interactions (Ollerton et al., 2003; Vázquez et al., 2009). The importance of abundances as linkage rule is also evident at the level of the entire networks. Specifically, species abundance predicted network *nestedness* and rewiring, often in combination with other ecological factors such as the sugar amount in the nectar, that reflects the role of abundant and rewarding generalist plants interacting with numerous pollinators in establishing the nested pattern and in creating new interactions (Bascompte, Jordano, Melián, & Olesen, 2003). Furthermore, it seems particularly relevant that even if single ecological traits determined some network indices before plant removal, instead, when several plants were removed, many indices of the studied networks were explained by complex combinations of predictors, such as the interaction of abundances with morphological match and with sugar rewards. This aspect could suggest a prominence of network complexity following the removal of generalist plants. The loss of single-driver ability and the need of combined factors for describing network assembly could reflect the increase of variance and of idiosyncrasies in pairwise interactions after generalist plant removals, clear indications of a networks in an altered state (Tylianakis & Coux, 2014).

Since the experiment stopped when four species were removed, we do not know if the observed linear trends in network indices and in loss of species and interaction would also progress linearly when the other remaining plants are taken away from the system. Theoretical studies have used various approaches, with models assuming linearity (Bascompte, Jordano, & Olesen, 2006a) and nonlinear models (Holland, Okuyama, & DeAngelis, 2006), which could generate conflicting results (Bascompte, Jordano, & Olesen, 2006b). Nevertheless, some theoretical studies consider linear functional responses as uncommon trends in mutualistic interactions (e.g. (Okuyama & Holland, 2008). Thus, it could be expected that, when other additional plant species are removed, the trend would become nonlinear, for instance as observed in co-extinction models (Dunne et al., 2002).

Conclusion

In this study we showed that pollinators occurrence and species interactions are more sensitive to the disappearance of generalist plants than the expectations from network co-extinction models. When the key plants are removed, the network structure is altered and changes, the loss of species and interactions increases, and opportunism in interaction establishment becomes more prominent. This gives strong support to proposals indicating that conservation of species interaction networks should be centred on the generalist species pool (Biella et al., 2017; Montoya, Rogers, & Memmott, 2012). However, this generalist-based conservation view should consider the dynamics and re-organization of interactions and the asymmetrical responses between plants and pollinators, which compensate for an even more detrimental collapse of species networks.

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Supporting information

Table S1 – The sequence of plant species removed during the experiment, and their relative floral abundance.

Appendix S1 – Sampling completeness estimation.

Fig. S1. Network- and species- level indices that did not change significantly in response to plant removal.

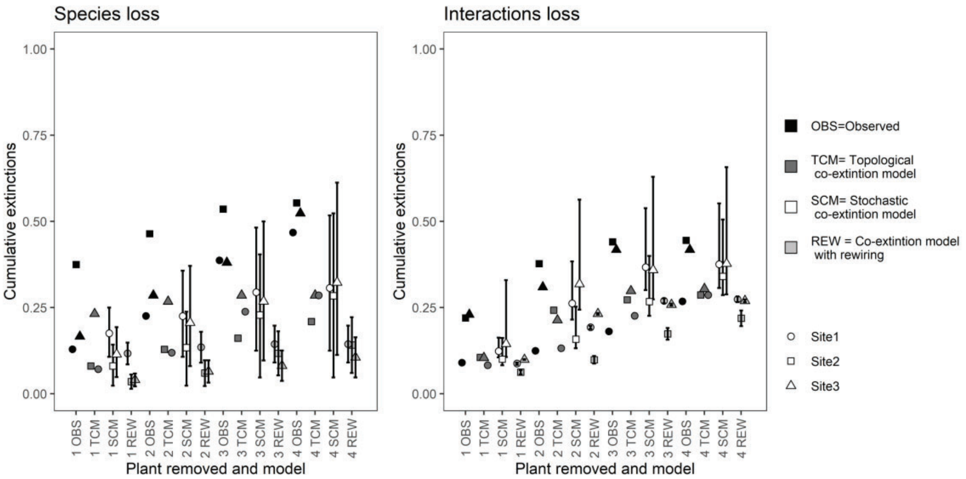
Fig. S2. Comparison between the observed real networks and the indices predicted by each co-extinction model

Fig. S3. Network *connectance* and *nestedness* indices predictions by probability models for each site.

Fig. S4. Network *specialization* and *modularity* indices predictions by probability models for each site.

Fig. S5. Qualitative indices of *Beta* diversity and its components predictions by probability models for each site.

Fig. S6. Quantitative indices of *Beta* diversity and its components predictions by probability models for each site.



Cumulative proportion of extinctions of species and of interactions over the sequential plant removal as observed in the real networks ("OBS") and as predicted by TCM, SCM and REW co-extinction models for each site. In SCM and REW, the symbols and lines indicate the mean and 5%-95% quantiles of 103 simulations. Statistical tests are presented in the Results.

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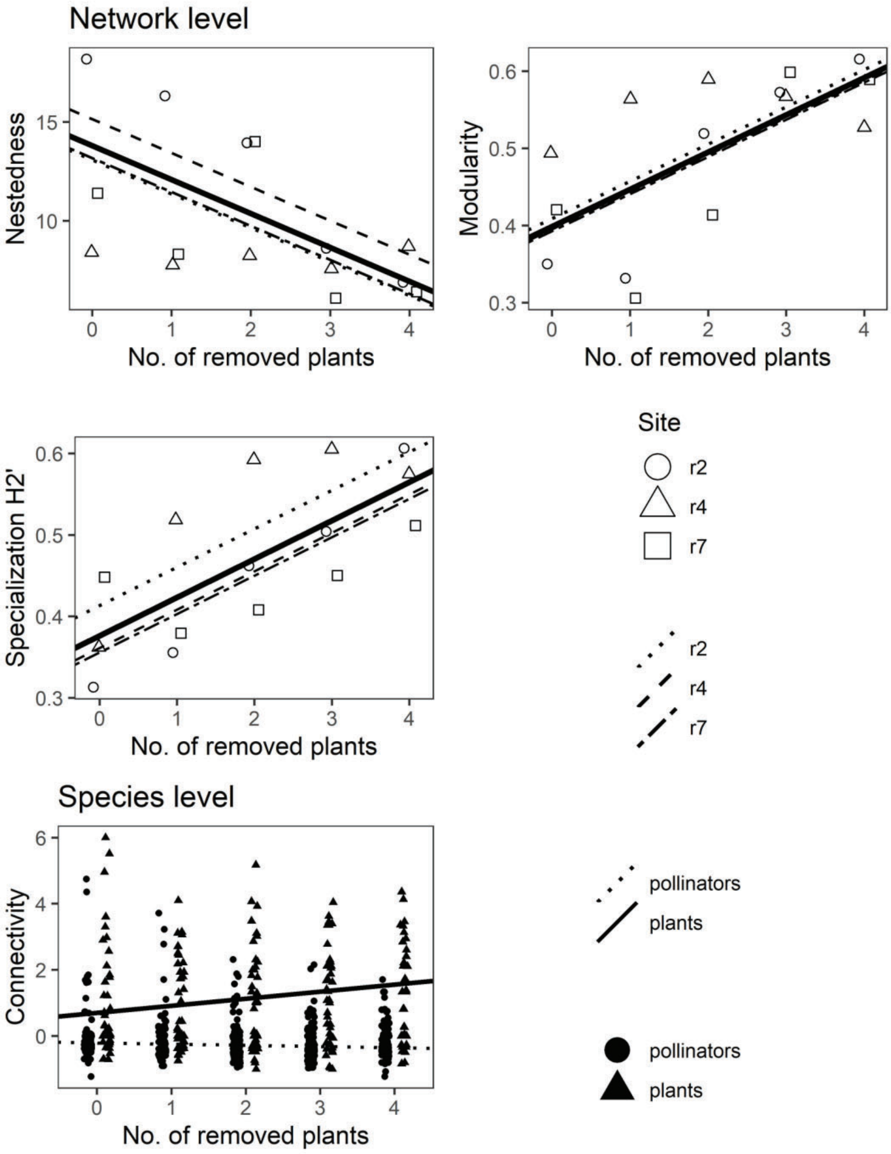

Responses of network- and species- level indices to the removal of generalist plants. The significances of predictors are expressed in Table 1. The solid line is the average trend significantly predicted by the models. See Fig. S1 in Supporting Information for other indices used in this study that resulted as not significant (i.e. connectance, stochastic robustness, participation).

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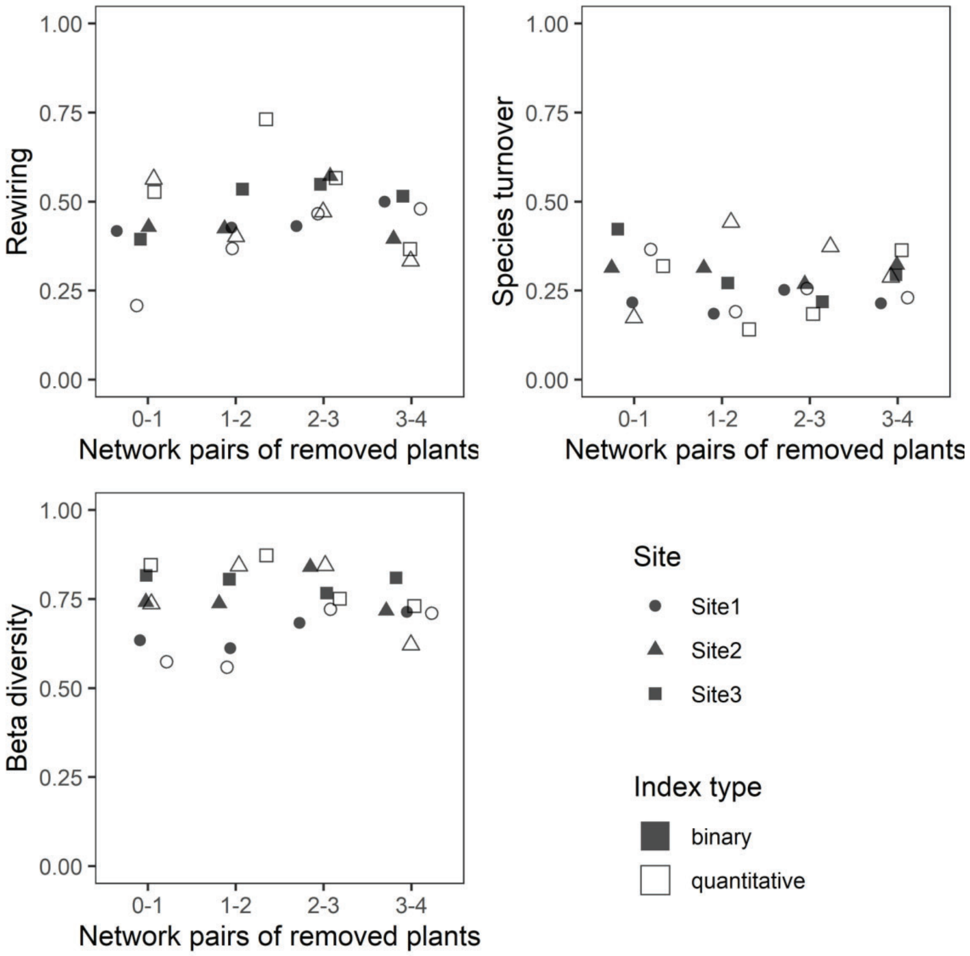

Fig. 3. Total Beta diversity and its components of species rewiring and turnover between network pairs after each stage of plant removal. Both the binary indices and their quantitative counterparts are plotted. Significances of predictors are included in Table 1.

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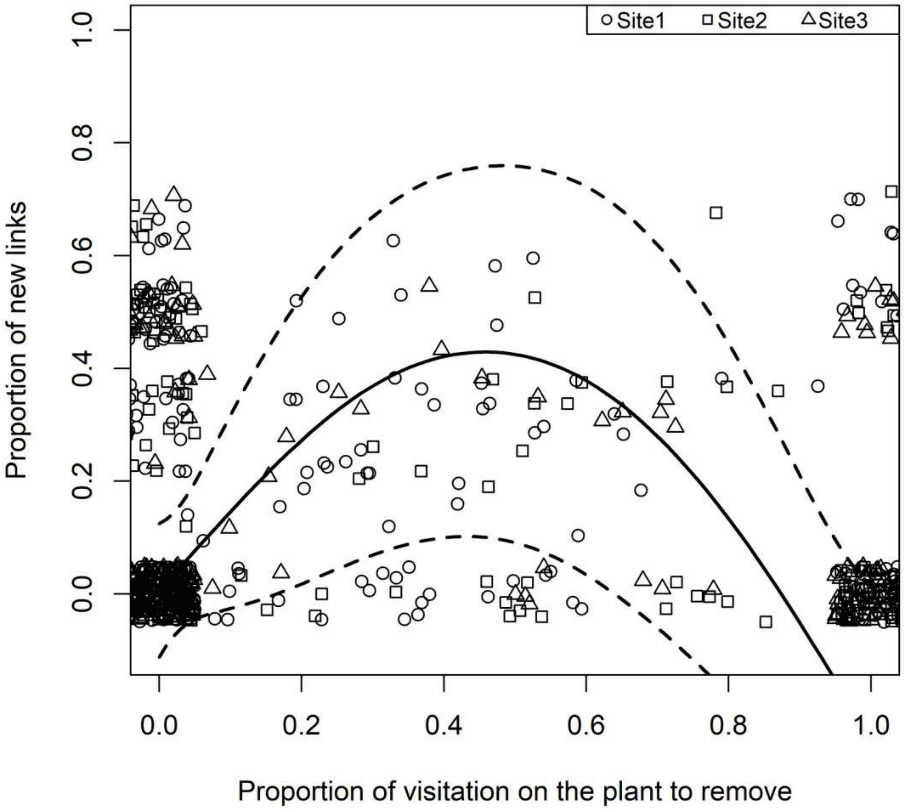

Pollinator tendency of establishing new links after plant removal in relation to the strength of the pollinator visitation to the plant being removed. Both axes are calculated as proportions (see Material and Methods), the solid and dashed lines indicate the estimated nonlinear relationship and the confidence intervals respectively, while single points and darker areas represents the raw data (notice that they have been jittered to decrease overlaps).

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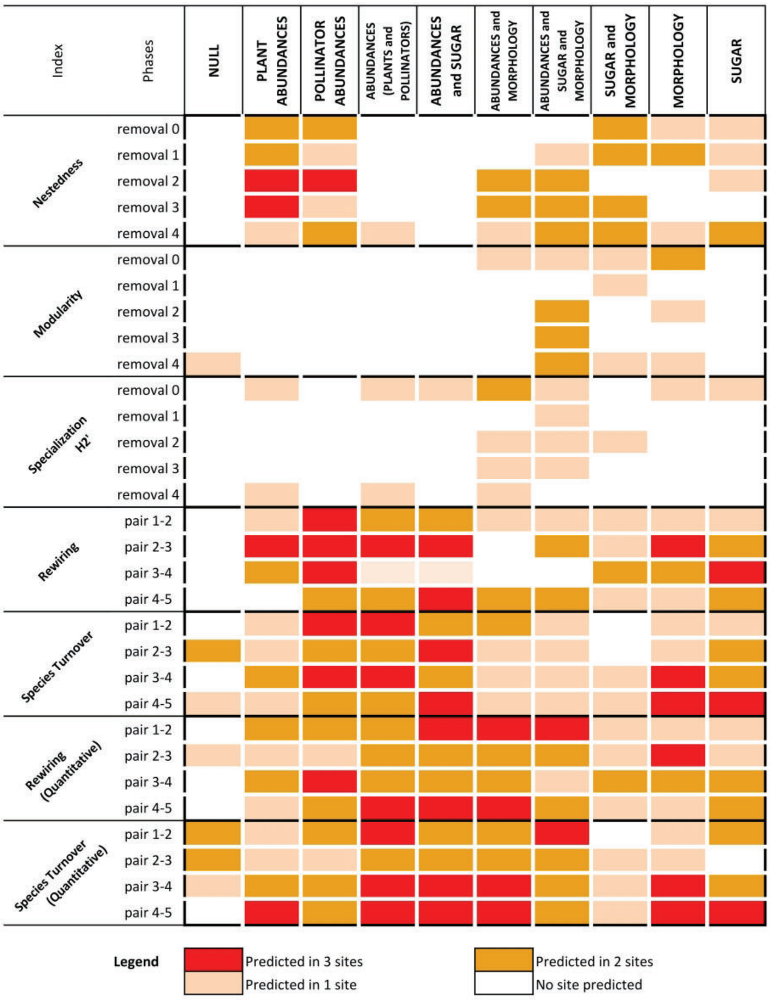

Heatmap indicating the overlap between the observed main network or Beta-diversity indices and the confidence intervals of 103 simulations generated from probability matrices (specified in columns in the figure and described in the Material and Methods). Colours symbolize the number of sites being correctly predicted, as shown in the legend. See Fig. S3-S6 in Supporting Information for all indices used and site-specific predictions..

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