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A conservation perspective on the mechanisms that influence plant-pollinator interactions

Ph.D. Thesis

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Annotation

Several aspects of plant-pollinator interactions are presented in the thesis. It contains a review on the open questions of plant-pollinator interactions from single species to complex networks. The following sections document novel results. Firstly, the conservation of complex pollination networks is addressed through the hierarchy of species' importance. In addition, the habitat requirements and interactions of a threatened rare pollinator species are explored. In the following chapters, the results from manipulative approaches applied in the field to plant-pollinator interactions are presented. The effect of pollinator's population decline on pollinators' foraging for pollen is investigated. Moreover, the way plant species loss impact several aspects of pollinator visitation is presented. Lastly, the impact of species removal on plant-pollinator network topology and on species ability of establishing new interactions is investigated.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Paolo Biella

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[Sorry for not writing all names, but this is not a gift card...]

List of papers and author's contributions

The thesis is based on the following papers:

I. **P. Biella**, J. Ollerton, M. Barcella & S. Assini, 2017. Network analysis of phenological units to detect important species in plant-pollinator assemblages: can it inform conservation strategies?
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Paolo Biella analyzed the dataset, conceived the focus of the manuscript with SA and JO and wrote the drafts. The other authors contributed at data collection and at improving the manuscript.

II. **P. Biella**, G. Bogliani, M. Cornalba, A. Manino, J. Neumayer, M. Porporato, P. Rasmont, P. Milanesi, 2017. Distribution patterns of the cold adapted bumblebee *Bombus alpinus* in the Alps and hints of an uphill shift (Insecta: Hymenoptera: Apidae).
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Paolo Biella collected the data and digitalized the records, contributed at designing the research with MC, PM and GB, analyzed the data with PM and wrote the drafts. The other authors contributed at data collection (MC, AM, JN, MP, PR), at digitalization (MC), and at improving the manuscript.

III. **P. Biella**, N. Tommasi, A. Akter, L. Guzzetti, J. Klecka, A. Sandionigi, M. Labra and A. Galimberti. Integrative approach reveals a constancy in the foraging strategy of a social pollinator after a strong reduction of the colony's workforce. Manuscript

Paolo Biella designed the experiment with JK, collected the data with JK and AA, analyzed part of the molecular data (the other part was by NT, LG, AG, and AS), analyzed statistically the data, conceived the focus of the ms with ML and AG and wrote the drafts. All authors contributed at improving the manuscript.

IV. **P. Biella**, A. Akter, J. Ollerton, S. Tarrant, Š. Janeček, J. Jersáková, J. Klecka. Experimental loss of generalist plants reveals alterations in plant-pollinator interactions and a constrained flexibility of foraging. Manuscript

Paolo Biella designed the experiment with JK and JO, collected the data with JK and AA, analyzed the data and wrote the drafts. Flower-related data were analyzed in the laboratory by SJ, JJ, AA. The data of a previously collected dataset were collected by JO and ST. All authors contributed at improving the manuscript.

V. **P. Biella**, A. Akter, J. Ollerton, Š. Janeček, A. Nielsen, J. Klecka. An empirical attack tolerance test on real plant-pollinator networks: plant removal impact network structure but plants and pollinators respond asymmetrically. Manuscript

Paolo Biella designed the experiment with JK and JO, collected the data with JK, AA and SJ, contributed at designing data analyses with AN and JK, analyzed the data and wrote the draft. All authors contributed at improving the manuscript.

Co-author agreement

I acknowledge the major contribution of Paolo Biella in the research paper presented in the Chapter 1 of this thesis.

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Introduction

Pollination: from single species to complex networks

The pollination of plants is a key aspect of ecosystem functioning: many plant species rely on animals for achieving cross pollination, and many insects gain energy and nutrition by feeding on plant rewards. At least two methodologies are undertaken for studying pollination. The first one is based on autecological approaches devoted to the pollination ecology of single plant species, chosen for their peculiarity (e.g. remarkable phenotypes or strict specializations, Anderson and Johnson 2008), for their biogeographical value (e.g. endemisms Macior 1986), or as a case-study for investigating flower-pollinator co-evolution and selection pressure (e.g. deceptive orchids Jersáková et al. 2006). The second one acknowledges the fact that species co-occur with others, thus establishing complex relations of facilitation and competition for the pollination service with the neighbouring species (Lázaro et al. 2014).

Community-level approaches are therefore important to shed light on interaction structures (Jordano et al. 2006; Vázquez et al. 2009) and their dynamics (e.g. year-to-year changes, Alarcón et al. 2008). Such an approach can also provide insights on facilitative aggregations of plants (Verdú and Valiente-Banuet 2008; Cranmer et al. 2012), the foraging strategies of insects (Miller-Struttmann and Galen 2014), the importance of phenology (Olesen et al. 2011), plant-pollinator mismatches (Fabina et al. 2010), the effects of invasive plants (Albrecht et al. 2016) and the robustness of complex interactions (Evans et al. 2013).

Interactions of a pollinator can be sampled by monitoring flower visitation and by observing the contacts of flower's reproductive structures with the pollinator's body. Unfortunately, in the field is actually challenging to sort out what is a pollinator (the pollen is at least carried on the body) from an opportunistic flower visitor (the animal does not collect any pollen). However, after visitation the pollen deposition can be studied by counting pollen grains on the stigmas, counting pollen tubes growing within the style or even recording the seed set (Alonso et al. 2012). Nevertheless, these approaches are practical in situations where few species are studied because of the amount of technical expertise and time involved.

When multiple species are active in a system and interact in an intricate way, the plant-pollinator network can be derived by recording interactions indirectly, such as by means of identifying the pollen on the body which summarizes the foraging bout (Bosch et al. 2009; Bell et al. 2017). However, traditional morphological identification is time consuming, inaccurate when distinguishing related taxa, and highly dependent on a local reference collection (Galimberti et al. 2014). Some limitations of classical palynology can be overcome by using molecular markers, which vary between species but are conserved within a taxon. Technological advances are making these molecular identification tools cost-effective, and the accuracy of identification can be high (MacGregor et al. 2018). Thus, pollination interactions can be constructed from DNA-identified pollen (Pornon et al. 2017; Lucas et al. 2018).

If we consider interacting species as nodes connected by links when they interact (“bipartite networks”), than the implementation of network analysis in studying such community-level relationships is a powerful tool. Advances in network analysis has led to a fast raise of community-level pollination ecology studies in the last few decades (Olesen et al. 2010). New aspects have been explored with methodological approaches based on individuals (Tur et al. 2015), on integrating network structure with pollen transfer rates (Bosch et al. 2009), on functional traits (Bartomeus 2013), on phylogeny (Chamberlain et al. 2014) and on data deriving from molecular identification of species (MacGregor et al. 2018). Many general and recurrent features of such networks have been described so far, such as truncated power-law degree distributions (Jordano et al. 2003), nestedness (Bascompte et al. 2003), compartmentalization (Olesen et al. 2007), asymmetric patterns of interactions (Bascompte et al. 2006), centralized structures and small-world properties (Olesen et al. 2006; Fang and Huang 2012). Some of these are presented in detail in the next sections.

Most of the patterns that emerge from plant-pollinator networks are consistent despite year-to-year changes in species composition (Alarcón et al.

2008), but they change for the effect of phenology and of the dynamic re-wiring, i.e., switching interaction partners (Olesen et al. 2008; Kaiser-Bunbury et al. 2010; Biella et al. 2017b). Most of the network studies consider plant-pollinator networks as fixed, static, and robust entities, but in reality they show dynamic properties that leads to frequent changes in the interactions. By investigating those aspects of plant-pollinator interactions that still remain unclear, it will be possible to shed further light on the processes that control the assembly and structure of plant-pollinator networks and on how to integrate them in a community-level conservation framework.

On the need for conserving plant pollinator interactions at a community level

Anthropogenic activity and environmental change drive declines in richness and abundance of species (Newbold et al. 2015), thus threatening the functionality of ecosystems (Cardinale et al. 2006). Most conservation efforts are still centred on maintaining species diversity, enriching species lists in protected areas, or targeting single species. Nevertheless, it is clear that species co-occur with others and interact with several species. In doing so, they provide a service to the ecosystem by providing an ecological function (Brodie et al. 2018). In fact, conservation actions devoted to increase biodiversity or support single species populations might not necessarily translate into making a more robust ecosystems. Thus, there is a strong need for integrating the ecological roles of species into conservation plans by considering their interactions (Pigot et al. 2016; Brodie et al. 2018).

A key aspect of integrating species interactions into conservation is to identify and describe all interactions in an effective way and also to quantify their contribution to the system (e.g. stability) or its productivity. In community-level pollination networks, it is now possible to identify species' topological position and this could also unveil species' roles in the ecosystem. Specifically, since the work on metabolic networks of Guimerà and Nunes Amaral (2005) and later on plant-pollinator networks of Olesen et al. (2007) and Dormann and Strauss (2014), it has become clear how to quantify species contribution to network cohesiveness. It is believed that network cohesiveness is based on a hierarchy of species, with “hubs” being more important than others, which not only prevent structural breakdown, but also assure the functionality of the system (Reis et al. 2014). In chapter 1, network analysis was used as a tool for finding the hierarchy of key species through the entire summer season in a dataset of two plant pollinator assemblages. This approach contributed to understanding network analyses and functional roles of species, for informing conservation practices. “What-makes-a-hub” features were also described, revealing that a network’s important species are mostly generalists but also that they interact with several specialist species as well.

Generalism in pollination and the role of rare species

The community-level approach to the study of complex pollination interactions unravelled a key feature of pollination ecology: the prevalence of generalism (Waser et al. 1996). Nevertheless, several definitions of specialism and of generalism exist in pollination ecology; some of the concepts have been summarized in Ollerton et al. (2007), as follows. The *ecological* generalist/specialist refers to the high/low number of effective pollinators visiting a plant. A *functional* generalist/specialist refers to a high/low diversity of functional groups of pollinators, which are species with similar functional traits and life histories and even with some degree of phylogenetic relation (usually a higher taxonomic level as a Family or above). In addition, a *phenotypic* generalization/specialization refers to the (un)adaptations for pollinating or being pollinated (e.g. radial or zygomorphic flowers, specialist rewards, special collecting apparatus or primitive chewing mouthparts, scents, nectar chemistry and others). An additional concept is the *evolutionary* generalization/specialization, which refers to the process of evolving towards generalization/specialization (Armbruster and Baldwin 1998; Armbruster 2006). To these definitions, it is possible to add an additional concept of generalization/specialization which relies on the concept of niche overlap: a *niche* generalist/specialist, which is a species with a high/low pollination or foraging niche overlap with other co-occurring species (Blüthgen et al. 2006). In other words, a niche generalist plant species is a species which shares pollinators with others. Furthermore, it is also possible to extend these definitions to the flower visitors (rather than strictly referring only to pollination and to efficient pollen-carriers), so that a plant within a given plant and flower-visitor network is a generalist if it has many kinds of visitors and/or has high niche overlap with others. Vice versa, within the network framework, Olesen et al. 2010 arbitrarily defines a specialist simply as a species with number of links equal or less than 2.

The mechanism of generalization is that a plant being visited by a high amount of pollinators increases the chances of having the pollen dispersed (Brosi 2016). In turn, once these generalist pollinators are active in a patch, they can collect resources from a wide range of plants. This strategy could provide nutritional benefits from multiple sources (Alaux et al. 2010; Filipiak et al. 2017) and therefore likely sustain pollinators' populations by providing nutrition (Faegri and van der Pijl 1979). These generalists will eventually contribute to plant reproduction because it is believed that generalist pollinators are actually visiting few plant species for each foraging bout (Brosi 2016). This is especially likely to occur in individual foragers performing flower constancy (visits to the same plant species during a foraging bout) or some degree of fidelity (visits to the same plant species during multiple bouts).

However, being a specialist might be more complex. In fact, a specialist plant is visited by a few species that might be specialist pollinators or generalist ones. While the latter has been discussed above, the former assure plant reproduction when either the pollinator's population size or foraging behaviour is sufficient to allow a meaningful pollen flow. From the pollinator's point of view, a specialist results from adaptation to use the specialist plant's phenotype. For example, in the case of an oligolectic bee which has a narrow diet, a plant allocates secondary metabolites into the rewards to avoid overexploitation by some opportunistic visitors, and thus only those pollinators evolved to process difficult metabolites can efficiently utilize these resources (Praz et al. 2008). The same applies to complex or prohibitive flower morphologies, such as long tubes in corollas, so that only specialized pollinators can access the rewards (Faegri and van der Pijl 1979).

Conversely, specialization might actually simply be the result of neutral processes, such as the stochasticity of detecting an interaction when population size is low. In other words, given randomness in finding a species/interaction, less abundant species (or have shorter phenophase) have less chance being interacting than those who are more dominant (or have longer phenophase) (Kallimanis et al. 2009). This opens a question, which is how to overcome this limitation as set by the low local population size, especially for the rare species.

An important component of the local species assemblage are indeed the rare species (Ohlemüller et al. 2008). These are often in need of important effort of conservation, due to relatively small population size compared to other generalist species and/or narrow and exclusive niche. For example, climatically restricted species are expected to have lower tolerance to a changing environment (Thuiller et al. 2005). Rare species are also difficult to study in the field because of their low detectability (Edwards Jr et al. 2004). This causes issues because tracking the few individuals of a given rare species is actually challenging. This has direct implications for studying the interactions, also in pollination, where the contribution of rare species to an ecosystem service is difficult to investigate.

A possible workaround to this problem may be, firstly, offered by detailed characterisation of the habitat a rare species is actually occupying and in which areas it is most likely to be found, based on a set of ecological variables such as habitat and climate (Ohlemüller et al. 2008; Biella et al. 2017a). This can be performed by precisely describing what ecological factors and what range of the environmental variables predict the occurrence of the species. Once the plausible areas of occurrence are defined, focusing on other aspects (such as trophic interactions) could be possible, for example with repeated surveys in key areas of expected occurrence of the species (Fois et al. 2018). Knowing where a species is likely to occur will overcome the problem of detectability. Thus, this framework would not only help the conservation of rare species but also help to further understand what can be done to shed light on the interactions of these rare species (Duffy and Johnson 2017). This framework, which consists of defining the ecological niche of a rare species and then to investigate its trophic interactions, was tested with a rare alpine endemic bumblebee subspecies, see chapter 2. The results confirmed that the rarity is due to climatic specialism (a narrow climatic ecological niche). Yet, when focusing on the entire occurrence range, it turned out that the bumblebee is a pollinator generalist which collects resources from about 50 plant species. Another result of the mentioned research is that a steady decline of the altitudinal occupancy has been detected, which might have strong implications for the extent of the populations and of the survival of this species in view of the decline of pollinators.

Pollinator decline and how foraging relates to population size

Pollinators are responsible for the ecosystem service of plant pollination, that has an estimated economic value of $361 billion worldwide (Lautenbach et al. 2012). However, wild pollinators are facing steady declines. For example, in Central European countries, the number of colonies of European managed Honeybees declined between 1985-2005 (Potts et al. 2010). Unfortunately, information on population trends of wild pollinators is rather scarce and dispersed. Nevertheless, pollinators and plants from the Netherlands, the UK, and Belgium were found to decline severely since the 1950s (Carvalheiro et al. 2013). Furthermore, historically common bumblebee species have declined, for example as shown by current Clover-pollinating bumblebees assemblages that are very different than past ones, as revealed by comparing historic data with recent surveys (Bommarco et al. 2012). These negative trends are related to factors such as climate change (Kerr et al. 2015), to several agricultural practices, the reduction of natural areas and the spread of new diseases (Goulson et al. 2015).

The way the declines in pollinator populations affect the pollination rate is yet unclear. For example, variation in generalization level of plant's pollination niche across two consecutive years was related to a 25% density diminishment in the abundant pollinator species (Lázaro et al. 2016). Therefore, declines in pollinator's population size can cause interaction extinction (i.e., the absence of an interaction that was observed previously to the reduction in population size). Interaction extinction rate is likely related to population size, as species abundance is correlated to the number of interactions (Olesen et al. 2008).

Thus, a situation in which the environmental conditions cause a gradual abundance decrease may cause profound changes in plant-pollinator networks before any actual extinction occurs (Santamaría et al. 2016). This has been shown in a few studies: plant-pollinator interactions decreased two or three times faster than species richness when surveying a gradient of increasing habitat fragmentation (Sabatino et al. 2010; Burkle and Knight 2012); furthermore, interactions were found to disappear when the pollinating birds' reached a population density that was below a threshold (Anderson et al. 2011).

Thus, pollinator population densities may modify the pollinator foraging. This is particularly true for taxa with high resource demands. In the case of the bees (Hymenoptera: Apiformes), the pollen is needed in order to feed the larvae and trigger ovary development in reproductive females. Social pollinators like honeybees or bumblebees are also subject to multiple stressors that affect the life cycle of their colonies and thus the size of their populations (Goulson et al. 2015). For instance, pathogens, like the trypanosome *Crithidia bombi,* can kill more than half of a bumblebee colony's stressed workers and up to 70% of the workers can die due to parasitoid conopid flies (Müller and Schmid-Hempel 1993; Schmid-Hempel 2001). Furthermore, under a diet containing field-realistic concentration of pesticides, a given bumblebee colony can produce 1/3 less workers than the normal amount (Laycock et al. 2012). In addition, up to 45% of the newly born bumblebees' workers do not last more than 4 days which is much less than the normal life expectancy (Gill et al. 2012). In honeybees, the survival is strongly reduced by 50% from the synergistic effects of both poor nutrition and pesticide exposure (Tosi et al. 2017). Therefore, it seems clear that populations of pollinators are subjected to strong declines for either single causes or for the interplay of multiple factors.

At the individual forager level, intra-specific competition can lead to a higher specialism at low density of foragers (Fontaine et al. 2008). This was expected because the Optimal Foraging Theory predicts that foraging behaviour is related to consumer density, by means of increasing competition for resources at higher foragers' abundance (Emlen 1966; MacArthur and Pianka 1966). Conversely, when a colony of social pollinators is subjected to a strong and fast reduction in size, an overall generalist foraging strategy should be triggered so that the system can still gather enough resources for the colony's larval demands, despite fewer active foragers (Kaluza et al. 2017). This is because social insects need a high amount of resources in order to feed the larvae. Thus, variations of populations size can trigger either specialization or generalization, but it is not clear which of the two strategies. In chapter 3, the way a realistic decline in population density will affect pollinator's foraging was tested in the field with commercial colonies of the bumblebee *Bombus terrestris.* However, since a limited adaptability by foragers was found in this research, a decline in population size might ultimately cause species extinctions, with serious implications for the network of interactions among trophic levels and mutualisms.

Species extinctions and manipulative tests in plant pollinator networks

Pollination studies showed that pollination networks are stable in their architectural features despite a significant turn-over of pollinator fauna over time (Alarcón et al. 2008). Indeed, this stability of the network structure against species compositional changes is a remarkable feature which suggests structural stability.

The ability of networks to not collapse after species loss has been investigated in several works, which also related this aspect to network robustness. By using simple simulation models, several extinction scenarios have been explored, namely random removal, systematic removal from the most connected species to the least connected one, and systematic removal from the least connected species to the most connected ones (Dunne et al. 2002; Memmott et al. 2004; Kaiser-Bunbury et al. 2010). The general view is that plant-pollinator networks are highly sensitive to species loss because of the high amount of secondary extinctions (Memmott et al. 2004). Furthermore, if plants are the target of primary extinctions, the number of secondary extinctions will be higher than those caused if pollinators are the target of primary extinctions (Santamaría et al. 2014).

However, these studies assume static responses by individuals and species of pollinators, which does not reflect the ecological reality of these complex, dynamic assemblages. In other words, in most of these studies networks are considered to be static entities, a view that contrasts the fact that many pollinators are dynamic generalist foragers and that several plants have rather unspecialized phenotypes or provide lot of rewards (Waser et al. 1996). In other simulations that allowed the pollinators to use randomly new resources after plant removal, the number of species extinctions decreased (Kaiser-Bunbury et al. 2010). This would imply that the network is more stable after perturbations if pollinators are able to use new resources and establish new links, a property named "rewiring" of network interactions.

Dietary adaptation of consumers within a food web has been suggested to be important to the long term stability of complex communities (Kondoh 2003). This may mean that some plant species could be "rescued" from extinction following the loss of pollinators because of adaptive behavioural switches in food choice in response to qualitative and quantitative resources changes. In the absence of adaptive foragers, food web complexity leads to a destabilisation of the assemblage, whereas with adaptive foragers, high richness in a food web acts as a buffer to environmental fluctuations (Kondoh 2003). When species are lost, new interactions could be built up (re-wiring process) and this softens the impact of species loss by indirectly increasing the connectance that is the proportion of realized links (Kaiser-Bunbury et al. 2010). Therefore, short term behavioural response in pollinators' foraging implies switching floral resources, although this aspect has been rarely explored with real plant-pollinator networks in the field.

It is only recently that experimental tests aimed at manipulating real plant-pollinator communities have been performed. These involved only relatively small manipulations in the field, typically by removing one species (Lopezaraiza–Mikel et al. 2007; Brosi and Briggs 2013; Ferrero et al. 2013; Goldstein and Zych 2016). Despite that, several questions remain unanswered, such as how multiple losses will affect the complex pollination interactions in natural ecosystems and the network structure. There is a strong need to employ experimental approaches systematically in order to investigate how networks are assembled and if they are robust. In chapter 4, an experimental approach was taken to examine the effect of abrupt removal of the floral resources provided by the most generalist plant species from three grassland plant-pollinator assemblages. These data were explored in order to determine how the pollinator guild would respond to the sudden loss of floral resources, in terms of visitation, pollination effectiveness, resource use related to plant traits, and their implications on the plant assemblage. Network structure is affected by the rate of species loss. It was shown from computer simulations that removing species from the least to the most connected increased the robustness of the system, when nestedness also increased (Burgos et al. 2007); conversely, in a random removal sequence, the robustness is low if generalist interactions dominate the network (Pocock et al. 2012). In general, predictions based on simulated networks point out that robustness is proportionally related to pollinator/plant ratio ("network asymmetry") when pollinators are removed (and is negatively related to it when plants are removed) (Pastor et al. 2012). This might indicate that pollinator/plant ratio is related to the redundancy of a network (Memmott et al. 2004; Ramos-Jiliberto et al. 2012).

Furthermore, the robustness in networks has been associated to nestedness because in nested interaction patterns the least-linked species connect directly with the generalist pool, diminishing the probability of their extinction (Jordano et al. 2006; Burgos et al. 2007). This relates nestedness with redundancy. The nested structure of such assemblages contains a central core of generalist species that interact with each other, and with other specialised species. 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). The importance of these core generalist species lies in their central, dominant ecological role within a community, as they are often the most abundant species (Dupont et al. 2003; Martín González et al. 2010). Furthermore, by providing a significant amount of floral resources or pollination services, their presence may facilitate the ecological functioning of the entire community. Using numerical simulations, Saavedra et al. (2011) showed that network survival decreases more when removing the strongest contributors-to-nestedness rather than when a weak contributors-to-nestedness gets lost. In turn, these strong contributors-to-nestedness are expected to be those most prone to extinction from the networks. Such astonishing fragility of the important key nodes in the framework was unexpected. Thus, by taking out the most important species and testing changes in network structure, resource utilization and pollination efficiency will provide an important assessment on how key species contribute to real plant-pollinator interactions and even how detrimental their loss is for the stability of the system. Using field-based approaches would also serve as proof-of-concepts in order to verify if results obtained from numerical simulation adhere to the reality of the ecosystem dynamics. In chapter 5, the effect of the sudden removal of core generalist plants on plant pollinator networks and also on species rewiring was investigated. The ecological factors driving these changes were also examined in order to provide a mechanistic view on plant-pollinator interactions and their rewiring.

Mechanisms of plant pollinator interactions

At least four aspects influence the mechanism of network functioning and assembly. These factors are: the phenology of species, their abundances, the matching and mismatching of functional traits and the role of resource amount.

wWhen included as a predictor of interactions, the phenology of species plays a role in determining network structure (Martín González et al. 2012). The clearest example is that when studying the entire season, phenology-driven replacements of species and interactions influenced network compartmentalization, a feature usually named “modularity” (Morente-López et al. 2018). Furthermore, the overlap in phenology between plants and pollinators also increased the probability of linking (Olesen et al. 2011). Conversely, phenological uncoupling between two interacting species determined a large part of the amount of unrealized links (Olesen et al. 2011). Unfortunately, most of the network studies overlook the phenological component by either creating cumulative networks which sums all interactions observed over large areas/time periods or focus on a short time snapshot period taken as a putative peak of the phenological activity (see Hegland et al. 2010). The way to include phenology in the static bipartite networks is presented in chapter 1, by focusing primarily on flower abundances in order to identify phonological units upon which build bipartite networks (Fantinato et al. 2016).

Another set of important predictors could be searched in the species functional traits, that are defined as phenotypic features related to fitness (Violle et al. 2007; Volf et al. 2016). In systems where such fitness depends on other species, the traits of both interacting partners need to match. In food-webs, traits of both predators and prey determine who eats whom (Klecka and Boukal 2013): in the same way, we need to pay attention to pollinator and plant traits for explaining the structure of plant-pollinator networks (Eklöf et al. 2013).

Some studies explored the morphological-matching in a subsample of the pollinator fauna and the flowers they visited, such as those studies restricted on a target group of pollinators (Corbet 2000; Goldblatt and Manning 2000; Borrell 2005; Janeček et al. 2015). A positive relationship is generally reported between mouthpart length of a pollinator and nectar depth of a flower. In addition, other traits also play a role: corolla tube width determines plant fitness (Muchhala 2007); flower color variation affects visitation rates (Bradshaw Jr and Schemske 2003); even nectar volume and petal size play a role in interactions with pollinators although probably their variation does not exclude all pollinators at once (Schemske and Bradshaw 1999). As for the pollinators, the time needed for handling flower resources influences plant fitness (Temeles et al. 2009), as well as proboscis length do (Kulbaba and Worley 2012; Anderson et al. 2014).

Still, understanding how entire-community interactions are shaped by traits remains a key problem. This is exemplified by the recent work of Fornoff et al. (2017), in where only 2 out of 20 plant traits played a role in predicting visitation and pollinator richness. This limited predictability of pollination network interactions from functional traits (Bartomeus et al. 2017) might be due to the often opportunistic nature of these complex community-level interactions (Ponisio et al. 2017), together with prominent generalism (Waser 2006). In this extremely dynamical foraging behavior, the fact the interactions happens simply because they are possible seems to prevail over strict mechanisms based on trait matching.

However, some topological features of the plant-pollinator networks generated by nectar-producing plants and nectar-searching animals have been predicted by matching proboscis length and corolla depth (Stang et al. 2006, 2007): well predicted were the degree of generalization, the nestedness and the specialization asymmetry. Despite that, predictability of interactions by plant traits was quite low (Stang et al. 2009). However, rather than singularly, species traits operate hierarchically in shaping network interactions, so that a given trait determines species interactions only if other traits have already given way (Junker et al. 2013). In other words, visitation or pollinator richness is explained better when traits are considered combinations and interactions with other traits (Fornoff et al. 2017). In fact, flower traits seem to be organized in a hierarchical sequence that determines flower visitors and thus network structure: for example, in order of importance: flowering phenology, flower height, nectar-tube depth and floral reflectance, pollen-mass, sugar, anther position, phylogeny, display size, and abundance (Junker et al. 2013).

Furthermore, in mutualistic webs, species tend to organize interactions into groups: species that are more likely to interact among themselves are clustered together. Such units are named compartments or modules (Guimerà and Nunes Amaral 2005). In food-webs, causal effects of traits such as body-size and phylogenetic-relatedness were found on the compartmentalization of the interactions (Rezende et al. 2009). However, it is not yet well known how traits are distributed within modules in plant-pollinator networks, although some studies started to investigate this topic (Vizentin-Bugoni et al. 2014). Species qualitative features as the localization of rewards in the flower (superficial, half-way, deep) and the feeding mode of pollinators (chewing, siphoning, sponging) explained well the species organization into modules (Biella et al., unpublished). Also flower syndromes, i.e. a set of phenotypical traits given by coevolution among plants and visiting pollinators (Faegri and Van Der Pijl 1979), have been investigated for predicting network compartmentalization (Martín González et al. 2012). However, even though some plant traits were statistically related to modules plant composition, it often happens that they are not exclusive. In other words, if plants with high pollen production characterized a module with high visitation by bees and beetles, yet also another module with much lower production of pollen could be highly utilized by bees and beetles (Martín González et al. 2012). Therefore, it seems clear that how species traits determines network modularity deserves further investigation. In general, except for some case-studies, the way the plant and pollinator traits interplay when building networks has yet to be clarified.

The resource amount also play a role in the interactions. The framework of the Optimal Foraging Theory predicts that foraging behavior depends on energy content, handling time and encounter rate. These parameters vary among species but they may be predictable by species traits, as single pollinators respond to (some) flower traits, abundance and rewards (Rodríguez-Gironés and Santamaría 2006). Moreover, the handling time during a flower visit is affected not only by the size of proboscis and by body size but also by the tube length of the flower and by the amount of reward (Harder 1983). For example, different times are required for honeybees to handle different flower morphs (Giovanetti and Aronne 2013). Interestingly, honeybees prefer foraging on morphs requiring lower handling times when facing equally-rewarding flowers (Sanderson et al. 2006). In addition, foraging on long-tube flowers caused bumblebees with shorter proboscis to forage slowly than long-proboscis ones, while experimentally reducing flower tube-length increased the shorter-proboscis bees' handling time (Balfour et al. 2013). Therefore, not only the amount of resources but also the relative role of insect traits possibly rule plant-pollinator interactions.

The role of plant's functional traits and also the rate of resource consumption was investigated in chapters 4 and 5, in order to elucidate some mechanistic features on the forces that might have taken place after removing the most important generalist plants from the plant assembly.

Objectives of the thesis

Despite the enthusiastic development of studying plant-pollinator interactions in recent decades, several aspects remain unclear and deserve investigation, especially at a community level. In this thesis I explored the following aspects:

In chapter 1, I explored the possibility of informing conservation by means of network analyses of plant-pollinator interactions. In this study, the research questions were to: (a) explore the plant pollinator network topologies and interpret their implications for conservation; and to (b) identify the important species (hubs) that make the networks cohesive and also what features characterize them. These questions were investigated by considering the seasonal phenology of the species on a dataset of two season-wide plant-pollinator surveys, by means of a workaround that overcomes the conflict of using static networks for studying dynamical communities such as interactions in time.

In chapter 2, the ecological niche and potential distribution of a rare pollinator taxon of the Alps was characterized. The aims were related to its distribution, by (a) asking what environmental variables described the presence of the taxon; (b) estimating the suitable surface and quantifying changes in historic vs. present altitudinal occupancy; and (c) listing the plant species visited in the distribution area. The study used a dataset that was assembled especially for these scopes, by integrating entomological collections from multiple sources with literature's reports.

In chapter 3, the resources utilization by a social pollinator with declining population size was investigated by using molecular identification of pollen pellets. The worker population of a bumblebee species was experimentally halved and the scope of the research was to determine possible adjustments in the collected pollen diversity and the compositional overlap with other foragers at both the individual level of the workers and of the associated bumblebee-plant networks.

In chapters 4 and 5, I investigated the consequences of species loss on the pollination guild and on the interaction networks after a field manipulation of species-rich areas by sequential removal of the 4 most visited plant species, one at a time.

In chapter 4, the scope was focused on the pollinator guild as a coherent unit by characterizing the effect of plant removal: (a) on the general visitation in the sites and on the visitation of each plant-species (i.e., flower visitor's abundances); (b) on pollinators foraging redistribution by using plant traits similarity to the removed plants; (c) on the pollination effectiveness to plants and on the amount of standing crop of nectar resources with the removal sequence.

In chapter 5, the focus shifted to the plant-pollinator networks by investigating how the sequential removal of the generalists plants: (a) will produce alterations in networks structures; (b) will trigger reorganizational responses as changes in species level networks indexes and also as interaction shifts ("rewiring") at network's and at species' level. Furthermore, (d) what ecological drivers influenced the network structure, rewiring and pair-wise interactions were determined.

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Chapter I

Network analysis of phenological units to detect important species in plant-pollinator communities: can it inform conservation strategies?

Biella et al. (2017)

Community Ecology 18: 1-10. DOI: 10.1556/168.2017.18.1.1

Network analysis of phenological units to detect important species in plant-pollinator communities: can it inform conservation strategies?

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Chapter II

Distribution patterns of the cold adapted bumblebee Bombus alpinus in the Alps and hints of an uphill shift (Insecta: Hymenoptera: Apidae).

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Distribution patterns of the cold adapted bumblebee *Bombus alpinus* in the Alps and hints of an uphill shift (Insecta: Hymenoptera: Apidae).

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most probably, the rising temperature. The period from 1983 to 2012 was the warmest over the last 800 years in the Northern Hemisphere (IPCC, Intergovernmental Panel on Climate Change 2014). A warming of 0.2°C/decade occurred worldwide in the period from 1984 to1998 (IPCC, Intergovernmental Panel on Climate Change 2014), but high-altitude areas warmed even more at a rate of 0.4 °C/decade in the period from 1979 to1998 (Pepin and Seidel 2005). The temperature timeline in the Alps shows that greater warming rates and fewer cooling events have been the rule since the 1980s (Beniston et al. 1997).

Many rare species are retracting their distribution into marginal areas where their optimum niche is assured (Casey et al. 2015), and bumblebees are among them (Manino et al. 2007; Kerr et al. 2015). From our analyses, it is clear that *B. alpinus helleri* became restricted to the higher elevations of the Alps (upper-alpine altitudinal belt). Therefore, shrinkage in their altitudinal range raises serious concerns for the fate of the taxon when considering both the upper limit in the Alps and the reduction of land surface as altitude increases.

Conservation and management implications

Specific conservation actions have not been designed yet, despite the fact that *Bombus alpinus* is included in the European Red List of Bees (Nieto et al. 2014), is a Vulnerable species (Rasmont et al. 2015b), is considered extinct in some parts of its historic area of occurrence, and that it has been argued that it will disappear worldwide by 2100 (Rasmont et al. 2015a).

At a regional and local scale, short-term actions might be the following: (i)exchange of males between areas of proven occurrence inside protected areas, which would overcome the bumblebees' limited dispersal ability (Lepais et al. 2010); (ii) guaranteeing top-quality habitats in the available remnants, given the loss in altitudinal range.

The latter action could take place by limiting the activities which compete with habitat availability and quality. We propose the integral protection of areas surrounding glaciers, at least downwards to 2,400 m a.s.l., in order to limit human activities that could alter the habitat. This would be feasible, as half of

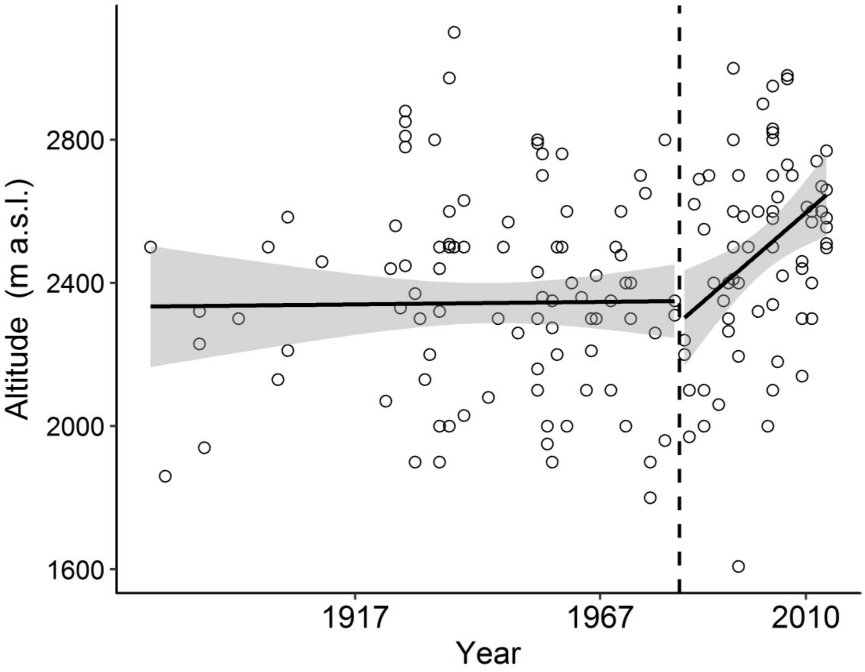

Figure 4. Long-term altitudinal records of *Bombus alpinus helleri*. Linear regressions (black lines), 95% confidence intervals (in grey). The dotted line shows the breakpoint year of changing slope, at the year 1984.

the suitable surface for this endemic bumblebee lies within protected areas. This is particularly urgent because, as clearly documented, the suitable areas for ski-pistes and the ones for high-altitude species are likely to overlap more as both shift to higher altitudes in response to climate change (Brambilla et al. 2016).

Other human activities to be considered concerns livestock. Conservation managers and decision-makers should carefully evaluate the real need for livestock and their density above the treeline

Simple models on cattle impacts are feasible (Barcella et al. 2016) and recent work suggests that low densities of livestock are the best for ecosystem functioning (Lázaro et al. 2016). In alpine prairies, livestock in high density competes with the bumblebees’ life cycle by removing the food sources (Lázaro et al. 2016) and even decreasing the habitat quality (Fleischner 1994). Biella (2015) presents a case in the Italian Central Alps, but this is a widespread situation.

Given that these activities are directly impacting ecosystems at high altitudes and therefore also *B. alpinus helleri*, short-term mitigation actions that would limit them are urgently needed.

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Electronic supplementary material

https://link.springer.com/article/10.1007/s10841-017-9983-1#SupplementaryMaterial

Figure S1 Study area (thin black lines indicate the borders of European countries, thick grey lines indicate the Alpine region, highlighted in grey).

List of data providers of the records used for SDM of B. alpinus helleri

Online resources (reported here)
A list of flower species visited by Bombus alpinus in the Alps

Species	Author	Species	Author
Androsace vitaliana	Pittioni 1942	Rhododendron ferrugineum	Manino A. et al.
Anemone sulforata	Pittioni 1942	Rhododendron ferrugineum	Kosior A.
Arnica montana	Pittioni 1942	Rhododendron sp.	Rasmont P.
Campanula cochleariifolia	Beaumont 1958	Salix hastata	Schedl 1982
Campanula scheuchzeri	Pittioni 1942	Salix helvetica	Schedl 1982
Campanula scheuchzeri	Schedl 1982	Saxifraga aizioides	Pittioni 1942
Campanula scheuchzeri	Manino A. et al.	Saxifraga aizioides	Schedl 1982
Cerastium uniflorum	Neumayer J.	Saxifraga aizioides	Beaumont 1958
Cirsium spinosissimum	Pittioni 1942	Saxifraga bryoides	Neumayer J.
Cirsium spinosissimum	Biella P.	Saxifraga oppositifolia	Pittioni 1942
Cirsium spinosissimum	Manino A. et al.	Saxifraga oppositifolia	Schedl 1982
Cirsium spinosissimum	Cornalba M.	Saxifraga oppositifolia agg.	Neumayer J.
Erica sp.	Beaumont 1958	Saxifraga oppositifolia agg.	Kreisch W.
Geum (syn. Sieversia) reptans	Schedl 1982	Sedum acre	Manino A. et al.

Hedysarum hedysaroides	Neumayer J.	Sempervivum tectorum	Pittioni 1942
Loiseleuria procumbens	Pittioni 1942	Silene acaulis	Schedl 1982
Loiseleuria procumbens	Rasmont P.	Silene acaulis	Manino A. et al.
Lotus alpinus	Manino A. et al.	Silene acaulis	Bossert S.
Lotus alpinus	Rasmont P.	Silene acaulis	Lindner
Papaver alpinum	Pittioni 1942	Silene acaulis	Rasmont P.
Pedicularis rosea	Manino A. et al.	Silene vulgaris	Pittioni 1942
Pedicularis rostrato-capitata	Neumayer J.	Soldanella alpina	Schedl 1982
Pedicularis verticillata	Rasmont P.	Soldanella alpina	Manino A. et al.
Phyteuma globulariifolium	Manino A. et al.	Thymus sp.	Manino A. et al.
Phyteuma globulariifolium	Neumayer J.	Trifolium alpinum	Manino A. et al.
Phyteuma globulariifolium	Kreisch W.	Trifolium badium	Biella P.
Phyteuma hemisphaericum	Pittioni 1942	Vaccinium gaultherioides	Manino A. et al.
Phyteuma hemisphaericum	Dalla Torre K.	Vaccinium myrtillus	Schedl 1982
Phyteuma sp.	Pittioni 1942	Vaccinium myrtillus	Rasmont P.
Phyteuma sp.	Schneller B.	Vaccinium uliginosum	Pittioni 1942
Rhododendron chamaecystus	Pittioni 1942	Viola calcarata	Pittioni 1942
Rhododendron ferrugineum	Pittioni 1942		

Chapter III

Integrative approach reveals a constancy in the foraging strategy of a social pollinator after a strong reduction of the colony's workforce.

Under review

Integrative approach reveals a constancy in the foraging strategy of a social pollinator after a strong reduction of the colony's workforce.

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Abstract

Pollinators are overpowered by environmental stressors, resulting in population declines. This situation could trigger, in the remaining foragers, density dependent individual responses as higher specialism at lower forager density. Conversely, in social insects, the survived foragers could behave as generalist for compensating the less amount of incoming resources to the nest. Therefore, population decline could induce changes at both the individual foraging and the sites' networks of feeding. To test that in the field, colonies of the model species Bombus terrestris were experimentally manipulated by removing half of the workers in order reproduce a commonly detected situation of population decline. To identify which plants were foraged, DNA metabarcoding on pollen pellet samples was performed by targeting at the ITS2 region using High Throughput Sequencing (HTS). A ROC approach (Receiver Operating Characteristic) was used for filtering of sequencing reads, and taxonomical assignments were based on a molecular reference dataset of the plant species found at the studied area. Despite a high plant diversity in the pollen pellets (i.e. mainly polyfloral with at least 34 species), only minor changes in the diet breadth or in the feeding networks were found after removal. The constancy in feeding strategy indicates that foragers may not expand their diet and may not compensate for the lack of resources when the population size is halved, with plausible implications for assimilation of resources and fitness. DNA metabarcoding and network analyses revealed that, in the field, pollinators may actually not shape their foraging to the stressing environment.

Introduction

Pollinators are responsible of the ecosystem service of pollinating plants. However, wild pollinators are facing steady population declines due to climate change (Kerr et al., 2015) and several "pollinator-unfriendly practices". Some of these are related to agriculture, such as general intensification, the use of monocultures, the use of harmful agrochemicals (Ollerton et al., 2014; Rundlöf et al., 2015), the reduction of natural ecosystems nearby fields (Osgathorpe et al., 2011), and the use of synthetic fertilisers that reduce key food resources for pollinators by causing shifts in the vegetation (Isbell et al., 2013). Moreover, land use change (Jha, 2015) and the lack of flower diversity, for instance due to overgrazing or frequent mowing, also have negative effects on the extent of pollinators' populations (Buri, Humbert & Arlettaz, 2014; Lázaro et al., 2016). Additionally, the spread of parasites and diseases (Smith, Sax & Lafferty, 2006) and the overwhelming competition from domesticated bees (Lindström et al., 2016; Norfolk, Gilbert & Eichhorn, 2018) alter the dynamics of wild pollinator populations. Declining pollinators might undermine both the extent of their wild populations and the sexual reproduction of wild plants and crops.

For detecting the interactions among a pollinator and a plant, different sampling approaches can be applied, such as the direct observation of an insect's behaviour (Biella et al., 2017) or the analysis of plant pollen found on the insect (Bosch et al., 2009; Galliot et al., 2017). Investigating these aspects in richly biodiverse habitats might be challenging mainly due to the required high sampling effort. Nevertheless, detecting all interactions of a pollinator's trip and drawing pollination networks is possible from the analysis of pollen on an insects body (Galliot et al., 2017). Furthermore, data from pollen analyses can also reveal the rarest interactions, normally undetected in observation surveys (Bosch et al., 2009; Pornon et al., 2017). Yet, morphology-based identification of pollen lacks a uniform discriminatory power and requires great taxonomical knowledge (Müller et al., 2006; Williams & Kremen, 2007; Galimberti et al., 2014). However, the potential benefits of pollen studies highlight the need to improve methods alternatively to the morphological analyses. In this context, DNA-based approaches, such as DNA barcoding and DNA metabarcoding, represent reliable approaches (Taberlet et al., 2012).

By using integrative approaches (e.g. DNA metabarcoding applied to ecological questions), the resource usage by declining pollinators can be explored in more depth. The social pollinators as the bumblebees (Hymenoptera: Anthophila: Apidae, *Bombus*) are subjected to multiple stressors that ultimately causes population reductions (Kerr et al., 2015). For instance, pathogens, such as the trypanosome *Crithidia bombi,* can kill more than half of a bumblebee colony's stressed workers (Schmid-Hempel, 2001). In addition when fed pesticides, up to 45% of the newly born bumblebee workers do not live longer than 4 days, which is much less than the normal life expectancy, and 50-55% of the workers are unable to return to their nest (Gill, Ramos-Rodriguez & Raine, 2012). In honeybees, survival is strongly reduced by 50% from the synergistic effects of both poor nutrition and pesticide exposure (Tosi et al., 2017). Such variation in pollinator population densities may modify the foraging. On one hand, at the level of individual forager, intraspecific competitive interactions can lead to a higher specialism at low density of foragers (Fontaine, Collin & Dajoz, 2008). On the other hand, when social insects' colony is reduced, a generalist strategy is expected in order to gather enough resources despite the few foragers (Kaluza et al., 2017). Therefore, being a generalist or a specialist could depend on variations of populations size.

In this work, we experimentally manipulated commercial colonies of the bumblebee *Bombus terrestris* (Linnaeus, 1758) by removing half of the colony's workforce for reproducing a situation of population decline due to the environmental conditions. We aimed at exploring if bumblebees could change the resource use by investigating (i) how individual bees are able to adjust their foraging to such a treatment by studying the plant diversity in collected pollen pellets and (ii) the changes in the associated bumblebee-plant networks. To achieve this goal, we applied a DNA metabarcoding approach. Due to the high precision and sensitivity of this tool, we employed a ROC method (instead of a fixed cut of threshold) for the filtering sequencing reads and a molecular reference dataset of the local plant species. These increased the power of the sequencing protocol and provided biologically reliable data. In this way, we were able to investigate in detail how bumblebees utilize plant resources for pollen after a treatment consistent with the previously documented reductions of bumblebee populations. Our experimental design provided new insights into the ways social pollinators respond to environmental or anthropic events by interacting with plant resources in the context of pollination ecosystem services.

Material and methods

Study area, experimental set-up and samples collections

The experiment was conducted in a meadow near Český Krumlov, 18 km southwest of České Budějovice (Czech Republic) in two locations 750 m apart (site 1 48°49'30.52"N, 14°19'4.02"E; site 2 48°49'53.53"N, 14°18'57.91"E). The study sites belong to a 62 hectare natural area located at an altitude of 600 m a.s.l. and is constituted of forest, isolated trees, and shrubs and is mainly covered by species rich calcareous grasslands managed by occasional extensive grazing. Around this zone, a mosaic of rural areas and urban settlements occur.

The experiment and the collection of samples were carried out on sunny days without strong wind or rain, in summer 2016. Number of used colonies lies within the range used in other studies about bumblebees foraging (Baude et al., 2011; Leonhardt & Blüthgen, 2012; Geslin et al., 2014; Ruedenauer, Spaethe & Leonhardt, 2016), and also the number of samples matched other studies on DNA metabarcoding of pollen (Gresty et al., 2018; Lucas et al., 2018). Four commercial colonies of *Bombus terrestris* were bought from a private company (Koppert s.r.o., Nove Zamky). At each of the two sites, two colonies were placed. One colony was used as control and the second one was used for testing the treatment, which consisted of manually removing half of the worker population of that colony.

The experiment took place as follows. All colonies were marked and placed in the field under shade to prevent overheating. After 4 days for each colony, the pollen pellets were collected from the corbiculae of the legs of 18 bumblebee workers anesthetized with CO_2 just before entering the nest, ("before" phase, 6th-11th July). After this sampling, the nests to be treated by worker removal were sedated with CO_2, the bumblebees were counted, and half of the worker population was removed and killed. Later on ("after" phase, 20th-23rd July), pollen pellets of 18 workers for each colony were collected in the same way as the "before" period. Overall, 72 samples were collected in the period before treatment, and another 72 samples were collected in the period after treatment. Pellets were collected with sterile tweezers and placed in Eppendorf tubes, marked with codes and placed in refrigerator at -20 °C.

Local botanists provided an accurate check-list of the flowering plant species at the study area (i.e., 112 plant species). Those species that were not available in public nucleotide databases (i.e., NCBI and BOLD) were sampled (i.e., 54 plant species, one or two young leafs each, stored at −20 °C) to create a complete DNA barcoding reference dataset. Reference ITS2 sequences for the remaining species were directly retrieved from GenBank NCBI prior to accurate validation of the accessions (i.e., availability of voucher details and complete overlapping with the DNA barcoding region sequenced in bumblebees' pollen pellets).

Overall, the final reference dataset encompassed 1196 ITS2 sequences.

DNA analyses and taxonomical assignments

Reference ITS2 DNA barcodes for the sampled plant species were obtained as described in (Mezzasalma et al., 2017) and deposited in EMBL GeneBank.

For each bumblebee, one pollen pellet was grinded with a Tissue Lyser LT (Quiagen, Hilden, Germany) prior to freezing the sample in liquid nitrogen. The total DNA was extracted by using the EuroGOLD Plant DNA mini kit (EuroClone, Pero, Italy), following the manufacturer's instructions and using a final elution volume of 70 µl.

Pollen load identification was performed through a DNA metabarcoding approach targeting the nuclear internal transcribed spacer 2 region (ITS2). This locus was successfully used for characterizing pollen-mixed samples in several recent studies (Chen et al., 2010; Keller et al., 2015). DNA libraries for each sample were prepared following Illumina guidelines (16S Metagenomic Sequencing Library Preparation, Part #15044223 Rev. B) with modifications for ITS2 sequencing. The ITS2 region was amplified using primers S2F and S3R (Chen et al., 2010) with the addition of the Illumina overhang adapter sequences, namely

S2F_Seq: 5’TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATGCGATACTTGGTGTGAAT 3'

S3R_Seq: 5’GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACGCTTCTCCAGACTACAAT 3'.

Before amplification, DNA extracts were normalized by means of Quantitative real-time PCR (qPCR) Ct values with the same amplification primer pairs and the same protocols described by (Bruno et al., 2017). PCR reactions contained 12.5 µl of KAPA HiFi HotStart ReadyMix PCR Kit, 5 µl of each primer 1 µM (forward and reverse) and 2.5 µl DNA (maximum volume of DNA per sample with 5ng/µl DNA concentration). Samples were initially denatured at 94° for 5 min, then amplified using 40 cycles of 94° for 30 s, 56° for 30 s, and 72° for 45 s. A final extension (72°) of 10 min was performed at the end of the programme to ensure complete amplification. All PCR amplifications were prepared under an UV PCR cabinet to avoid contamination. The success of amplification was tested on a 1.5% agarose gel-electrophoresis. A 100 bp mass ladder (GeneDirex 100 bp DNA Ladder RTU, FroggaBio Inc., Toronto, ON, Canada) was used to confirm the successful normalization of the amplicon concentration within the samples.

Index PCR and library sequencing were performed through the Illumina MiSeq instrument using MiSeq Reagent Kit v3 (2 x 300-bp paired-end sequencing). The library preparation and the sequencing process were conducted at BMR Genomics (Padova, Italy). Raw Illumina reads were paired and pre-processed using *USEARCH* 8.0.1623 (Edgar, 2010). Reads were filtered out if ambiguous bases were detected and lengths were outside the bounds of 250 bp. Moreover, an expected error of 1 was used as an indicator of read accuracy. Pollen pellet composition was obtained using *--cluster_fast* algorithm from *VSEARCH.2* software (https://github.com/torognes/vsearch) (Rognes et al., 2016) with a 99% sequence identity. The cluster centroid was chosen as the representative sequence of the cluster. The taxonomic assignment of the representative sequences was carried out using the *BLAST* algorithm

(Altschul et al., 1990) against the reference DNA barcoding dataset of the study area, accepting only assignments with Max Identity and Query Coverage > 98%. OTU representative sequences showing assignment values of MI and QC < 98% with the use of this database were assigned using the GenBank NCBI database.

Selection of OTUs

The need of identifying false positives in data originated from the metabarcoding approach has been recently underlined (Bell et al., 2018). In order to exclude false positives in the OTU assignment step from the dataset, the ROC (Receiver Operating Characteristic) framework was used to quantify a trade-off of acceptance or rejection of OTUs within the analyzed pollen samples. The ROC framework assesses the true positive rate and the true negative rate of a test (Metz, 1978), based on the Youden index. This approach can improve the reliability of OTU assignments by establishing defensible thresholds for rejection or acceptance (Serrao, Reid & Wilson, 2017). This is a well-accepted methodology for threshold detection, since it is used in several biological fields, including DNA- and environmental DNA-based studies (Nutz, Döll & Karlovsky, 2011; Serrao, Reid & Wilson, 2017).

In the samples of this study, some OTUs were represented with only a very low number of reads. This would hint at the presence of false positives. Therefore, in order to find reliable thresholds, we followed the suggestions of (Serrao, Reid & Wilson, 2017) which employs ROC curves instead of arbitrarily cut-off values for excluding OTUs from the samples.

Specifically for each sample independently, a categorical variable “negative” was assigned to the OTUs with 0 number of DNA reads and “positive” was assigned to the OTU with reads >0. A GLM (Generalized linear regression) with an overdispersed Poisson distribution (quasipoisson) was performed independently on each sample in order to estimate the distribution of reads related to positives and negatives; the amount of reads per OTU was response variable and "positive" or "negative" was the predictor variable. On the values estimated by the regressions, the *pROC* package (Robin et al., 2011) in the R environment (R Core Team, 2017) was used to estimate the per-sample cutting threshold and thus to identify which OTUs were false positives. In our samples, the obtained range of cutting thresholds was at 0.81% - 3.4% of the total amount of reads in the samples, with an average across samples of 2.3%. Those OTUs with a number of reads below the thresholds were excluded from the dataset and considered as false positives.

Data analyses

For each commercial bumblebee nest and at each level of the treatment (“before”, “after”), matrices of bumblebees and plants were analysed to investigate changes in the foraging behaviour of bumblebees and in the insect-plant interaction networks.

In our networks, a given plant species or a given bumblebee is considered to be a “node”. We tested for changes in pollen collected by calculating several node-level indexes, as follows: (a) the “degree”, that is number of plant species in a pollen pellet; (b) RR, the “resource range”, estimates the fraction of used resources to the total available (Schoener, 1989) and is computed here as $1-\frac{R-r}{R-1}$, where R are the available resources (= plants) and r are the used ones; (c) PG, the “proportional generality”, is the quantitative diversity of consumers in relation to the potential one of resources; it is computed as the ratio between the power of the quantitative Shannon diversity H for consumers p and that for the abundances of resources q: e^{H_p}/e^{H_q}; (d) PDI, the “Paired Difference Index”, is the quantitative counterpart of RR and it compares the strongest quantitative interaction with all remaining interactions (Poisot et al., 2012); it characterizes the decay of performance as drift from the optimal resource; it is calculated here as $1-\frac{\sum_{i=2}^{R}(P_{max}-P_i)}{R-1}$, where P_{max} is the maximum of all quantitative interactions, P_i is the quantity of interaction with the i plant, and R is the number of available resources (=plants); (e) *d’* index, which is a measure of specialization based on niche overlap among species (Blüthgen, Menzel & Blüthgen, 2006) and is calculated as $\sum_{j=1}^{R}(p'_{ij}\, ln\frac{p'_{ij}}{q_j})$, where R is the number of resources, p'_{ij} is a species’ i interaction with partner j as proportion of the sum of interactions of i, q_j is the sum of interactions of partner j divided by the total of all interactions; (f) the “closeness centrality” which indicates how a plant is near the core of the interactions based on the path lengths of the network; $CC = \frac{R-1}{\sum_{i:i\neq v} d(v,i)}$, where R is the available plants and $d(v,i)$ is the geodesic distance between plant v and i (Freeman, 1978). Indexes (a), (b), and (f) are calculated from the binomial interaction matrices (presence or absence of a plant in a sample), while indexes (c), (d), and (e) are based on the quantitative interaction matrix including the number of DNA reads of a certain plant species in a pollen pellet. Using DNA reads as a proxy of a quantitative amount of pollen was decently supported in Bell et al. (2018) and was already applied to networks in Pornon et al. (2017); these indexes include normalizations by matrix total.

For testing changes in these indexes, each one was analysed with generalized linear mixed-effect models with library *lme4* (Bates et al., 2015) in the R environment with a given index as response variable, treatment as a predictor variable (“before”, “after” worker removal), and nest identity as the random intercept. Poisson distribution or Gamma distribution with the log link function were used, depending on the response variable.

To test whether the ecological networks changed after the treatment, the interaction matrices included either binary interaction matrices or the count data of the DNA reads, such as in (Pornon et al., 2017), standardized by the total of the matrix. For each nest, the network structure was studied by means of 9 indexes: (a) Links density LD (Bersier, Banašek-Richter & Cattin, 2002), which is based on the quantitative measure of the plants’ and bumblebees’ contribution to the network, is computed as $LD = \frac{1}{2}\left(\sum_{j=1}^{s} \frac{b_{j.}}{b_{..}} 2^{H_q} + \sum_{i=1}^{s} \frac{b_{i}}{b_{..}} 2^{H_p}\right)$, where s is the number of species in the networks, $b_{..}$ is the total sum of the matrix, $b_{j.}$ is the sum of the interactions of bumblebees j, H_q is calculated as $-\sum_{j=1}^{s} \frac{b_j}{b_{j.}} log_2 \frac{b_j}{b_{j.}}$ with b_j as an interaction (and similarly for plants H_p and plant species i). (b) Connectance C (Bersier, Banašek-Richter & Cattin, 2002), which is the proportion of realized links calculated as $= \frac{L}{I*J}$, L is the number of interactions, $I,$ and J is the number of plant and animal species, respectively, and can vary from 0 to a maximum of 1. (c) Nestedness based on Overlap and Decreasing Fill (NODF) and (d) the weighted counterpart WNODF (Almeida-Neto et al., 2008), is based on decreasing fill and on paired overlap on the matrix. Between pairs of columns and pairs of rows, it detects the degree of nestedness N_p by comparing the marginal totals and the proportion of filled matrix cells located at the same position. Thus, for a matrix with i plants and j bumblebees, $NODF = {\sum N_p} / {\left[\frac{i(i-1)}{2}\right] + \left[\frac{j(j-1)}{2}\right]}$. It ranges from 0 to 100 (fully nested). (e) Modularity Q, and (f) the quantitative counterpart Q_w, computed by the algoritm *DIRTLPAwb+* (Beckett, 2016), namely a measure of the nodes partition into separate modules (or clusters of interactions); Q is computed as $\frac{1}{m}\sum_{i=1}^{r}\sum_{j=1}^{c}\left(A_{ij} - \frac{k_i d_j}{m}\right)\delta(\mathrm{g_i}, \mathrm{h_j})$, where A_{ij} is the interaction matrix of r rows and c columns, m is the number of links, k is the node degree for a plant with label h , and d is the node degree for a bumblebee with label g, while the Kronecker function $\delta(\mathrm{g_i}, \mathrm{h_j})$ is 1 if nodes i and j belong to same module or 0 otherwise. Q and Q_w range from 0 to its maximum 1. (g) Interaction Diversity H_2' is a measure of generalization of network-level interactions. It is computed as $\sum_{i=1}^{r}\sum_{j=1}^{c}(p_{ij} * lnp_{ij})$, with r and c referring to rows and columns of the interaction matrix between a plant species i and pollinator species j, and p_{ij} is the proportion of the number of interactions in relation to the respective row total. Its possible maximum and minimum are obtained from the distribution of interaction totals of the matrix and used to normalize the index to vary between 0 and 1 (perfect specialisation) (Blüthgen, Menzel & Blüthgen, 2006); (f) Generality and (g) Vulnerability are mean effective numbers of partners, that is of plants for bumblebees (Generality G) and of bumblebees for plants (Vulnerability V), calculated from the weighted interaction matrix and thus weighted by the marginal totals; they are calculated as $\frac{1}{l/s}\sum_{j=1}^{s} b_{ij}$, where a node i is interacting with a node j, and b_{ij} is the sum of quantitative interactions between i and j, and the total number of links in the network is l and that of nodes is s (Bersier, Banašek-Richter & Cattin, 2002).

Changes in these indexes of network structure were tested by means of random permutations of the data, which test whether the difference between the observed networks is significant with respect to random expectations. To reach this goal, the interactions (matrix cells) of both networks were swapped randomly between the two networks (“before”, “after”), following (Farine, 2017) and (van Borkulo et al., 2015), for 10000 times for each of the two networks. After each swap, the value of the difference was recalculated. The statistical significance was obtained by comparing the observed difference to the distribution of differences from the random permutations.

The node and the network indexes were calculated with the packages *bipartite* (Dormann, Gruber & Fründ, 2008) and *vegan* (Oksanen et al., 2018) in R.

Results

Sequencing, filtering, and taxonomic assignment of pollen loads

Illumina sequencing of pollen samples yielded 18,473,760 raw reads. After pair-ending and quality filtering, 5,600,000 reads were used for the subsequent analyses and were clustered in 167 OTUs. Of these, 51 OTUs (30.5%) showed high similarity with fungi accessions and were excluded from the dataset. The remaining 116 OTUs were assigned to 44 plant taxa and specifically 90 OTUs (53.9%) to the species level and 26 OTUs (15.5%) to the genus level. The ROC selection excluded 25 OTUs corresponding to at least 10 plant species. Therefore, the filtered list of plant species encompassed 34 taxa (91.2% with species identity) with a mean of 2.25 taxa per sample, st. dev. = 1.54, min. 1 and max. 10. The most frequent families in this list were Fabaceae (23.5%), Asteraceae (14.7%), and Rosaceae (11.8%). Ten taxa were not initially included in the floral checklist.

Monofloral pollen pellets were 37% (53 samples), while 63% (90 samples) were classified as polyfloral. Monofloral pollen loads were mainly composed of seven taxa: *Tilia cordata* Mill. (Tiliaceae), *Rhinantus major* L. (Orobanchaceae), *Rubus caesius* L., *Filipendula ulmaria* L. (Rosaceae), *Lotus corniculatus* L. (Fabaceae), *Papaver somniferum* L. (Papaveraceae), *Plantago* *media* L. (Plantaginaceae). Among the polyfloral pollen loads, 44 samples were composed of two taxa, while the other 46 samples of more than two taxa.

Changes after treatment, node- and network-level responses

Taxa composition of the samples changed over the study period, both in the control and in the treated nests (Supplementary Figure S1).

As for the nodes' indexes (a plant species or a bumblebee in the network), despite some variations, the degree and proportional generality were low but plant closeness was high. PDI and RR were both low, while d' spanned over a wide range of the specialization-generalization gradient (Fig. 1). Changes after treatment were not significant, except for the quantitative Proportional Generality Index, which changed significantly only in the control nests (Table 1 and Fig 1).

For the network analyses (Fig. 2, Table 2) in the treated colonies, none of the binary indexes changed significantly. On the other hand, only two of the quantitative indexes (i.e., the linkage density and vulnerability of plants) significantly decreased in one treated colony, while they increased in the other treated colony.

Discussion

Previous studies on the foraging activity of bumblebees mainly focused on altering a diet and investigating adjustments in foraging in laboratory conditions (Fontaine, Collin & Dajoz, 2008; Baude et al., 2011; Ruedenauer, Spaethe & Leonhardt, 2016). Noteworthy, our study investigated how reductions in colony size would affect the resource utilization by these key pollinators when free to forage in the field.

The identification of pollen using DNA metabarcoding was realiable, because the plant list found in the pellets matches other central European surveys (Teper, 2004; Kleijn & Raemakers, 2008). However, the integrative approach yielded several plants (29% of the taxa) that were not found in the botanical survey of the natural area, but rather occur in the neighbouring landscape mosaic (namely in gardens, crop fields and road margins).

Table 1 – Individual level foraging indexes tested for significant changes after halving the colony workforce, by GLM. Statistical significance is highlighted in bold.

	Type	**Treated nests**	**Control nests**
(a) Degree	Binary	χ^2= 0.11, df=1, p = 0.74	χ^2= 0.53, df=1, p = 0.467
(b) Resource range	Binary	χ^2= 0.897, df=1, p = 0.344	χ^2= 0.157, df=1, p = 0.692
(c) Proportional generality	Quantitative	χ^2= 1.41, df=1, p = 0.235	χ^2= 5.113, df=1, p = **0.024**
(d) PDI, Paired Difference Index	Quantitative	χ^2= 0.322, df=1, p = 0.571	χ^2=0.151, df=1, p = 0.698
(e) d', Complementary specialization	Quantitative	χ^2= 0.2, df=1, p = 0.655	χ^2= 0.2, df=1, p = 0.648
(f) Plant closeness centrality	Binary	χ^2= 1.28, df=1, p = 0.258	χ^2= 2.092, df=1, p = 0.148

Furthermore, the 34 plant species that were found highlight how polylectic bees normally rely on a wide set of flowering species. This is true for honeybees, several bumblebees, and other polylectic wild bees (Müller et al., 2006; Galimberti et al., 2014; Saifuddin & Jha, 2014).

Consistently with the literature (Teper, 2004), more than 63% of the pollen load samples recovered from the bumblebees were polyfloral. These are considered particularly beneficial for larvae alimentation in polylectic bees. Pollen is the main source of nutrients and secondary metabolites for bees, such as C, N, S, P, K, Na, Mg, Fe, Zn, Cu, vitamins, and antioxidants (Filipiak et al., 2017). Since the amount of micronutrients in the pollen is variable among plant taxa (Müller et al., 2006), an unbalanced and un-nutritious diet can limit development and fitness when plant diversity in the field is low (Filipiak et al., 2017). Therefore, stocking polyfloral pollen pellets in the nest is considered to

Table 2 – Site level netwok indexes tested for change during the experiment time (before and after the removal of half workers’ amount) by 10000 random permutational swaps of interactions between the networks before and after the treatment. Statistical significance is highlighted in bold.

		Site 1 Treated			**Site 1 Control**		
	Type	**before**	**after**	**p**	**before**	**after**	**p**
Links Density	Quantitative	3.00	1.87	0.006	2.79	3.35	0.335
Connectance	Binary	0.17	0.15	0.221	0.21	0.16	0.050
NODF	Binary	17.21	11.55	0.054	11.56	9.62	0.482
Weighted NODF	Quantitative	12.77	12.45	0.925	9.76	10.31	0.881
Modularity	Binary	0.42	0.50	0.090	0.44	0.47	0.581
Weighted Modularity	Quantitative	0.65	0.75	0.151	0.67	0.60	0.248
H2'	Quantitative	0.85	0.92	0.365	0.86	0.79	0.362
Generality	Quantitative	1.67	1.47	0.388	1.40	1.76	0.089
Vulnerability	Quantitative	4.34	2.27	0.004	4.19	4.94	0.459

		Site 2 Treated			**Site 2 Control**		
	Type	**before**	**after**	**p**	**before**	**after**	**p**
Links Density	Quantitative	2.94	5.57	0.043	3.70	4.97	0.224
Connectance	Binary	0.25	0.25	1.000	0.22	0.24	0.677
NODF	Binary	7.68	7.96	0.926	9.06	10.73	0.625
Weighted NODF	Quantitative	8.33	5.18	0.613	9.58	9.91	0.960
Modularity	Binary	0.48	0.47	0.829	0.50	0.47	0.634
Weighted Modularity	Quantitative	0.68	0.26	0.015	0.60	0.37	0.046
H2'	Quantitative	0.87	0.83	0.768	0.86	0.74	0.310
Generality	Quantitative	1.34	1.17	0.381	1.37	1.38	0.969
Vulnerability	Quantitative	4.55	9.97	0.032	6.02	8.55	0.199

be an adaptive advantage in order to overcome this risk and thus strengthen the nutraceutical value of the diet and immunocompetence (Alaux et al., 2010). Conversely, monofloral pollen are made from those plant species which are exceptionally high in nutritional value. Our data confirms that bumblebees were able to collect multiple types of pollen, providing both polyfloral and monofloral pellets to their larvae, possibly depending on the nutritional value of the foraged plant taxa.

Over the study period, the pollen changed in plant composition, since the plant list from the “before” period was different from the “after” workforce removal period in all colonies, both treated and control (Supplementary Figure S1). Although major phenological shifts were not detected in the field, it is possible that several plants shifted their flower determination status while still blooming, namely by ceasing the phenological stage of the pollen production or starting it if previously not developed (Lloyd, 1980). Despite that, substantial changes in the foraging strategies in the bumblebees were not triggered during the treatment period, as indicated by the individual node level indexes and by indexes of the binary networks. Therefore, the bumblebees changed the set of visited plants, but not the way they utilized them.

Although it was shown that a higher abundance of foraging bumblebees can lead to the usage of more plants and thus to higher generalism (Fontaine, Collin & Dajoz, 2008), our study did not find higher specialism at lower abundances of foragers (“after” removal period). In fact, choice experiments revealed that plant usage was only slight at higher bumblebee densities (Geslin et al., 2014). Furthermore, higher forager densities did not change bumblebee foraging behavioural traits (Baude et al., 2011). On the whole, density dependent foraging strategies in pollinators deserve further study in order to clarify how resources are collected in relation to foragers density, at least under field conditions.

When foragers of social pollinators are few, colony level strategy should shift towards higher generalism for compensating for the consequent lack of incoming resources. However, in the treated colonies, changes in the structure

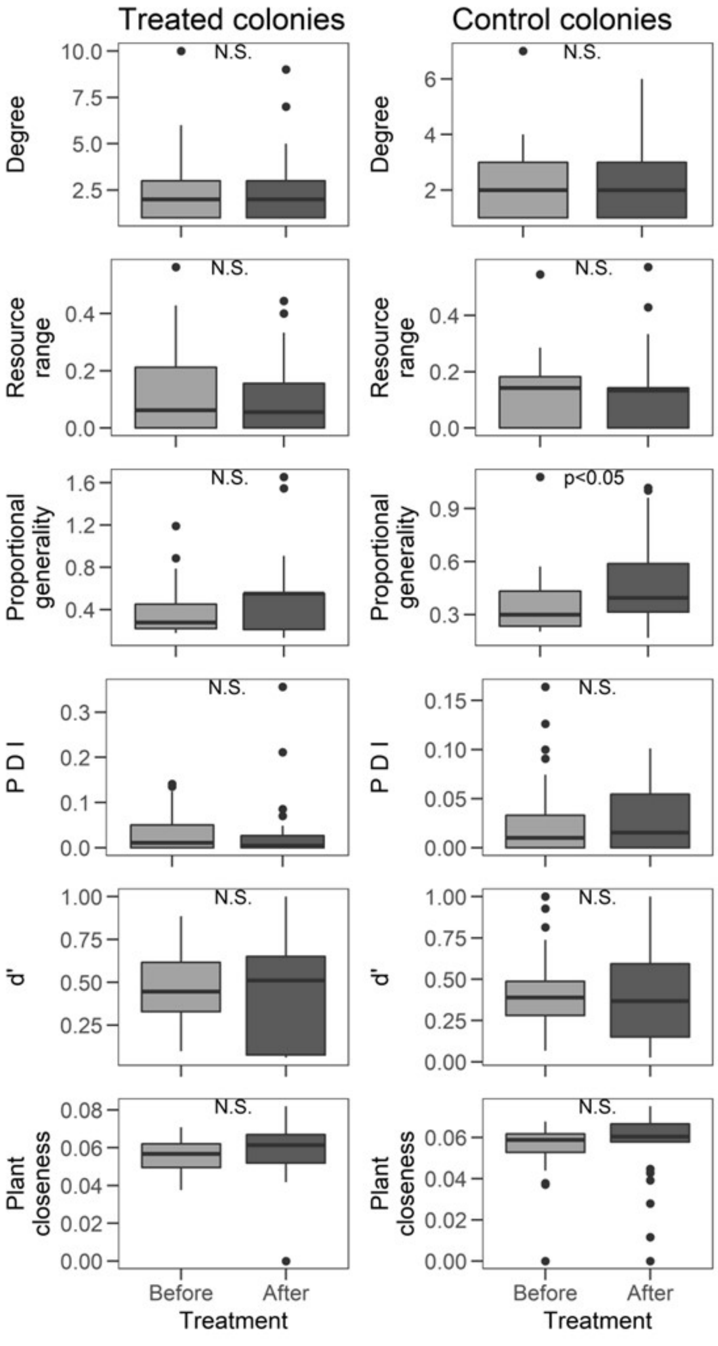

Figure 1: Individual level indexes of foraging (“a”-“f” panels) tested for change during the experiment in both treated and control bumblebee colonies. “N.S” signifies not statistically significant.

of the networks were not observed despite the reduction in bumblebee abundance (indexes of binary networks in Table2, Fig. 2). Nevertheless, in quantitative networks, the link density and plant vulnerability changed significantly in the treated nests. They are both related to the quantity of the resource used by foragers. However, they did not change in the same way in both treated nests: if they increased in one, they decreased in the other one. This among-colonies variability suggests a lack of a unique strategy for coping with reduced colony size in the treated nests. The explanation of this might rely on two aspects of bumblebees' biology. Firstly, bumblebees are primitively eusocial, which implies that colonies' performance tend to rely more on individual choices of single foragers than on social information (Leadbeater & Florent, 2014), and therefore a unique, shared strategy in all colonies cannot be expected. Furthermore, workers of the used model taxon *Bombus terrestris* have almost null contact with larvae development (Goulson, 2003), and foragers might not have acquired any information on the rate of development of the larvae nor on the amount of incoming resources. Thus, by relying on individual quality assessment (Ruedenauer, Spaethe & Leonhardt, 2016), the lack of direct feedback between larvae and forager could unpair the foraging choices and the colony growth rate.

These aspects could have prevented the bumblebees from the treated colonies from adapting their foraging strategy to the new conditions of a reduced workforce and thus from coping with less resources arriving to the nest. In fact, individual bumblebee foraging changed only slightly in the treated nests: the network nodes' indexes revealed small and not significant changes in specia lism/generalism, in the number of gathered plants, in the proportion of the available resources actually collected, and in the centrality in the plant network (importance based on the position in the network).

In this framework, DNA metabarcoding of pollen samples sheds light on a previously unresolved aspect of the bumblebee foraging dynamic, related to resources collection and to density dependent foraging in the field. After applying a realistic reduction of the bumblebee workforce, the actual foraging strategies were maintained by this social pollinator even at a reduced density of foragers. This implies that bumblebees are in fact not adapting their foraging to

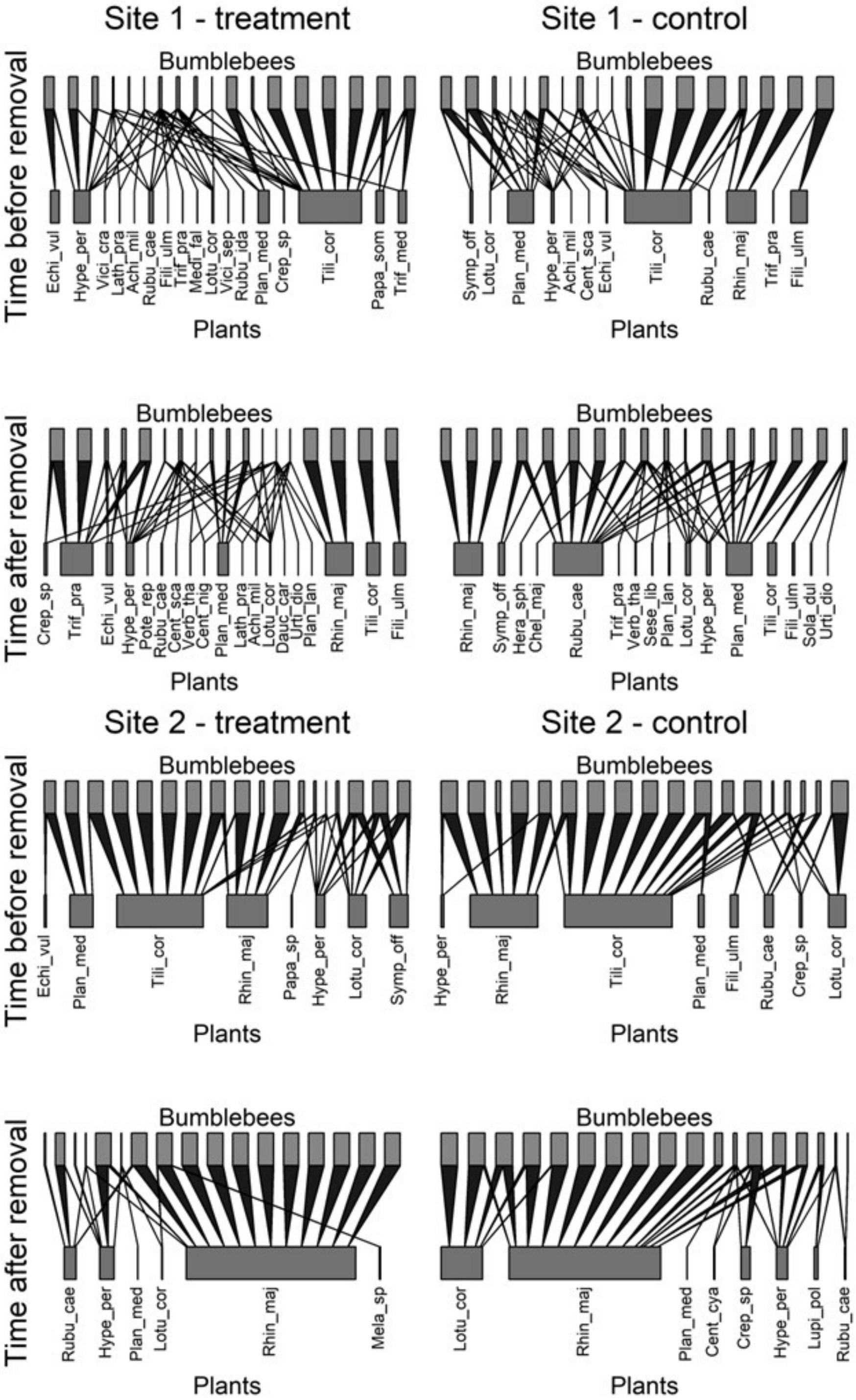

Figure 2: Bumblebee-plant networks during the experimental time: before (plot's panels "a" and "c") and after (plot's panels "b" and "c") the removal of half workers' amount.

the lack of incoming resources induced by less workers being available. This aspect might have consequences on the extent of pollinator populations, since having less resources could impact development, fitness and ecosystem service.

Conclusion

Bumblebees' foraging for pollen was investigated after an experimental removal of half of the colonies' workforce settled in species-rich grasslands within a land-use matrix. In this framework, DNA metabarcoding of pollen samples sheds light on a previously unresolved aspect of the bumblebee foraging dynamic, related to resources collection and to density dependent foraging in the field. After applying a realistic reduction of the bumblebee workforce, the actual foraging strategies were maintained by this social pollinator even at a reduced density of foragers. This implies that bumblebees are in fact not adapting their foraging to the lack of incoming resources induced by less workers being available. This aspect might have consequences on the extent of pollinator populations, since having less resources could impact development, fitness and ecosystem service.

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DNA Deposition

The raw sequences obtained in this study were submitted to the European Nucleotide Archive (https://www.ebi.ac.uk/metagenomics/). Study accession number is: PRJEB27433. Accession numbers of DNA barcoding sequences are available in GenBank NCBI system under the accession numbers LS973890-LS973943.

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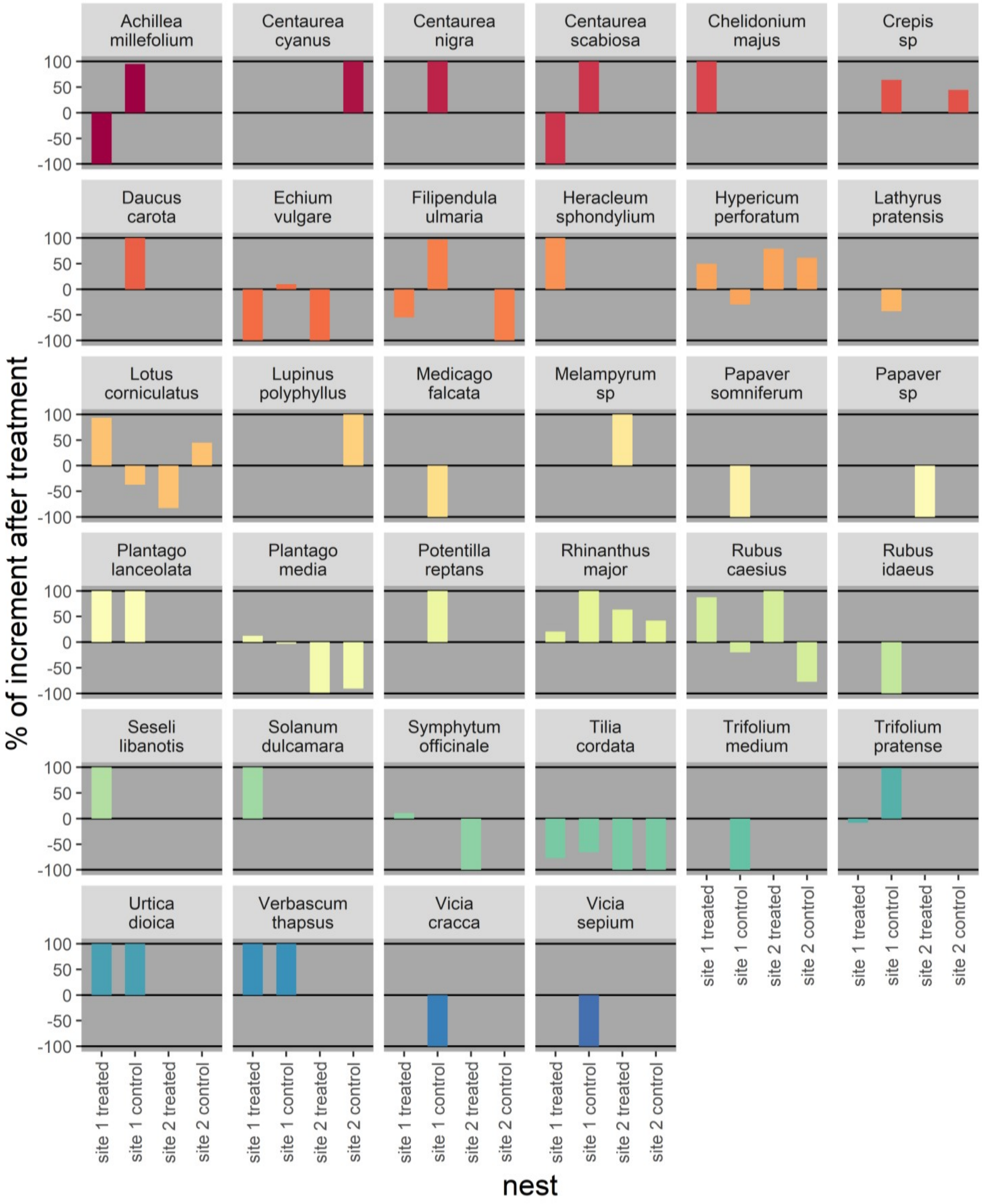
Supplementary information

Supplementary information, Figure S1 – Relative change in sequencing reads for each plant species during the experiment.



Chapter IV

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

Under review

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

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Abstract

Species extinctions undermine ecosystem functioning, with the loss of a small subset of functionally important species having a disproportionate impact. However, little is known about the effects of species loss on plant-pollinator interactions. We addressed this issue in a field experiment by removing the plant species most frequently visited by insects, then measuring the impact of plant removal on flower visitation, pollinator effectiveness and insect foraging in several sites. Our results show that total visitation decreased exponentially after removing 1-4 most visited plants, suggesting that these plants facilitate others by maintaining high flower visitor abundances. Nevertheless, we found large variation in changes of visitation among plant species. Plant traits mediated the effect of removal on flower visitation; while visitation of plants which had smaller inflorescences and more sugar per flower increased after removal, foraging of flower visitors was constrained by not switching between flower shapes. Moreover, pollinator effectiveness fluctuated but was not directly linked to changes of flower visitation. In conclusion, it seems that the loss of generalist plants alters plant-pollinator interactions by decreasing pollinator abundance with implications for pollination and insect foraging. Therefore, generalist plants have high conservation value because they sustain the complex pattern of plant-pollinator interactions.

Introduction

Overall community-level dynamics and ecosystem services are often disproportionately affected by a subset of the local species pool 1,2. This core of functionally important species contains, among others, generalists, which play a dominant ecological role within the community as they are often among the most abundant species3,4 and interact with a majority of other species5, mostly by complex facilitation-competition interactions6.

Generalist plants offer floral resources, mostly sugars from nectar and proteins from pollens, to a wide spectrum of pollinators and thus help to sustain those pollinators' populations7. Pollinators of generalist flowers forage both as specialists and as generalists, creating a nested pattern of interactions in the entire plant-pollinator network, which , it has been argued, increases the robustness of the whole community8. Furthermore, visitation by a large number of different pollinators increases the chances of having the pollen dispersed9. In turn, when generalist pollinators are foraging in a patch, they can collect resources from a wide range of plants. This strategy could provide nutritional benefits from multiple sources10,11, but may also lead to pollination of several plant species because it is believed that generalist pollinators are visiting only a few plant species during each foraging bout9. Therefore, conservation of abundant generalists may be important, because their persistence can sustain most of the complexity of interactions taking place in a community12.

Despite their functional importance, little is known about the effects of local extinction of generalists. The consequences of species loss have traditionally been investigated by removing species at random. However, in nature this is rarely the case as species do not go extinct randomly, but rather non-random losses are the rule13–15. In the context of plant-pollinator interactions, the loss of plants or pollinators and its consequent effect on the interacting assemblages has been studied mostly by simulation models16,17. Experimental tests in the field have started only recently and have been so far limited to relatively small manipulations, for instance removing either one invasive plant species18,19 or a single native plant species20. Other studies have excluded one species or a small set of pollinators21,22. Furthermore, only simulation-based studies have tested the effects of excluding several species sequentially17,23, while the few field experiments removed only one species and led to disparate results. For instance, removal of one species of bumblebee led only to small differences in the interaction networks after manipulation21, while a study removing one invasive plant species found a pronounced effect of removal18. In fact, removing either a pollinator or a plant has different implications. Specifically, by removing a generalist pollinator, more resources will be available for the other pollinators; conversely, the removal of an abundant plant induces an immediate reduction of resources available to the pollinator guild. However, the consequences of the loss of multiple species for plant-pollinator interactions remains an open question.

In this study we experimentally tested the effects of the removal of generalist plant species on plant-pollinator interactions. We focused on pollinators as a guild rather than on individual pollinator species by investigating the effects of the removal of the main flower resources on visitation, nectar consumption, effectiveness of pollination and shifting resource use by the pollinator guild. The experiments comprised two parts. Firstly, a pilot project in the United Kingdom experimentally manipulated a plant-pollinator community by removal of the most generalist plant (from here on called the "pilot study"). This provided a proof-of-concept and suggested ways in which the pollinator assemblage might react to such perturbation. Secondly, in the Czech Republic, we conducted a larger experiment, where we sequentially removed four of the most visited plant species over a short time period to determine how the plant-pollinator interactions would respond to a more profound loss of resources (from here on called the "sequential removal" experiment).

The aim of our experiments was to test whether removal of generalist plants (1) led to a decrease in the overall visitation i.e., the abundance of pollinators in the sites; (2) caused species-specific changes of the visitation of individual plant species, which could be explained by shifts of pollinators driven by traits of the plants, and (3) whether pollination effectiveness (determined by the number of pollen tubes grown in the pistils after visitation) and the amount of used nectar resources ("standing crop") changed as a consequence of plant removal.

We hypothesised that the pollinator guild could respond to the removal of major floral resources in three ways: (a) they could shift food source and distribute evenly on the remaining plant species; (b) they could shift preferably towards a subset of the plants, possibly on the basis of plant traits; (c) they could stop foraging at the site (or emigrate) due to the lack of the main food source. The scenario (a) might be expected if pollinators are generalists able to use any resources. In addition we hypothesised that under scenario (a) and (b) plant reproduction, measured as number of pollen tubes, would increase, whereas under scenario (c) it would decrease.

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 (data in S1 Table).

Surveys of insect visitation were undertaken at two stages: (i) before flower removal in order to determine the plant-pollinator interactions prior to the experiment; (ii) in the days following the removal of inflorescences of the plants species with the highest visitation, i.e. the total abundance of flower visitors on the plant. Inflorescences of the target plant (*Knautia arvensis*) were removed from the whole surface of the entire site. To indicate which plant to be removed, flower visitor abundance was used because it would not be practical to wait for species-level identification of flower visitors needed to decide which was the most generalised plant. We later confirmed that the plant with the highest abundance of flower visitors was also the one with the highest species richness (see Results). During the same period, the nearby Scrub Field, which hosts a vegetation similar to the treated site, 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 of the experiment (i.e., before and after removal) 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 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 each survey of an area of approximately 600 m^2). This method allowed for a large area to be surveyed owing to the relatively low pollinator density where floral resources are patchy and sparsely distributed. Insect specimens were identified either in the field or by experts. Sampling permission was obtained from The Wildlife Trust for Bedfordshire, Cambridgeshire, Northamptonshire and Peterborough.

Sequential removal

The sequential removal experiment was performed in the vicinity of Český Krumlov (South Bohemia, Czech Republic) in the summer 2015. No sampling permits were required for this project in the Czech Republic, because no protected species were affected and the study was conducted on public land. Three experimental sites were chosen (Site 1: 48° 49' 26.8" N, 14° 16' 26.2" E, area ca. 1500 m^2; Site 2: 48° 49' 51.63" N 14° 17' 34.12" E, ca. 1800 m^2; Site 3: 48° 49’ 35.07’’ N 14° 18’ 8.2’’ E, ca. 1600 m^2). The mean pairwise distance between the sites was 2.01 ± 0.95 Km. These experimental sites were small dry meadows surrounded by trees or bushes and with a dense forest on at least two edges. These were intended as physical barriers clearly separating the experimental sites from other grassland habitats in the surroundings. The entire surface of each experimental site was treated by removing the target plant species (see below).

An untreated control site was located at 48° 49' 26.8" N, 14° 16' 26.2" E, which consisted of habitat and plant community very similar to the treated sites. It was not feasible to pair each experimental site with a control site because of the lack of sufficiently similar sites in the vicinity of the experimental sites, so we opted for a single control accompanying the three experimental sites.

An alternative design such as one where we would manipulate one half of a site and use the half as a control would violate the independence of treated or untreated plots given the flying ability of pollinators and the lack of separation of the plots by physical barriers or by distance.

The sampling of insects was based on walking six transects (10 m x 1 m) in a randomized order in each site between 9:00 and 17:00 hours. Transects were set up for sampling pollinators and to count plant abundances in order to account for heterogeneity in plant distribution within the sites. The size and number of transects was set proportionally to fit within the small size of the selected sites. All transects were usually sampled twice during each day. Sampling was postponed in the case of rain or strong wind. All insects found while visiting flowers were sampled by a handnet or a mouth aspirator. The floral abundance of plant species was recorded by counting the number of open flowers or compound inflorescences on each plant within each transect. These data were recorded during all stages of the experiment, so that changes in plant phenology across the experimental period would be recorded as changes in the number of flowers, which is the appropriate measure of plant abundance in the context of plant-pollinator interactions. It was not possible to collect further details on the flower sexual maturation stage (pollen presentation or stigma receptivity) because that usually requires destructive methods[25], which could affect the flowering community in the sites, and it would also not be feasible to collect such data for the entire community given the large number of species present.

In the experimental sites, all flower visitors feeding on flowers were sampled for two days prior to any manipulation (hereafter the “Before” period). At the end of the sampling days, the captured specimens were counted for each plant and thus we were able to determine the plant species with the highest visitation, i.e. the total abundance of flower visitors on the plant. We call these highly visited plants “generalist”, although we did not evaluate the diversity of the visitors, in accordance with the literature (see [20]) and the outcome of the pilot study (see Results). This most generalist plant species was then removed from the entire site by cutting all inflorescences in the entire site, as was done in the pilot study. We only removed flowers and left the stems otherwise intact so that the vegetation structure remained unaltered. Twenty four hours after the removal, the sites were sampled again over two days. After we had counted the abundances of the visitors for each plant, we determined the next plant species with the highest visitation, the inflorescences of which were then removed. The flower visitors were sampled again after another twenty four hours for two days. We repeated this procedure until the fourth plant species was removed, which was followed by the last sampling period. Throughout the experiment, we verified that inflorescences of the removed plants were still absent in the sites. Site 1 and 2 were sampled from 25th June to 12th of July, Site 3 was sampled from 2nd to 17th of July. The control site was sampled synchronously to each of the experimental sites (the same days and during the same hours), but no manipulation of the plant community was performed there (data in S2 Table). For each site, we analysed data on total flower visitation of all plant species by the entire guild of flower visiting insects, data on insect species identity from this experiment are yet not available. The sequence of the plant species removal for both pilot study and sequential removal is detailed in Table 1.

Pistil collection and pollen tubes

Pollinator effectiveness was tested by counting pollen tubes in the pistils of flowering plants in two of our experimental sites. Growth of pollen tubes provides information about the effectiveness of the pollination service because it links pollen deposition and seed production[26], although it may confound cross and self pollination. We collected pistils from on average 40 flowers of each sufficiently abundant flowering plant after each insect sampling period and preserved in formalin-acetic acid-alcohol (FAA) at room temperature. Flowers were collected outside (but nearby) the transects to avoid depletion of flower abundances in the transects. Later, in the laboratory, the flowers were dissected under the microscope and pistils were prepared for softening and staining, following the technique of [27]. After softening the pistils in 4M NaOH, they were stained with 0.1% aniline blue in 0.1M K_2HPO_4 for 12 hours. Then, the pistils were washed and mounted in a drop of 50% glycerine on glass slides and covered with cover slips for observation under a fluorescence microscope. Pollen tubes were visible and counted for most of the species. However, in a few cases, pollen tubes were impossible to visualize properly. For these species the number of pollen grains on the stigmas were counted assuming that only germinated grains with tubes still attached would remain on the stigma after the preparation process. All processes were carried out at room temperature. After the observation, the edges of the cover slips were sealed with clear nail polish and stored at 4°C for future reference. Data were successfully obtained for 10 species because other plant species did not yield any countable pollen tubes (data in S3 Table).

Table 1 - Plant species removed during the experiments in the treated sites, with details on the plant family and the raw number of specimens found on that given plant.

Treatment period	Species removed	Family	Number of specimens
Pilot study			
Before removal	*Knautia arvensis*	Caprifoliaceae	54 insects (of 11 species)
Sequential removal experiment			
Before removal			
Site 1	*Anthriscus sylvestris*	Apiaceae	256
Site 2	*Veronica teucrium*	Plantaginaceae	103
Site 3	*Aegopodium podagraria*	Apiaceae	141
1 sp. removed			
Site 1	*Rubus caesius*	Rosaceae	315
Site 2	*Agrimonia eupatoria*	Rosaceae	96
Site 3	*Veronica teucrium*	Plantaginaceae	26
2 spp. removed			
Site 1	*Knautia arvensis*	Caprifoliaceae	83
Site 2	*Centaurea scabiosa*	Asteraceae	96
Site 3	*Knautia arvensis*	Caprifoliaceae	45
3 spp. removed			
Site 1	*Galium mollugo*	Rubiaceae	39
Site 2	*Securigera varia*	Fabaceae	26
Site 3	*Origanum vulgare*	Lamiaceae	55

Nectar content of flowers and other functional traits

We determined the standing-crop of nectar in flowering plant species at two sites of the sequential removal experiment in order to assess the amount of unused floral resources and its changes after plant removal. To do so, we collected flowers in each site after the pollinator sampling on the same day (data in S4 Table). A 100µl Hamilton capillary syringe was used for the collection and for washing the nectar into distilled water. Flowers were selected randomly but outside the sampling transects in order to avoid impoverishment of flower resources in the transects; only flowers in full anthesis were sampled. Nectar samples were stored in a cool bag in the field and in a -20°C freezer in the laboratory. Sugar analysis of nectar was done using high performance anion exchange chromatography with pulsed amperometric detection using a Dionex ICS-3000 system and CarboPac PA1 analytical column. Nectar amount was expressed as milligrams of sugars per flower. Because the method is not sensitive when the amount of sugar is extremely low, several nectar samples from a known number of flowers (of a given species on a given day) were merged in one unique sample which was analysed as described above; afterwards, the amount of sugar per flower was calculated by dividing the quantity of sugar by the number of flowers included in the sample. Data were extracted from an average of 45 flowers per plant species.

Furthermore, we measured several functional traits for each plant species in the two sites. The daily production of nectar was measured with a similar methodology as for the standing crop but the only difference was that flowers were bagged for 24 hours before sampling nectar: several inflorescences were bagged in the morning and sampled the next day in the morning, yielding nectar data on average 45 single flowers per species . This is an appropriate method used for comparing the cumulative secretion of nectar over a standard amount of time which covers the entire daily rhythm in several different species. Other traits were: plant height (linear distance between the ground and the top of an inflorescence, measured on an average of 12 plants per species); inflorescence maximum size (the maximum dimension of the inflorescence, measured on an average of 10 flowers per species); dominant colour of the corolla (categorical variable: “white”, “blue”, “pink”, “yellow”); flower shape coded according to [7] as follows: bell- or funnel-shaped, dish- or bowl-shaped flowers, flag-shaped flowers, gullet-shaped flowers, head- or brush-shaped, tube-shaped flowers; unclear cases were checked with [28].

Statistical analyses

All data were analysed by means of generalized linear mixed-effect models (GLMM) using the library *lme4* [29] in the R environment [30].

Site level visitation

For the sequential removal, the overall visitation was derived by calculating the sum of flower visitor abundances across plant species for each transect, separately at each sampling event. Thus, the number of flower-visiting insects per transect was the response variable, treatment was a categorical predictor describing the number of removed plants ("Before", "1 sp.", "2 spp.", "3 spp.", "4 spp."). Transect identity nested within site identity was used as a random effect on the intercept and a Poisson distribution was used. We used plant visitation as an offset in the model to account for possible confounding effects caused by factors other than our experimental manipulation, e.g. phenology, decrease of insect abundance due to sampling, etc. Thus, the plant visitation in the control site at a given time (geometric mean across the control site's transects) was included in the model as an offset. In the experimental sites, a multiple comparison tests were performed using the package *multcomp* with the function *glht*[31] to compare a given treatment level with the preceding treatment level (e.g. "1 sp. removed" vs "Before", "2 spp. removed" vs "1 sp. removed", and so on) in order to test the significance of relative increases or decreases in pollinator abundances.

For the data from the pilot study, the visitation was analysed in a slightly different fashion because flower visitors were collected along a single transect and insect species were taxonomically identified. Thus in a GLMM with Poisson distribution the number of visits by individual flower visitor species at each plant species was used as a response, treatment was a predictor ("before" and "after" removal of *Knautia arvensis*). Species identity was used as a random factor on the intercept. The analysis included visitation in the control site as an offset for the same reasons as for the sequential removal (see above). Specifically, visitation by an insect species on a plant species in the control site was used as an offset for visitation in the same insect-plant combination in the experimental site. For those insect species that were not recorded in the control site, the mean visitation recorded on that plant species across all visiting insect species at a given experimental time was used. Data from the control site were also analysed in a similar way (but without an offset); i.e. we also tested whether plant visitation changed between the sampling periods in the control site. For the control site, changes in the visitation on the species that was removed from the treated site were explored with a similar GLMM model, but without plant species as the random intercept.

Plant species level visitation

To test the effect of treatment on each plant species in the sequential removal experiment, plant-level visitation (visiting insect abundance for each plant species at each sampled transect, including unvisited plants) was the response variable and treatment was a predictor variable of the removal events (“Before”, “1 sp.”, “2 spp.”, “3 spp.”, “4 spp.”). An offset of plant abundance measured as the number of flowers per transect was included, to account for possible variation of the amount of flowers during the experiment (changes in plant abundance or phenology), as suggested in[32]. Transect identity within site identity was used as a random intercept. Plant species identity was used as another random factor affecting the slope of the treatment; i.e. we assumed that different plants may respond to the removal treatment in a species-specific way. Poisson distribution was used in the GLMMs.

Pollination effectiveness

We tested whether the number of pollen tubes in pistils of each plant species, i.e. viable pollen grains deposited and successfully germinating, changed during the experiment in the sequential removal sites. The number of pollen tubes was used as a response variable, the number of removed plants (coded as a categorical variable: “Before”, “1 sp.”, “2 spp.”, “3 spp.”, “4 spp.”) and the plant’s mean visitation across each transect were used as predictors. Random slopes of predictors were included with site identity within plant species as a random factor; i.e. we assumed that the effect of the predictors varied between plant species and sites. The GLMM was fitted with a Poisson distribution.

Standing crop of nectar

To test the variation of standing crop of nectar, the amount of sugars per flower of each plant species was used as a response variable, the treatment of the removal events (“Before”, “1 sp.”, “2 spp.”, “3 spp.”, “4 spp.”) and the plants’ mean visitation across transects were used as predictors as in the analysis of pollen tubes (see above). We used site identity within plant species as a random factor affecting the intercept, i.e. assuming that the mean amount of sugar per flower varies between sites and plant species (including the effect of the random factors on the slope was not possible due to a lack of convergence of the model). Gamma distribution was used, and the link function was the natural logarithm.

Traits analyses

To test if foraging during the treatment would be directed towards flowers being similar or dissimilar to the removed species, we assessed how visitation related to differences between plant-species’ traits and the removed plants’ traits in the two sites where traits were measured (data in S5 Table). To do that, the log-ratio difference between a given continuous plant trait value and the trait value of the removed plant species, $log(trait_{sp.\ i} / trait_{removed\ sp.})$, was calculated for each treatment level and used as a predictor variable. This measure of difference between quantitative values thus varies from negative values, through zero to positive values and has favourable statistical properties for analysis[33]. For categorical variables, a binomial variable was used, i.e. “same” as or “different” from the removed species. We aimed to compare visitation of individual plant species before and after each removal event (i.e. 1 sp. removed vs. before, 2 spp. removed vs. 1 sp. removed, etc.), so we reorganised the data for this analysis with the treatment coded simply as “before” and “after” removal. Plant traits used as predictors of visitation changes were flower colour, flower shape, inflorescence size, plant height and the daily production of sugars in nectar. We tested whether the change in visitation of individual plant species was affected by its traits by including interaction terms between the traits and the treatment variable. By including the treatment as predictor, the variation of visitation amount over the experimental time is included in the model. The number of flower of each plant species was included as an offset to account for possible variation of plant abundance across treatments. Transect identity within the site identity was used as a random intercept. Plant species identity was used as another random factor affecting the slope of the treatment effect. A Poisson distribution was used in the model. The control site was not included in this analysis because species were not removed in that site and thus it is not possible to calculate the trait similarities between the removed and remaining plant species.

Results

In the pilot study, the surveys identified a total of 13 insect pollinated plant species in flower and 25 pollinating insect species in the experimental site. The plant with the highest pollinator abundance and also the highest visiting species richnes was *Knautia arvensis* (details in Table 1). Most other plants were visited by fewer than 20 insect individuals and less than 8 species in both experimental phases, except *Centaurea nigra* which was visited by 41 individuals of 11 species before removal and 86 individuals of 11 species after removal of *Knautia*.

In the sequential removal experiment, the sites 1, 2 and 3 were surveyed for a total of 31, 26 and 23 insect pollinated plant species, respectively. The amount of insect specimens and the sequence of removed plants is detailed in Table 1.

Results from the pilot study

In the pilot study, visitation to plants changed only slightly after removing one species (Fig. 1). The treatment was not a significant predictor of visitation in the statistical model including an offset of the control's visitation when compared with a model without treatment variable (χ^2=0.194, df=1, p = 0.66).

Visitation changed only slightly in the near control site, the difference was not statistically significant (χ^2=0.85, df=1, p = 0.36) (Fig S1). In the control site, visitation to *Knautia arvensis* (the plant that was removed in the experimental

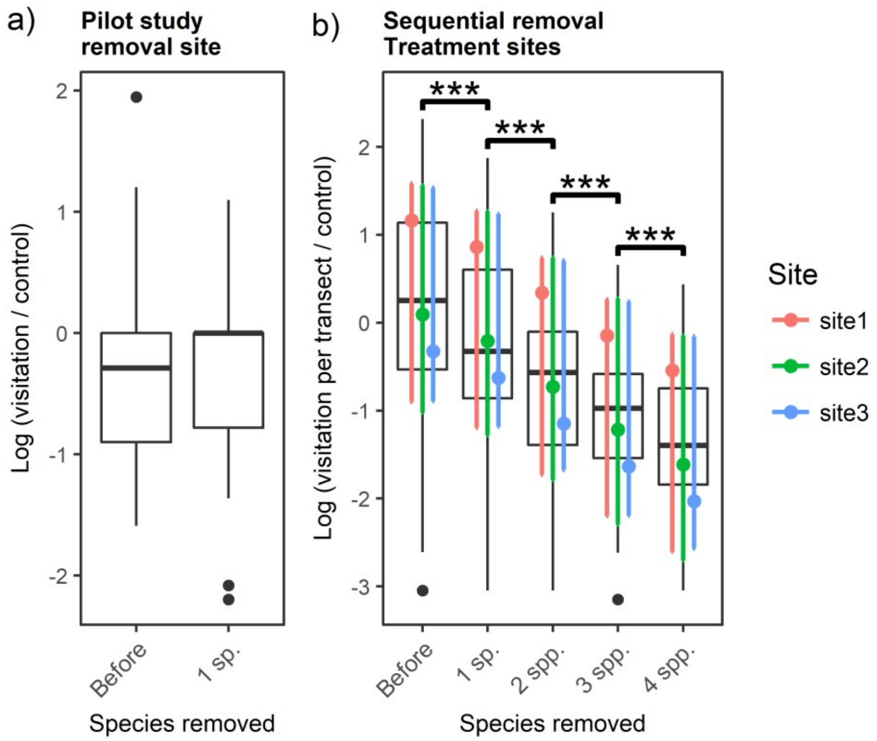

Figure 1 – Overall visitation in both pilot study and sequential removal experiment, including offsets of control sites' visitation. (a) Boxplots of raw data of the pilot study indicating the flower-visitor visitation (abundances) for each plant species; (b) Plot of the sequential removal experiment in where the boxplots indicate visitation across plant species of the same sampling event (i.e. a transect walk) and the estimated (modelled) means and confidence intervals are represented. All plots are on a logarithmic scale. When significant after multiple comparison test, it is indicated with the following codes: *** p<0.001, ** p<0.01, * p<0.05.

site) did not change over the study period when compared with a model without treatment variable (χ^2=0.852, df=1, p = 0.36).

General pattern of visitation in the sequential removal experiment

In the sequential removal sites, visitation per transect decreased sharply after the removal of selected plants. The treatment was a significant predictor when compared with a model without treatment variable (χ^2= 1605.3, df=4, p < 0.001), in the model including the visitation in the control site as an offset. The model without offset of the control site's visitation yielded very similar results.

Multiple-comparison test of treatment levels gave significant results in most cases (Table 2), with a similar decrease of total visitation after each stage of the removal experiment (Fig. 1).

Visitation of individual plant species

Visitation at the level of individual plant species was highly variable and significantly dependent on the number of plant species removed during the experiment (χ^2= 11.39, df=4, $p < 0.05$). The average trend in plant species-level visitation was non- linear across treatment levels and differed between sites. (Fig. 2). .

Plants showed high variation in visitation both within sites (among transects) and between sites (coloured lines and confidence intervals in Fig. 2). Nevertheless, the trend was not uniform across plant species, as idiosyncratic responses took place: visitation to some plants increased while in others it decreased in response to the same level of the removal treatment.

Plant traits

All plant-traits included in the models except colour and plant height were significant predictors of insect visitation (Table 3). Our results show that after the removal of a plant, the visitation remained stable in plants with the same flower shape, but decreased in plants with flower shapes different to the removed species. Furthermore, visitation increased in plants with relatively small inflorescences and high sugar content per flower (Fig. 3).

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=4, $p < 0.001$), but visitation was not (χ^2= 0.62, df=1, $p = 0.43$).

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

Table 2 – Multiple comparison statistics on the effect of treatment on general visitation, i.e. the total abundance of flower-visiting insects per transect, in the sequential removal experiment, with and without an offset with the visitation in the control site. The treatment of sequentially removing four most visited plant species is coded as "Before", "1 sp. removed", "2 spp. removed", "3 spp. removed", "4 spp. removed". Statistically significant effects ($P<0.05$) are highlighted in bold.

	Comparison	Estimate	Std. Error	P
Without offset of control site's visitation	1 sp. removed vs Before = 0	-0.071	0.039	0.228
	2 spp. removed vs 1 sp. removed = 0	-0.609	0.049	**<0.001**
	3 spp. removed vs 2 spp. removed = 0	-0.543	0.061	**<0.001**
	4 spp. removed vs 3 spp. removed = 0	-0.195	0.072	**<0.001**

	Comparison	Estimate	Std. Error	P
With offset of control site's visitation	1 sp. removed vs Before = 0	-0.302	0.039	**<0.001**
	2 spp. removed vs 1 sp. removed = 0	-0.519	0.049	**<0.001**
	3 spp. removed vs 2 spp. removed = 0	-0.488	0.061	**<0.001**
	4 spp. removed vs 3 spp. removed = 0	-0.397	0.072	**<0.001**

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).

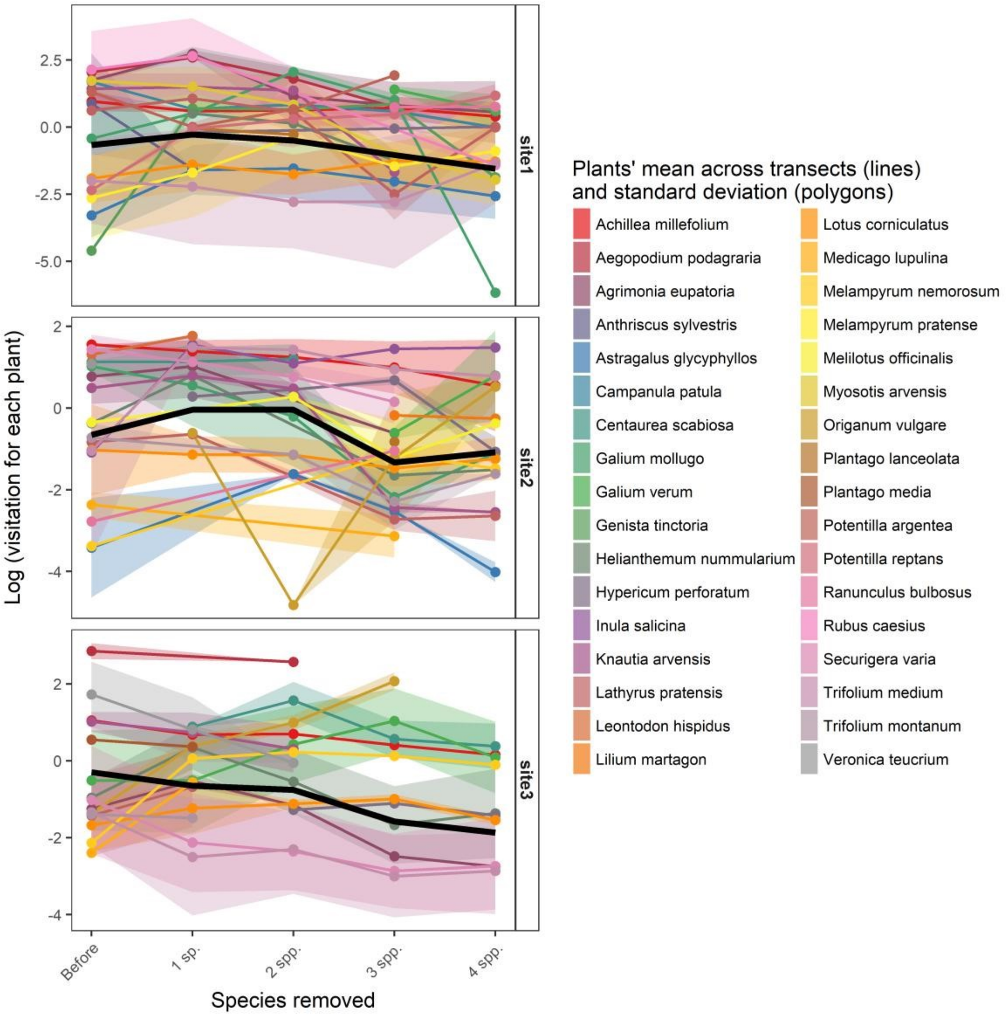

Figure 2 – Trends of flower visitor abundances ("visitation") on individual plant species in the sequential removal experiment. Visitation is shown on a logarithmic scale, coloured lines are estimated means across transects of a given plant species, coloured polygons are standard deviation around the plants' means obtained to indicate variation among transects, black line is the plant community average trend.

Table 3 – The effect of plant traits on changes of visitation after plant removal evaluated by single-term deletion of interaction terms from a GLMM model. Columns are used for interactions between treatment ("Treatment" referring to before-after removal of one plant species) and difference of trait values of individual plants from the removed species. Trait difference for colour and flower shape was classified as "same" or "different". Log-ratio difference was used for plant height ("Height"), inflorescence size ("Size") and amount of sugars in nectar ("Sugars"). ΔAIC refers to the change of AIC after removing the tested term from the full model. Statistically significant effects (P<0.05) are highlighted in bold.

	ΔAIC	χ^2	P
Treatment : Color	0.4	2.441	0.628
Treatment : Shape	14.8	16.845	**<0.001**
Treatment : Size	16.5	18.573	**<0.001**
Treatment : Height	-2.0	0.027	0.870
Treatment : Sugars	3.3	5.359	**0.021**

Discussion

We demonstrated that removal of several generalised plant species led to changes in overall flower visitation at the level of the entire community, but that visitation and pollination of individual plant species were affected in a species-specific way. We hypothesised that, at the community level, flower visiting insects may respond to the removal of the most visited plants according to one of three scenarios: (a) they may redistribute their visits equally among the remaining plants, (b) they may switch to a subset of the remaining plants depending on the traits of the plants, or (c) they may stop foraging at the affected sites. Our results strongly support the scenario (b) and partly also (c).

In the pilot study, total visitation did not change after the removal of the most visited plant. Flower visitors responded to the removal of their main food source by increasing their visitation to the next most visited plant, while visitation of the remaining plants was unaffected. The observed shift of pollinators between the two most important species happened because the second generalist plant assumed the role of the removed generalist plant[20]. There was no sign of insect emigration in the pilot study after removing one

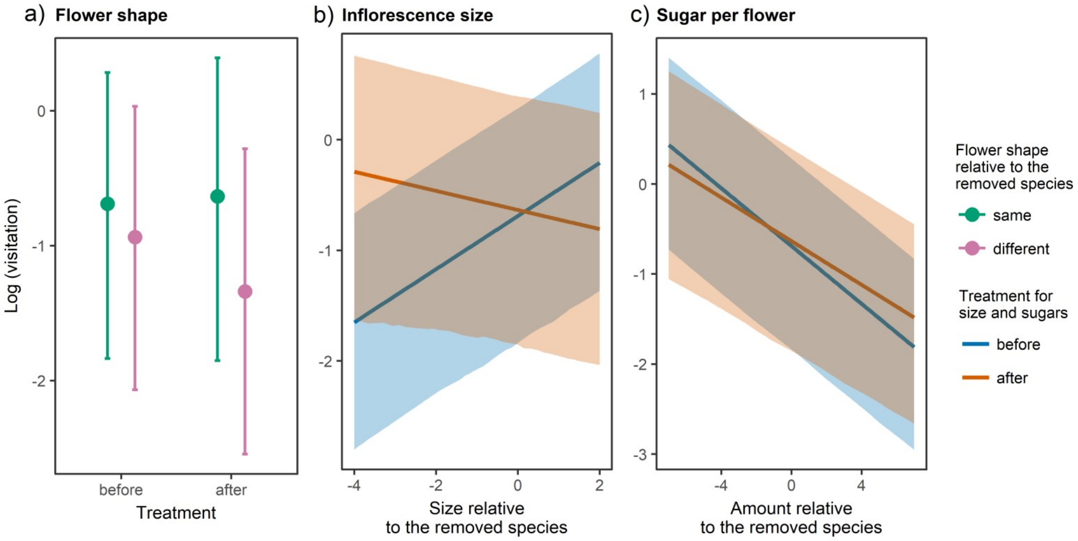

Figure 3 – Sequential removal experiment's flower visitor abundances as a response to plant functional traits expressed as the difference between a given plant trait value and the trait value of the removed plant species across treatment. Estimated (modelled) means and confidence intervals are represented. Only the statistically significant traits are presented (plots a-c).

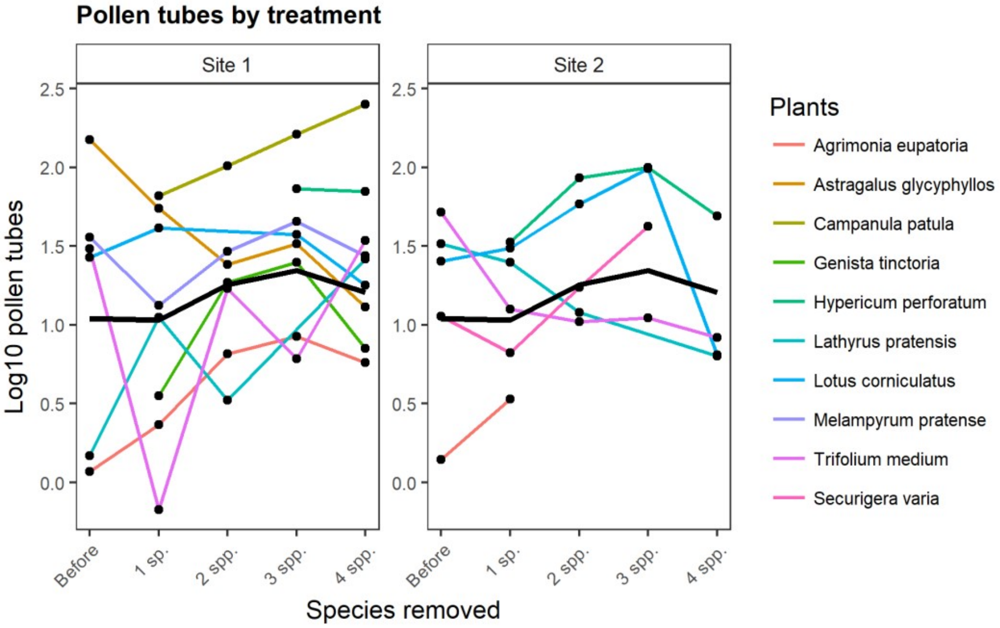

Figure 4 – Trends of the number of pollen tubes in two of the sites in the sequential removal experiment. The number of pollen tubes is shown on a logarithmic scale, coloured lines are the estimated means across transects of a given plant species, the black line is the plant community average trend.

dominant food source, because the nearby control site changed only slightly and the visitation did not increase on the plant species that was removed in the treated site. More pronounced changes occurred in the sequential removal experiment, where 4 plant species were removed, one at a time. In this latter experiment, pollinators distributed preferentially on a subset of the plants remaining in the experimental site after removal (scenario “b”), instead of visiting all other plants (scenario “a”) or being unable to use alternative resources (scenario “c”).

The main outcome is that some pollinators shifted between plant species but their resource use depended on how plant functional traits related to the removed plants’ traits. Data from both the pilot and sequential removal experiment thus support a conclusion that flower visiting insects react to the loss of important resources by selectively increasing their visitation to a subset of the remaining plants (scenario “b”).

Scenario “b” has implications for the entire plant community and reveals a key feature of complex interacting communities. That is, a few dominant, generalist species support the overall interactions structure by facilitating many other species, as outlined by [34]. This community-level facilitation likely took place 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 assemblage. This reinforces the hypothesis that complex community-level interactions are based on a core of a few important species, without which the system appears as altered and impoverished [12]. This pattern has important implications for both conservation of community-level interactions and functionality of these systems, as we discussed at the end.

Removal of multiple generalist plant species in the sequential removal experiment led to a decrease of visitation both at the site level (total visitation per transect) and on average also at the single plant species level. This might suggest that some pollinators did not find alternative resources as the removal of plants continued and insects stopped foraging at the sites. We did not collect any data on insect dispersal, so we do not know whether emigration increased after the removal. However, other aspects suggest that the remaining impoverished plant community was not affected by insect emigration. Specifically, pollination effectiveness fluctuated; the nectar consumption did not change as indicated by a nearly constant nectar standing crop; instead, both pollination effectiveness and nectar consumption were expected to decrease in scenario “c” as a consequence of a drop in local pollinator visitation.

Although previous simulation models of the consequences of species extinctions in plant-pollinator networks did not account for the redistribution of pollinators following the removal of key resources [23,35], our study shows that static responses in foragers are not the rule. Instead, these assemblages are dynamic and new interactions can be established after perturbations [17,24]. Adaptive foraging by consumers within a food web has been suggested to be important for the stability of complex communities[36]. This is because perturbations to the system are buffered by switches in interactions and use of new resources[24]. However, in our results, utilization of new resources investigated at the level of the entire pollinator guild was not random but was constrained by particular plant traits. Specifically, the flower visitors did not swap between flower shapes after removals of plants, and thus the flexible foraging of insects was constrained by flower morphology. The sugar content of nectar also affected the flower visitors because flower visitors were attracted by

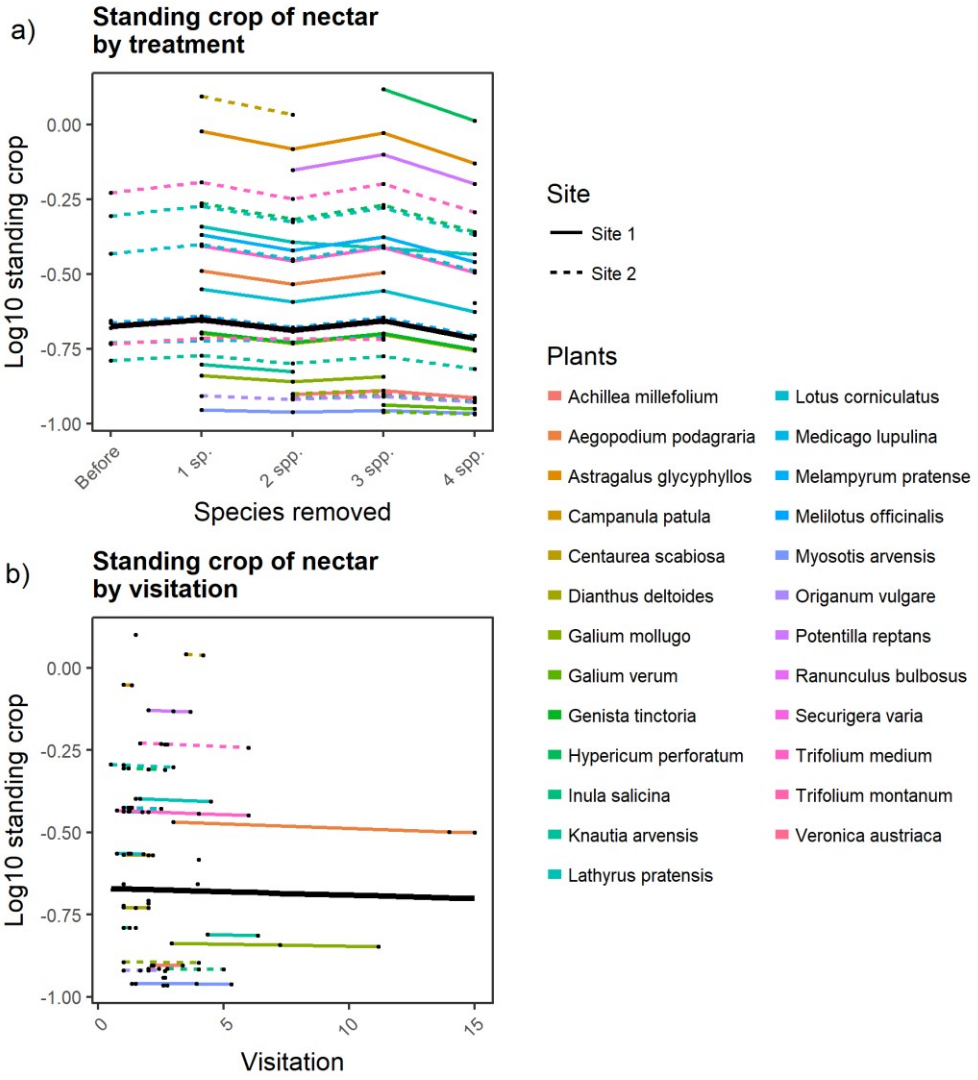

Figure 5 – Standing crop in both sites in logarithmic scale vs. Treatment (plot a) and flower visitor’s visitation (plot b), coloured lines are the estimated means across transects of a given plant species, black line is the community-average trend.

more rewarding flowers once the plant community was impoverished. Thus the energetic intake also constrained the process of utilizing new resources. Furthermore, smaller inflorescences also became more visited after removals. It is likely that they were underutilised in the original community because of their small floral display, so their visitation benefited from the overall reduction of flower abundance after the removals. Smaller inflorescences may also provide more resources per flower, and thus be more rewarding to flower visitors on a per-visit basis[38]. Taken together, our results imply that foraging can be flexible but also constrained within a specific plant-trait space[39]. These constraints would eventually limit the accessibility of new resources after perturbations.

Another aspect of the complexity that emerged after the experiments is that pollinator service varied across the treatment in a non-linear way, as shown by the fluctuating pattern of pollen tube numbers. This might be due to destabilization of the pollinators by the removal of key resources, which thus reacted in a fluctuating way. In fact, exclusion experiments have highlighted that in the absence of a dominant pollinator, other pollinators can compensate by becoming themselves more effective pollen vectors[22]. However, another work has shown that once the abundant pollinator is excluded, plant fitness and fidelity to flowers can decrease[21]. Although these two studies contradict each other, our results showed both responses: increasing or decreasing pollination effectiveness in different plant species. This resulted from species-specific responses as some plants benefited by receiving more pollen, while other species received less, after removing key plant species. Thus, an idiosyncratic, fluctuating trend of pollination was the outcome at increasing impoverishment of the plant assemblage.

Further variation was found when considering the responses in visitation on each plant species; some plant species had different trends compared to the average community trend after treatment (Fig. 2). Such plants could be highly competitive taxa, able to gain pollinator visitation above average while other plants were losing visitors. From this fact, it is possible to indirectly draw another conclusion about the most generalist plants. That is that they facilitate only a subset of species by keeping visitation on that subset high, while other plants are being limited and receiving less visitation. Such species-specific facilitation of pollination has been observed also in the case of invasive species (e.g.[19]). This could also be responsible for high spatial variation in pollinator abundances within sites (see the wide standard deviation around plant means in Fig. 2, which reflects differences between transects). That is, a given plant species had high visitation in one sampling transect and low in another transect, even on the same day. Although these patchy responses could be due to a local heterogeneity of abiotic factors[46], it seems more likely that the set of neighbouring plant species and their relative abundances caused variations in competition and facilitation[44]. In other words, in one patch the facilitation might predominate, while competition might outweigh facilitation in another patch, thus resulting in very complex overall patterns[6].

The consequences of species loss observed in our experiments have several implications for conservation of biodiversity. Generalist plants which are visited by a wide range of insects play a key role in plant-flower visitor interactions, and they serve as hubs in the interaction networks [5,20,50]. The removal of such generalist plants in our experiment led to important decreases of visitation and significant fluctuations of pollination effectiveness. We also showed that some of the responses were highly variable and species-specific. Furthermore, the insects changed their use of resources after the perturbation but their foraging flexibility was constrained by plant traits, which likely limited the utilization of new resources after plant removal. Thus, the stability of this system could depend on a small subset of important species whose loss has severe consequences for the entire community of plants and flower-visiting insects. We thus conclude that generalist plants play a key role in sustaining the complex pattern of interactions in the community[5,12] and may be more important than commonly thought for the conservation of species-rich ecosystems[51,52].

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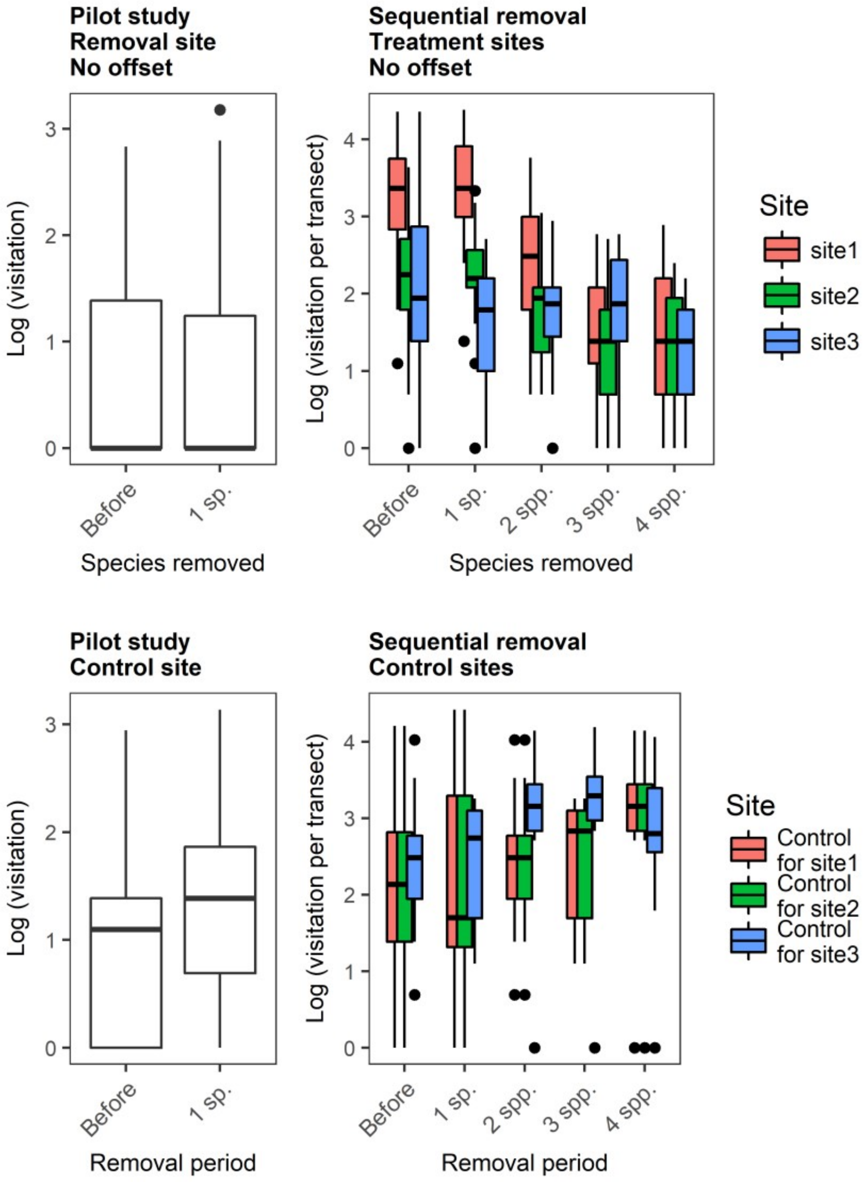
Supplementary material

Summery material Figure S1. Overall visitation in control sites of the pilot and sequential removal experiment.



Chapter V

An empirical attack tolerance test on real plant-pollinator networks: plant removal impact network structure but plants and pollinators respond asymmetrically

An empirical attack tolerance test on real plant-pollinator networks: plant removal impact network structure but plants and pollinators respond asymmetrically

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Abstract

Plant-pollinator network structure is hypothesized to bear several properties relating to robustness and redundancy, but to date this has mainly been examined using computer simulations. To test the effect of species loss on network structure and rewiring of interactions, we conducted a field experiment that assessed plant-pollinator networks during the sequential removal of four core generalist floral resources from natural species-rich communities. Hence, we performed an attack tolerance test by exploring the ways the system reacts after the sudden loss of important species. This manipulation caused decreasing networks' nestedness, increasing modularity and specialization, but there was no decrease in a robustness index calculated by the stochastic coextinction model. Network rewiring was high but without a specific trend and individual species tended to gain as many new links as were lost. Furthermore, the species of the two mutualistic levels reacted asymmetrically, because only plants increased their centralities after the removal of core plants, while pollinators didn't show any trend. The abundances of species were the main ecological drivers of network structure, of rewiring and of interactions, while morphological match and sugar amount occasionally played a role in the observed changes; however, unpredictability of network structures and randomness of the interactions also increased with plant removal. Therefore, removing a few core species from the community caused changes in network structure and increased unpredictability, although it did not translate into a network collapse. We conclude that although the observed flexibility of plant-flower visitor interactions made the networks robust to consequences of species loss, focusing conservation on species playing key roles in maintaining network structure might be a promising future practice.

Introduction

Species establish intricate essential interactions in the ecosystem they inhabit [1–3]. Specifically, most plants are dependent on insects for pollination and flower visiting insects rely on nectar or pollen as an important source of nutrition [4,5]. Therefore, exploring the patterns of plant-pollinator interactions may illuminate the ability of the system to keep functioning and avoid a collapse when exposed to some form of disturbance leading to species or interactions loss, either natural or caused by human activities [6–8]. Better insights into the responses of plant-flower visitor networks to species loss will allow a more efficient conservation based on the ecological role of species [9,10].

Our efforts to understand the consequences of species loss caused by human activities and conversely to adopt restoration measures to re-establish the lost diversity of species and their interactions has to be based on understanding the root mechanisms underlying the structure of interactions [11,12]. Theoretical models have provided some general insights into the responses of ecological networks to disturbances and species loss, but their real-world implications remain unclear because of the limitations of the models and lack of comparable empirical data. Observations of the impacts of human activities on species turnover and interaction network structure are needed to move forward. For doing this, ecological or habitat alteration gradients are the ideal laboratory for understanding the assembly and disassembly of interaction networks [13,14]. However, the heterogeneity of habitat types and human impacts often prevents from drawing clear conclusions employable in conservation [8,15]. Thus, field manipulations appear an ideal approach from which mechanisms governing network structure could emerge and be used in ecological conservation.

In this study, we performed an attack tolerance test on real plant pollinator networks. An attack tolerance test is usually aimed at testing whether the functionality of a system is maintained after knocking down its important components. In plant-pollinator networks, this topic has been investigated by relating the amount of co-extinctions with robustness and redundancy [16,17]. However, this has been addressed only theoretically with numerical simulations of sequential species extinctions in a trophic level and afterwards calculating the fraction of species that lost all interactions [6,7]. Conversely, empirical tests are scarce or aimed at removing only one species [18–20]. In this study, we performed a sequential removal of generalist plant species in the field to investigate how the plant-pollinator community responds to a profound loss of resources (see also chapter 4 of this thesis). We previously reported that removal of 1-4 most visited plants led an exponential decrease of total flower visitation in the affected sites, suggesting that generalised plants facilitate others by maintaining high flower visitor abundances (see also chapter 4 of this thesis). Here, we test the impact of the loss of generalised plants on the structure of plant-pollinator networks.

It can be expected that after removing key abundant food sources, pollinators will expand their diet and thus higher generalism will be observed [21]. Consequently, network modularity (i.e., compartmentalization of a network as result of selectivity for food sources) will decrease because foragers will use a broader range of the remaining resources [22] and thus higher interaction shifts (i.e., rewiring) will take place as result of adaptive foraging [23]. In contrast, an alternative expectation considers that if core elements are removed, the network can fragment into disconnected units [24]. Consequently a higher modularity and specialization can be expected, as interactions will happen uniquely within isolated compartments. Specifically, we investigated these expectations by asking whether (a) the sequential removal of generalist plants can cause alterations in the structure of plant-pollinator networks; (b) reorganization of network structure can emerge by changes in species level indices of specialization and of network cohesiveness; (c) individual species of flower visitors will shift towards using alternative resources (“rewiring” of interactions) instead of emigrating (species turnover). Moreover, we also tested (d) what ecological factors (species abundance, morphology, amount of sugar in the nectar) can explain the observed changes in network structure, rewiring and pair-wise interactions.

Material and methods

Study area and sampling design

The study took place near Český Krumlov (South Bohemia, Czech Republic) in three experimental sites and one untreated control site. The sites were located at a mean pairwise distance of 2.01 ± 0.95 Km (48° 49' 26.8" N, 14° 16' 26.2" E Site 1; 48° 49' 51.63" N 14° 17' 34.12" E Site 2; 48° 49’ 35.07’’ N 14° 18’ 8.2’’ E Site 3, 48° 49' 26.8" N, 14° 16' 26.2" E control site). All four sites were small dry grasslands rich in plant species and were similar in habitat type and plant community composition. Furthermore, the sites were surrounded by tall trees and bushes on the edges, so that they could provide a barrier between the sampling area and the surrounding landscape. Further details on sampling design and period can be found in chapter 4 of this thesis.

The treatment was applied to the entire surface of each experimental sites, but the control site was not treated. The treatment consisted of sequentially removing the most generalist plant species from the meadow by cutting its inflorescences, one at a time until four species were removed. We sampled flower visiting insects in six short transects (10 m long, 1 m wide) spread evenly across the area of each site. Before and after each removal, we sampled the flower visitors on each plant species flowering in the transects for two days. To decide which plant species to remove, we used flower visitors abundances yielded after each two-day sampling period as a proxy of generalization, as supported by [18], i.e., we removed flowers of the most visited plant in each step of the experiment.

The sampled insects were killed by ethylacetate, transported to the lab, pinned and stored dry until identification. We identified most of the insects to the species level either ourselves or with the help of several specialists and settled for genus or family level identification and sorting into morphospecies only when absolutely necessary. Hence, we obtained a set of highly-resolved networks suitable for detailed analyses.

In addition, we recorded the number of open flowers or compound inflorescences by counting them within each transect at all stages of the experiment.

Network topological indexes

We assembled interaction matrices for each stage of the sequential removal experiment (i.e., before removal, after 1 sp. was removed, etc.) in each site. For each matrix, we calculated a range of network-level and species-level indices. Some of the indices use only binary data (presence or absence of interactions), while others are based on quantitative data (frequency of interactions, strength); the latter ones are thus more informative.

We calculated network-level indices using the package *bipartite* for R [25] according to formulae described in [26]. The *Connectance* is the proportion of realised links among all possible links and it is a binary index. The *Links Density*, is the mean number of links per species but weighted by the number of interactions. Both range from 0 to its maximum 1. The weighted Nestedness describes the tendency of more specialized species to interact with the generalists pool. It was calculated with the quantitative *NODF* (Nestedness based on Overlap and Decreasing Fill) which compares the marginal totals and the proportion of filled matrix cells located at the same position. It ranges from 0 to 100 (fully nested). The Weighted *Modularity* is a measure of network's partition into clusters of interactions ("modules", "compartments", "sub-networks"); it was computed by the algoritm DIRTLPAwb+ [27] and ranges from 0 to its maximum 1. We also calculated several indices describing the level of specialisation of interactions in the networks. The Weighted H_2' is a measure of specialization of the network based on Shannon entropy which varies between 0 and 1 (perfect specialisation). It describes the tendency of networks' species to deviate from the species marginal totals and summarizes the amount of interaction overlap between species [28]. A different index of specialisation is the *Paired Difference Index* (*PDI*), which indicates the specificity of quantitative resource use of individual species [29]; we calculated the mean across species in each network weighted by species strength to obtain a network-level measure of specialisation. Weighted *Generality* (insects) and

Vulnerability (plants) are the weighted mean effective numbers of partners (that is of plants for the pollinators, and of pollinators for plants). We calculated these indices also for data from the unmanipulated control site collected at the same time as data in the experimental sites for comparison.

Species level indexes of network analyses were calculated as follows. The weighted connectivity and participation (also named c- and z-values) were computed with *rnetcarto* package for R [30] and account also for the strength of interactions [31]. They express modularity-related roles and measure the ability of a species to connect species between modules (connectivity) or to interact with species of the same module (participation). Weighted *Eigenvector Centrality* describes the species' position in the network proportionally to the centralities of the species it interact with. Thus, high eigenvector centralities are expect from species which connect species interacting with many others. It is computed from values of the first eigenvector of the interaction matrix [32]. We used the *igraph* package for R for the calculations [33]. As weighted measures of species specialization, *PDI* (see above) and *d'* were calculated in the *bipartite* package for R; the latter is a measure based on interactions overlap among species [28].

Network robustness

Network robustness was calculated for each site and removal stage by using the stochastic coextinction model (SCM) which allows quantitative data and overcomes some assumptions of the previously used topological coextinction model [34]. In brief, the SCM simulates extinction cascades by removing a species and tracking the number of species that become extinct afterwards (of either trophic levels) and thus it allows complex coextinction cascades (e.g. a plant c gets extinct after a pollinator b has gone extinct because plant a was removed from the network). For each species of a network, the probability of extinction is calculated as: $P_{ij} = R_i\, d_{ij}$. Hence, d is a measure of dependence of the population of *i* on the species *j* and is directly derived from the network as the interaction strength of *i* with *j* divided by the total interaction strength of *i* with all its partners. R is an intrinsic measure of demographic dependency of species *i* on the pollination mutualism with *j* (i.e. fitness). R values can be assigned as a constant or randomly generated or calculated empirically by the means of proxies [3,34].

For the plants of the studied sites, R was calculated as the amount of pollen tubes growing in the pistils after insect visitation. The details on the collection of these data (pollen tubes per plant species per removal stage) are included in chapter 4 of this thesis. These data were fitted to a regression (*glmer* with lme4 package in R, [35]) with treatment and visitation as predictors (pollen tubes were collected outside the transects ad thus a mean visitation for each plant species across the sampling transects accounts of the high variation in insect abundances among transects), plant species identity was random factor and the Poisson distribution was used. This procedure allowed to account for repeated measures on each plant species and also to estimate both mean values of pollen tubes among sampled plants (function *predict* in R) and also for those plants where pollen tubes were not sampled (function *simulate* in R). The R values for the pollinators of the studied sites were calculated as amount the sum of the sugars contained in a flower of the plant species visited by a pollinator, because it is expected that R relate to the intrinsic dependence of a pollinator on nectar [34]. The details on the collection of these data are included in chapter 4 of this thesis.

We run 10^4 simulations of cascading SCM. Following [3], the R obtained values where categorized in “low” (0-0.33), “low-medium” (0-0.66), “medium” (0.33-0.66) , “medium-high” (0.33-1) and “high” (0.66-1). Therefore, in each simulation, a R value was randomly generated within the range of the relative category. Afterwards, the coextinction cascades were triggered by removing the strongest plant species (species with the highest frequency of interactions), so that it would resemble the removal strategy applied in the field.

A similar procedure was performed also with data from the the control site. Since the proxies used to calculate R values in the removal sites were not directly available for the control site, the lower and upper bounds for the R values for a given species were taken from the corresponding removal site. When a given species was not found in the removal sites, 0 and 1 was set for the R value bounds.

From these simulations, a value of "stochastic robustness" was calculated in a similar fashion as the commonly used *robustness* function [17] of the *bipartite* package for R. In detail, a curve describing the number of surviving species was drawn based on all secondary extinction events following a given (primary) plant extinction and the area under the curve was calculated. As number of secondary extinctions, all extinction recorded as consequence of a primarily extinct plant were counted (both extinct pollinators and also the plants extinct after a pollinator's extinction). The mean number of secondary extinctions for each primary plant removal across all simulations was used for drawing the curve.

Components of interaction turnover

We quantified the turnover of interactions across the removal stages using the approach developed in [36] and used by [37] in time and by [38] in space. This method is based on calculating interaction turnover (the beta diversity of species interactions between pairs of networks sampled in different time periods or locations) and partitioning it into species turnover (i.e., the diversity of interactions in the pool of species that are not shared between the two networks) and rewiring of interactions (i.e. switching of partners in interactions among species which occur in both networks). These were calculated for all sites and consecutive pairs of treatment levels (before - 1 sp. removed, 1 sp. removed – 2 spp. Removed, and so forth).

Whittaker's beta diversity index $\beta_{int}=[(a+b+c) / ((a+b+c) / 2)] -1$ was used as in [36], where β_{int} is the interaction dissimilarity (beta diversity) between two networks, *a* is the number of interactions shared by them, while *b* and *c* are the number of interactions unique to each of them. This approach is based on a qualitative representation of the interactions (with binary data), and thus the beta diversity is based on the number of interaction links of the species belonging to each diversity component a, b, c. To account for the frequency of interactions, we used a quantitative estimation of beta diversity, where the sum of interaction frequency across the species in the *a*, *b*, and *c* components is used for the calculations, instead of using only the number of links as in [36].

Following [36], the beta diversities were decomposed into the component of species turnover and of rewiring. In brief, $\beta_{WN} = \beta_{ST} + \beta_{OS}$ where OS stands for rewiring, ST for species turnover and WN for total diversity. These components were extracted with the function *betalink* in the package *betalink* [39]. Values for these indexes range from 0 to 1; higher values indicate higher turnover or rewiring.

Species level rewiring

For each treated site, and consecutive pairs of treatment levels (before - 1 sp. removed, 1 sp. removed – 2 spp. removed, and so forth), an estimation of species' partner shifting (i.e., rewiring by establishing new links) was calculated by means of a new index. In detail, given a pair of network matrices M_a and M_b, then $r_i= (t+n_i-l_i) /2t$, where t is the total number of species which species i interacted with in the network pair, n_i is the amount of new links, i.e. species contacted by i only in M_b, and l_i is the amount of lost links, i.e. species contacted by i only in M_a but not in M_b. r is 0.5 when species i has same amount of new partners as the amount of lost ones; if $r \rightarrow 1$, species i tends to have more partners in M_b than the lost ones, and if $r \rightarrow 0$, species i tends to have less partners in M_b than the lost ones. $r = 1$ and $r=0$ results when i is a species with only new links (arriving at the site in the second unit of time, e.g. invaders) or with only lost links (leaving the site after the first unit, e.g. extinctions), respectively. Therefore, those species with ranges $0<r<1$ are truly rewiring, while species with values $r=0$ and $r=1$ are instead emigrating or immigrating.

This index has its quantitative counterpart, in where the interaction strength of the t, l, n components is used, instead of using only the number of links. So that l_i is the interaction strength of all links lost by species i in the new situation, n_i is the interaction strength of all links gained by species i, and likewise t is the strength of the (unique) species visited in each network of the pair. The interpretation is similar to the above one binary species level rewiring.

Trends in network structure and beta diversity component over the experimental time

The effect of species removal on network's indexes and beta diversity components were tested by means of generalized linear mixed effects models with the *glmmTMB* package in the R environment [35], where a given index was set as response and site was random intercept. For the indexes of beta diversity components, pairs of removal stages were used as categorical predictor variable. For all other indexes, the number of removed plants was used as numeric predictor. Gaussian distribution (with identity as link function) or beta (with logit as link function) was used depending on the range of the response variable.

As in [40], network size and amount of network indexes were included in the models in order to account for their possible effects on the networks at each treatment level. The total amount of interactions was included as predictor in the model to account for its possible indirect effect on quantitative network indexes and was calculated as the sum of the strengths in the matrix. In addition, network size was obtained as the number of animal species multiplied by the number of plant species (excluding non interacting species) and was included as predictor variable in the model in order to account for possible effect of network size on the indexes. Delta or z transformation of index values are other possibilities of standardization by matrix size, but they can cause biases [41]; and they are mainly used to test departure from randomness [1], while we aimed at testing the effect of a treatment in causing specific trends (i.e. increase or decrease of an index). These two predictors (networks size and interaction amount) were excluded from the statistical models if resulted to be not significant ($p>0.05$). Moreover, to account for effects of factors not due to the treatment, statistical regressions similar to those described above were performed with a given index in the control site as an offset as in chapter 4 of this thesis, for both network indices and beta diversity components.

For species level indexes in network analyses, plants were analyzed separately from pollinators. The effect of plant removal was tested by means of generalized linear mixed effects models, in where a given index was set as response variable, the number of removed plants was predictor and species identity within site was set as random slope. Gaussian distribution (with identity as link function) or beta distribution (with logit as link function) was used depending on the properties of the response variable. Species level rewiring was analyzed similarly but instead pairs of removal stages were used as categorical predictor variable; beta distribution (with logit as link function) was used because only the species truly rewiring ($0<r<1$) were analyzed.

Data from the control site were not included because not all species were shared with the removal sites, thus prohibiting a direct comparison of the species level data.

Drivers of interactions and simulation models

For each site and for each treatment stage, several probability-based simulation models were constructed to explore what ecological factors determined the observed networks indexes and beta diversity components over the study period. The probability matrices were the following. (1) NULL (N): the matrix is filled with 1s so that all species have the same probability of interactions; it serves to explore the possibility that randomness is driving interactions; (2) ABUNDANCES: the matrix is filled with either the amount of flowers of a plant species at an experimental time (P), or the abundance of the pollinator species calculated as total abundance of the interactions of an insect species over the entire study site (I), or on the element-wise multiplication of these two components, A = P*I; these are used to investigate the role of each component of species abundances; (3) MORPHOLOGICAL MATCH (M): the matrix cells are filled with 1 only when a morphological match between insect mouthparts length and flower's nectar allocation depth occurs [42], such as insect's "long mouthparts" – flower's "hidden nectaries", "intermediate mouthparts" – "semi hidden nectaries" and "short mouthparts" – "accessible nectaries"; like in [43], insects were categorized as having a long tongue (>9 mm), intermediate tongue (0.4-0.9 mm) and short (<0.4 mm); while plants were categorized as nectar hidden in flower structures (larger Fabaceae and flowers with tubular corolla), semi hidden nectaries (more open tubes, shorter tubes and smaller Fabaceae) and accessible nectaries (very short tubes or open flowers); (4) SUGAR

RESOURCES (S): this model assumes that the probability of plant-flower visitor interaction increases with the amount of sugar per flower the plant provides; i.e. the probability matrix is filled with standardised amount of sugar per flower for each plant species [44]. The amount of sugars per flower was measured sampling nectar from flowers bagged for 24h and measuring the concentration of different sugars with HPLC, the total amount of sugars per flower was then calculated (see chapter 4 of this thesis). We also build several simulation models which included a combination of these drivers by multiplying two or three of the probability matrices described above, specifically:: A*M is the combination of the A (abundances) probability matrix and the M (morphology match) matix; A*S is the combination of A and S (nectar's sugar resources) probability matrices; M*S is the combination of morphology and resources probability matrices; the A*M*S is the combination of the three probability matrices A, M and S. For each matrix, probabilities were obtained by dividing the cells of the matrices by the matrix sum. The obtained probability matrices were used to produce 10^3 simulated networks with *mgen* function of the *bipartite* R package [25]. This function generates matrices of identical size as the real (observed) network and fills a matrix by distributing the quantity of the interactions of the real network to the matrix cells according to the probability specified by a given probability matrix. Consequently, for each of the 10^3 simulated networks, network indices were calculated in the same way as for the real network (see above). Mean and confidence intervals of network indices were then calculated. Predictions of a given simulation model ("driver") were considered as consistent with the empirical observations when the 95% confidence interval of the model's index distribution consistently included the observed value of the network index. The same procedure was followed with calculating beta diversity and its components (see above).

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, a likelihood approach was used. Following [45], a multinomial distribution was calculated from the interaction frequencies of the observed network and the probabilities of a given probability matrix. Therefore, the Akaike information criteria (AIC) was used to evaluate the ability of each probability model to predict the likelihood of pairwise interactions. As in [46], in the AIC calculation, the number of parameters was set as the number of species of each probability matrix in order to weight each model's complexity, i.e. (no. of plant species + no. of insect species)*number of probability matrices (which were 1 in N, C_E, S, M, I, P, 2 in A=P*I and M*S, 3 in A*M, A*S and, 4 in A*M*S). The Δ AIC was obtained by subtracting the AIC of the best-fitting model from the AIC of each model and used to compare models.

Results

The plant-flower visitor networks of the experimental sites were species rich. Specifically, we found 28 entomophilous plant and 157 flower-visiting insect species in Site 1, 24 plant and 171 insect species in Site 2, 20 plant and 106 insect species in Site 3. Experimental removal of one to four most visited plant species caused a number of changes in the structure of the networks and interactions at the level of individual species.

Network and species index trends with treatment

Several indices describing the overall structure of the networks changed significantly in response to the removal of most visited plants (Fig. 1). Specifically, modularity and two measures of specialization (H2' and PDI) increased with the number of removed plants, while pollinator nestedness and generality decreased. The statistical results are summarised in Table 1. When we included values of these indices from the control site as an offset in the GLMM models, the trend in increasing specialization measured as H2' and pollinators' generality was no longer significant because these indices increased also in the unmanipulated control site during the same time period (Table 1). However, the trend of increasing modularity and specialisation based on PDI and decreasing nestedness was confirmed. In addition, there was a statistically significant increase in link density and plant vulnerability (the effective number of partners) when the values of these indices in the control site were included as an offset (Table 1, Fig. 1).

For species level analyses, plants and pollinators species differed in responses (Fig. 2). Additional statistical results are in Table 2. For plants, connectivity, eigenvector centrality and specialization (d') increased while PDI decreased with plant sequential removal. Conversely, pollinators maintained these indices nearly constant with plant removal. Participation did not change significantly in response to plant removal.

Table 1. - Statistics of the network level indexes and network interaction turnover components. Each row is a separate generalized mixed effect model and the test is obtained by comparing with a model without treatment as predictor, further details are in Methods. WN is total beta diversity, OS is the rewiring of interactions, ST is the species turnover between networks pairs. "W." stands for "weighted". *ΔAIC* is calculated as AIC_i - AIC_{min}. In bold the statistically significant predictors are highlighted ($P<0.05$). Significance of the models including the offsets of with the control site's indexes are given.

	Df	ΔAIC	χ^2	P	P with control offset
Connectance	1	1.274	0.726	0.394	0.719
Link density	1	1.933	0.067	0.796	**0.039**
W. NODF	1	6.032	8.032	**0.005**	**0.001**
W. Modularity	1	7.246	9.246	**0.002**	**0.007**
W. H2'	1	11.076	13.076	**<0.001**	0.073
W. Network's PDI	1	4.846	6.846	**0.009**	**0.003**
W. generality (pollinators)	1	18.177	20.177	**<0.001**	0.285
W. vulnerability (plants)	1	0.158	1.842	0.175	**0.035**
Stochastic robustness	1	1.819	3.819	0.051	0.350
WN (β)	3	0.604	5.396	0.145	0.110
OS (rewiring)	3	3.793	2.207	0.531	0.525
ST (turnover)	3	2.262	3.738	0.291	0.323
W. WN (β)	3	4.170	1.830	0.608	0.688
W. OS (rewiring)	3	2.581	3.419	0.331	0.698
W. ST(turnover)	3	1.400	4.600	0.204	0.890

Table 2 . Statistics of the species level indexes of the network analyses and of the interaction rewiring. Each row is a separate generalized mixed effect model and the test is obtained by comparing with a model without predictor, further details are in methods. ΔAIC is calculated as AIC_i - AIC_{min}. In bold the statistically significant predictors are highlighted (P<0.05). "W." stands for "weighted".

Pollinators				
	Df	Δ AIC	χ^2	P
W. connectivity	1	5.285	7.285	**0.007**
W. participation	1	1.509	0.491	0.484
W. eigenvalue centrality	1	11.879	13.880	**<0.001**
W. d'	1	1.855	0.145	0.703
W. PDI	1	1.961	0.039	0.844
r (rewiring)	3	2.538	8.538	**0.036**
W. r (rewiring)	3	0.193	6.193	0.103
Plants				
W. connectivity	1	10.439	12.439	**<0.001**
W. participation	1	1.857	0.143	0.705
W. eigenvalue centrality	1	6.384	5.065	**0.024**
W. d'	1	4.500	6.500	**0.011**
W. PDI	1	12.589	14.589	**<0.001**
r (rewiring)	3	4.164	1.836	0.607
W. r (rewiring)	3	4.726	1.274	0.735

Rewiring of networks and species with treatment

We detected a very high level of interaction turnover during the removal experiment. In the binary version, the overall interaction turnover (WN) ranged between 0.61 and 0.84, with a larger proportion of interaction turnover attributable to rewiring (OS, from 0.39 to 0.57) then to species turnover (ST, from 0.18 to 0.42), depending on site and sampling period (Fig. 3). In the quantitative version, these indexes had similar ranges. There was no statistically significant trend in the values of rewiring, species turnover, and total interaction turnover in response to the treatment (Fig. 3, Table 1). The species of the two levels rewired similarly after plant species removal (Fig. 4). There was a large interspecific variation in the relative loss or gain of interactions, but the average values changed little. Only the insect species binary rewiring significantly varied in response to the removal treatment (Table 2).

Drivers of network change

In the likelihood analysis of pairwise interaction frequencies between species, the abundances models usually provided the best fit, especially in the case of insect abundances model (I). However, the null model assuming that all interactions have the same probability, i.e. interactions happen randomly (NU), predicted the interactions as the removal of plants progressed, especially in two of the three sites. In addition, the model based on the amount sugars in nectar (S) also had low Δ AIC values. Other models were generally not predicting the interactions well.

The ability of the models to correctly predict network structure as described by the selected network indices was limited as none of the probability models generated distributions that predicted all network indices (i.e. 95% confidence intervals of the model's index distribution consistently including the observed network index). The details are shown in Table 4 and in Supplementary Figure 1-3. Connectance was poorly predicted at all plant removal stages. Conversely, some indices were predicted in all stages, at most sites (i.e. W NODF, W. Generality). Moreover, in the majority of the indices, both models of abundances (plant and insect) and also the multiplication of abundance with other models were contributing at explaining the network topology.

In most of the indices the predictors usually changed as the removal of plants progressed. Specifically, before removing plants some networks indices were predicted by models with low complexity (i.e. drawn from one probability matrix), while at following removal stages the indices were predicted by complex models (i.e. from the multiplication of probability matrices) in most of the sites, i.e. W. Network PDI, W. Generality, W. Modularity, W. NODF. Conversely, the models' fitting ability decreased with the progressing of plant removal for W. Vulnerability and W. H2'.

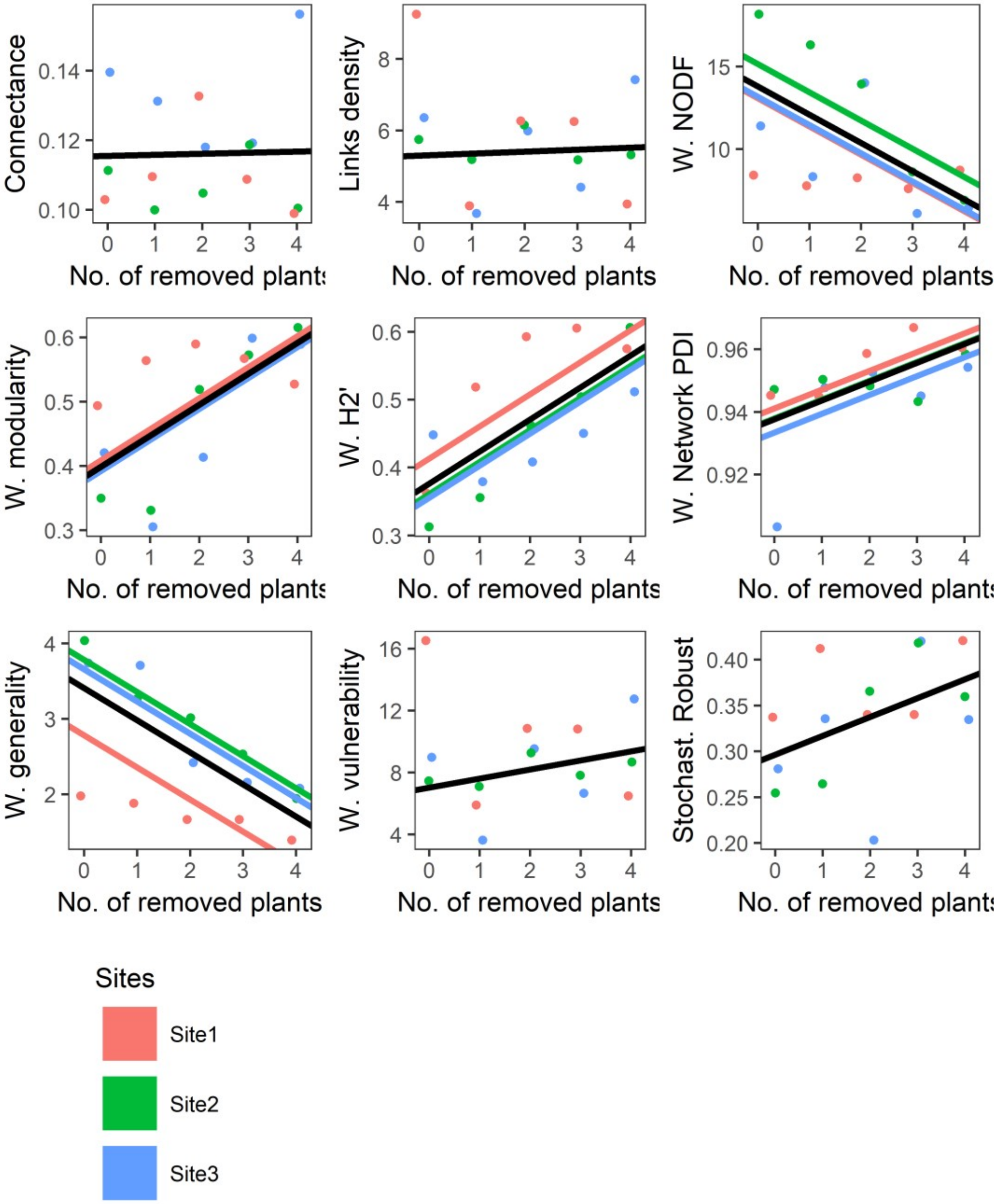


Figure 1 – Network indexes' responses to plant removal. "w." stands for "weighted". Significances of predictors are expressed in Table M. The black line is the average trend predicted by the models, colored lines are predictions for each site; plots with black line only were not significant models.

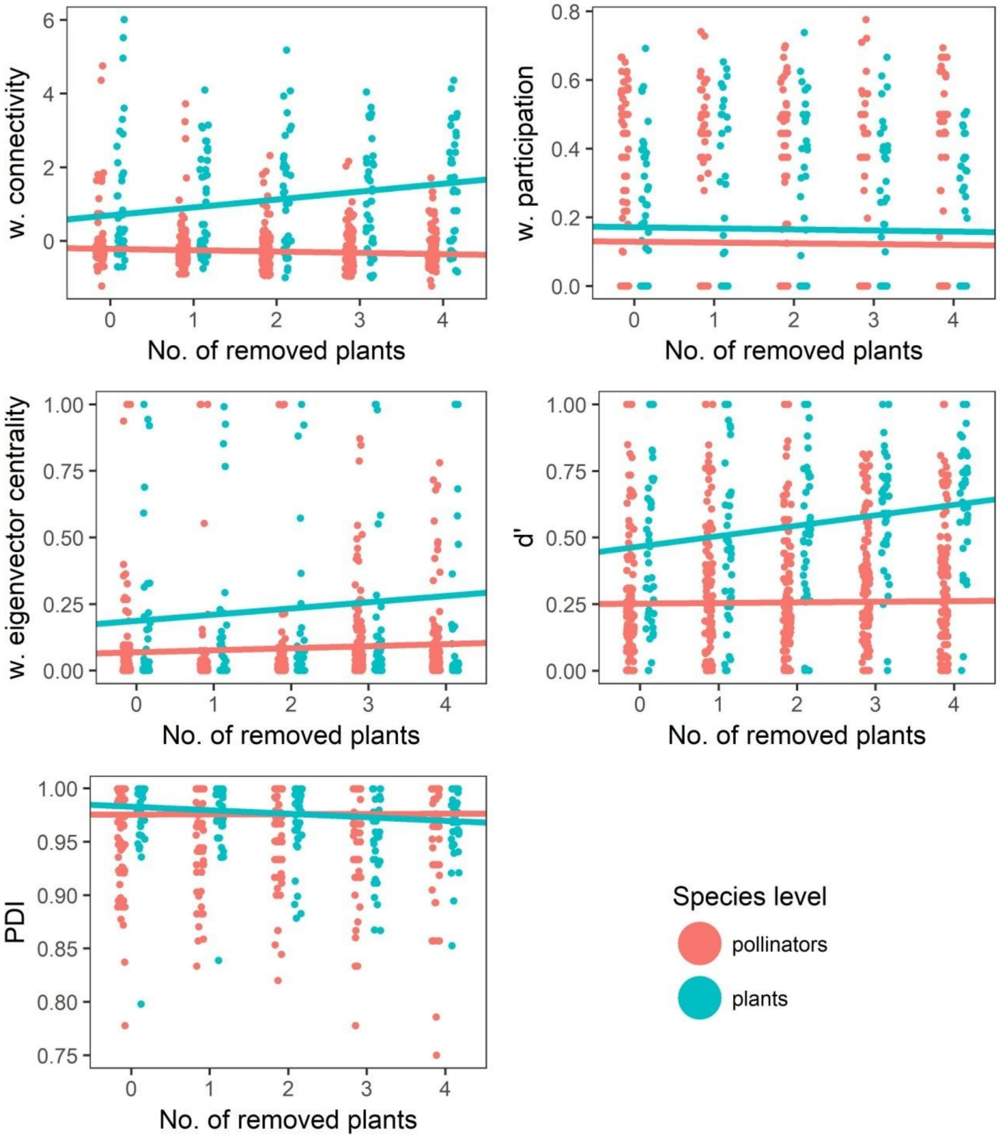


Figure 2 – Responses to plant removal of the species level indexes of the network analyses. “w.” stands for “weighted”. The two levels are spaced for graphic purposes. Significances of predictors are expressed in Table 2.

Remarkably, in specialization indices, a role was played by the abundances. So that pollinator’s W. Generality was predicted by the abundances of plants and of sugar amount. Similarly, plant’s W. Vulnerability was predicted by the insect abundances (at the first stages of the removal), and also W. Network PDI was determined by abundances (but at the last stages of the removal). Abundances of insects and of plants also fitted the W. NODF and also predicted decently the Link Density especially if in multiplication with other models. Morphological matching models well predicted W. Modularity but only before any removal happened. Morphology in interaction with sugar amount also predicted the W. NODF in most stages. W. H2’ was only partly described by models involving interactions of morphological match with abundances but only during the first stages of removal.

In the case of the interactions turnover, of the rewiring and of species turnover (WN, OS, ST), the models of abundances explained most cases, and especially the insect abundances and the multiplication of plant and insect abundances. The details are shown in Table 5 and in Supplementary Figure 4-5. This was also the case of the quantitative interaction turnover indexes (W. WN, W. OS, W. ST), especially for all models including the abundances (both the simpler and the more complex ones). In rewiring and also in species turnover indexes, the morphological matching and the sugar amount predicted several cases after the first plant removal, but the multiplication of these two models decreased their explanatory ability.

Table 3 – Likelihood of pairwise species interactions (ΔAIC). In bold, the probability models that best predicted the interactions are highlighted; the second important probability models are underlined. Models acronyms are described in the Methods.

Site	Species removed	NU	P	I	A	M	S	A*S	A*M	S*M	M*A*S
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

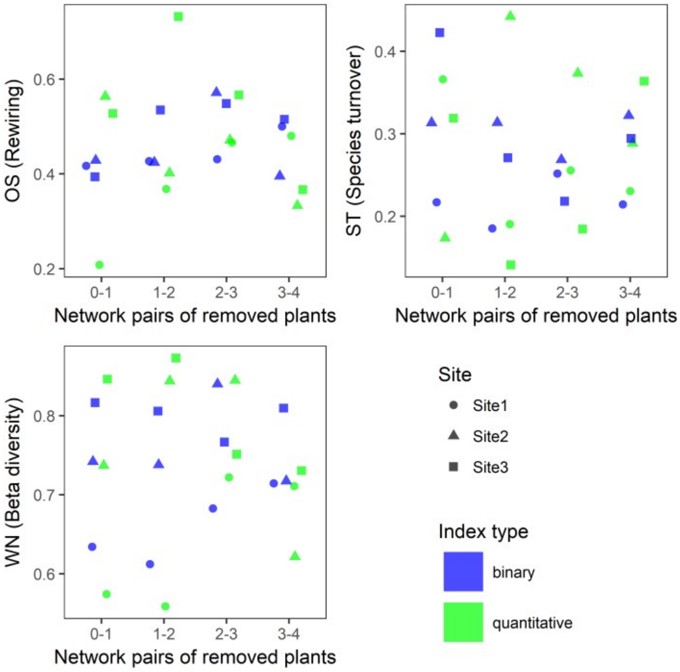


Figure 3 – Interaction turnover of pairs of networks after each stage of plant removal. Both the binary indexes and their quantitative counterparts are plotted. Significances of predictors are expressed in Table 1.

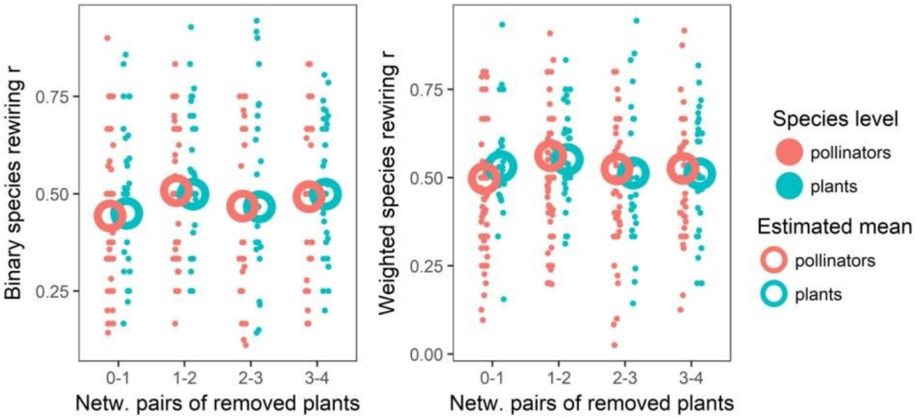


Figure 4 – Rewiring at species level, as responses to each stage of plant removal (pairs of networks before-after each removal stage). The two levels are spaced for graphic purposes. Significances of predictors are expressed in Table 2.

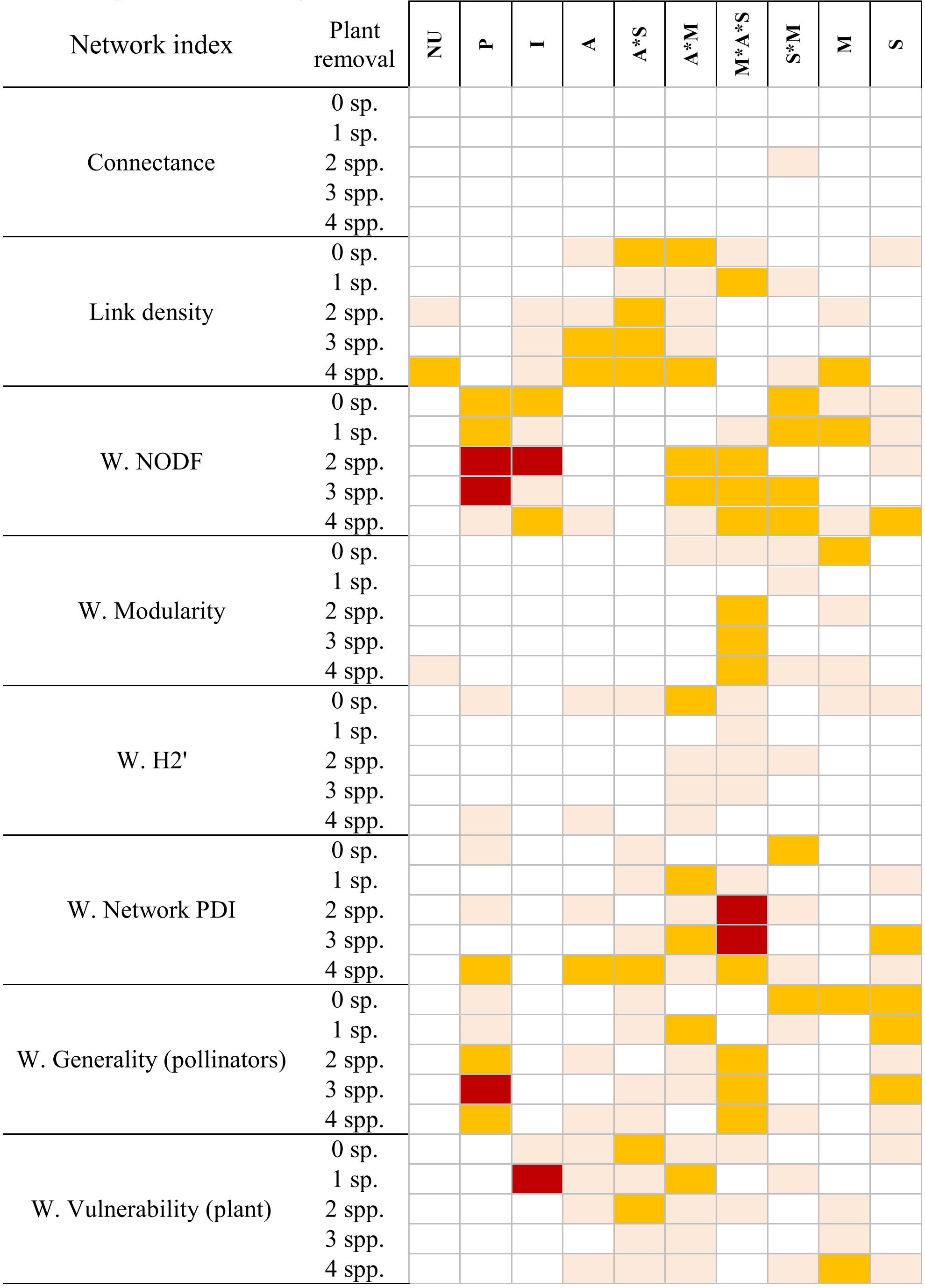
Discussion

The removal of the most generalist plants from the plant-pollinator assemblages caused changes in the structure of the network, both at the level of the topology of the entire networks and of interactions of individual species. This indicates that core generalist species are important for the distribution of interactions and for maintaining the structure of entire plant-pollinator assemblages, as previously hypothesised [24,47,48]. Sure enough, changes in network structure were recorded in a number of studies, and negative effects are generally reported in the context of high habitat degradation or lowered species richness [8,13,49,50].

In the species-rich networks of this study, the removal of core generalist plants caused a linear decrease in nestedness, that could be viewed as a symptom of higher instability [17], because specialists species may be less entangled into the generalist pool [51]. This could be corroborated by the observed linear increase in modularity, as an increasing emergence of sub-networked structure and, thus, symptom of network shredding [24]. However, in food webs it is believed that high modularity actually buffers the spread of alterations (e.g. extinctions) into the entire web [52]. Conversely, in mutualistic networks, compartmentalization is controversial. When high, it can prevent the access to alternative resources (e.g. visiting plants of other modules, or being visited by new pollinators), but when low, it can cause a cost in terms of inefficiency in handling resources by flower visitors or, for plants, in being visited by ineffective pollinators. Yet, by means of numerical simulations, it was shown tha lower modularity promotes the persistence of mutualistic networks [22]. However, in the manipulated networks of this study, the trends of a decreasing nestedness and increasing modularity were not directly related to any trend in the stochastic robustness index.

During the field manipulation, we have observed an increasing trend of specialisation with plant removal, possibly as a result of decreased pollinator abundances (see chapter 4 in this thesis). As previously observed, reductions in number of interactions are related to changes in network structuring [49,53].

Table 4 - Network indexes overlap between the observed values and the confidence intervals after 10^3 simulations by tfollowing probability matrices (NU= null, P= plant abund., I= Insect abund., A= abund., M= morphological match, S= sugar amount). Red is for overlap in 3 sites, orange is for overlap in 2 sites and pink is for 1 site.



Network index	Plant removal	NU	P	I	A	A*S	A*M	M*A*S	S*M	M	S
Connectance	0 sp.										
	1 sp.										
	2 spp.								▨		
	3 spp.										
	4 spp.										
Link density	0 sp.				▨	🟧	🟧	▨			▨
	1 sp.					▨	▨	🟧	▨		
	2 spp.	▨		▨	▨	🟧	▨			▨	
	3 spp.			▨	🟧	🟧	▨				
	4 spp.	🟧		▨	🟧	🟧	🟧		▨	🟧	
W. NODF	0 sp.		🟧	🟧					🟧	▨	▨
	1 sp.		🟧	▨				▨	🟧	🟧	▨
	2 spp.		🟥	🟥			🟧	🟧			▨
	3 spp.		🟥	▨			🟧	🟧	🟧		
	4 spp.		▨	🟧	▨		▨	🟧	🟧	▨	🟧
W. Modularity	0 sp.						▨	▨	▨	🟧	
	1 sp.								▨		
	2 spp.							🟧		▨	
	3 spp.							🟧			
	4 spp.	▨						🟧	▨	▨	
W. H2'	0 sp.		▨		▨	▨	🟧	▨		▨	▨
	1 sp.							▨			
	2 spp.						▨	▨	▨		
	3 spp.						▨	▨			
	4 spp.		▨		▨		▨				
W. Network PDI	0 sp.		▨			▨			🟧		
	1 sp.					▨	🟧	▨			▨
	2 spp.		▨		▨		▨	🟥	▨		
	3 spp.					▨	🟧	🟥			🟧
	4 spp.		🟧		🟧	🟧	▨	🟧	▨		▨
W. Generality (pollinators)	0 sp.		▨			▨			🟧	🟧	🟧
	1 sp.		▨			▨	🟧		▨		🟧
	2 spp.		🟧		▨		▨	🟧			▨
	3 spp.		🟥			▨	▨	🟧			🟧
	4 spp.		🟧		▨	▨		🟧	▨		▨
W. Vulnerability (plant)	0 sp.			▨	▨	🟧	▨	▨			▨
	1 sp.			🟥	▨	▨	🟧		▨		
	2 spp.				▨	🟧	▨	▨		▨	
	3 spp.					▨	▨			▨	
	4 spp.				▨	▨	▨		▨	🟧	▨

Table 5 – Network interaction turnover overlap between the observed values and the confidence intervals after 10^3 simulations by following probability matrices (NU= null, P= plant abund., I= Insect abund., A= abundances, M= morphological match, S= sugar amount). Red indicate overlap in 3 sites, orange indicate overlap in 2 sites and pink indicate overlap in 1 site.

Interaction turnover indexes	Network pairs before-after species removal	**NU**	**P**	**I**	**A**	**A*S**	**A*M**	**M*A*S**	**S*M**	**M**	**S**
WN	pair 1-2			🟥	▨	▨	🟧	▨	🟧		▨
	pair 2-3		▨	🟥	🟧	🟧			▨		▨
	pair 3-4		🟧	🟧	▨	▨			▨	🟧	
	pair 4-5		▨	🟥	🟥	🟥	🟧	🟧	▨	▨	
OS	pair 1-2		▨	🟥	🟧	🟧	▨	▨	▨	▨	▨
	pair 2-3		🟥	🟥	🟥	🟥		🟧	▨	🟥	🟧
	pair 3-4		🟧	🟥	▨	▨			🟧	🟧	🟥
	pair 4-5			🟧	🟧	🟥	🟧	🟧	▨	▨	🟧
ST	pair 1-2		▨	🟥	🟥	🟧	🟧	▨		▨	▨
	pair 2-3	🟧	▨	🟧	🟧	🟥	▨	▨		▨	🟧
	pair 3-4		🟧	🟥	🟥	🟧	▨	▨	▨	🟥	🟧
	pair 4-5	▨	▨	🟧	🟧	🟥	▨	▨	▨	🟥	🟥
W. WN	pair 1-2			🟥	🟧	🟥	🟥	🟥	🟥	▨	
	pair 2-3		🟧	🟧	🟧	🟧	🟧	🟧	▨	🟧	🟧
	pair 3-4		🟧	🟥	🟧	🟥	🟧	▨	▨	🟧	
	pair 4-5		▨	🟥	🟥	🟧	🟥	🟥	▨		
W. OS	pair 1-2		🟧	🟧	🟧	🟥	🟥	🟥	▨	▨	▨
	pair 2-3	▨	▨	▨	🟧	🟧	🟧	🟧	▨	🟥	▨
	pair 3-4		🟧	🟥	🟧	🟧	🟧	▨	🟧	🟧	🟧
	pair 4-5		▨	🟧	🟥	🟥	🟥	🟧	▨	▨	🟧
W. ST	pair 1-2	🟧	▨	🟧	🟥	🟧	🟧	🟥		▨	🟧
	pair 2-3	🟧	▨	▨	🟧	🟧	🟧	🟧	▨	▨	
	pair 3-4	▨	🟧	🟧	🟥	🟥	🟥	🟧	▨	🟥	🟧
	pair 4-5		🟥	🟧	🟥	🟥	🟥	🟧	▨	🟥	🟥

Likewise, in this study, the observed increases of some specialization indexes could also have played a role in changing modularity and nestedness, although this did not translate into a lower robustness. Thus, it is possible that re-organizations at species level interactions could cause departures from scenarios expected by (other) numerical simulations.

Actually, the studies exploring relations between network structures and stability are constained on a rather static set of plants and pollinator assemblages. Thus, dynamical re-organizations of interactions are usually limited or allowed just randomly [6,7,22]. In the studied sites, species interactions re-organized in a very dynamical yet asymmetrical way, by the fact that plant species changed their species level indexes but pollinators did not. In other words, while pollinators were rather constant in their within-network positions, plant species became increasingly centralized (higher connectivity), connected other more centralized species (eigenvector centrality) and became slightly more generalized (PDI). Furthermore, the network-level rewiring was high, although without a clear linear trend with progressing plant removal, and played a larger role than species turnover in the partitioning of the total interaction turnover, as in [37]. Even the species-level rewiring index showed fluctuating trends but they centred around the values indicating that on average the number of lost interactions was equal to the number of new ones. This high rewiring rate suggests that in this system the interactions are highly flexible, which apparently helps to maintain the stability of the plant-flower visitor networks modified by species loss [15,54]. Thus, both the increased plant centrality and the sub-constant but high level of rewiring could explain why network robustness, as evaluated by the stochastic coextinction model, increased as more plants were removed.

Mechanistic insights into the changing structure of the plant-flower visitor networks can be gained by fitting a set of simulation models with different combinations of potential drivers of network structure [37,43,45,46]. Interestingly, a different picture emerges depending on whether we focus on the ability of the models to predict a set of indices describing structural features of the networks or their ability to predict individual pairwise interactions.

In the analysis of network indices (Table 4), the most consistent fit to the observed data, i.e. most indices at least in some sites and removal stages correctly predicted, was achieved by the most complex model which assumed that the probability of interactions depends on the combination of plant and insect abundance, morphological match, and the amount of rewards (sugars per flower). This took place especially with the progressing of the plant removal, that may suggest a prominence of network complexity with generalist plant loss. Conversely, some of the simpler models predicted some of the indices well and others poorly. In particular, the model based solely on plant abundance predicted nestedness and insect generality particularly well. This reflects the role of abundant generalist plants, which interact with a large number of insect species and thus form the generalist core of the network which connects different modules [55] and drives the nested pattern typical for plant-pollinator networks [56]. It is worth to highlight that in this study, the plant abundance was measured as the number of flowers in the sampling transects, so this result is not biased by using the sums of interaction frequencies as a proxy for abundance. Furthermore, modularity was predicted well by morphological match before the start of the removal experiment, as expected based on the assumption that trait matching is responsible for modular structure in plant-pollinator networks [57,58], although this relation did not hold with the progressing of plant removal.

Our approach also included a model based on the amount of sugars in the nectar, that is a well known driver of pollinator interactions with plants [59]. In this study we have found that it can partly explain the pairwise interactions frequencies. Furthermore, it also played a consistent role in predicting insect generality, and also network nestedness when in interplay with morphological match. This is not surprising because it is the morphological matching that allows to gather resources more efficiently [60,61]. Therefore, it seems that the availability of this important reward played a role in shaping network nestedness, the pairwise interactions and also the insect generality foraging choices.

On the other hand, the occurence and frequency of individual pairwise interactions (Table 3) was best fitted by the model where insect abundance was assumed to be the main driver and the null model fitted the interactions best after three or four plant species were removed. The good performance of the model using insect abundance reflects that the most abundant insects, which had high probability of interactions with all plant species in the model, were also the most generalists in reality. In addition, the superior fit of the null model in the later stages of the experiment suggests that in impoverished communities, species tend to interact more randomly.

Different components of the interaction turnover were correctly predicted by several models. The total interaction turnover, the role of species turnover and the rewiring were best predicted by a combination of plant and insect abundance (Table 5). Moreover, also the combination of abundance and sugar amount consistently contributed to explain these indexes. The abundant species, and the richly rewarding plants, are mostly generalists so the disappearance or appearance of an abundant species brings about the loss or gain of many interactions. Generalists are also more connected within a network and, thus, more likely to rewire and to establish new interactions [47,62]. Furthermore, the null model consistently over-predicted the rewiring of the species, and this indicates that rewiring did not happen randomly or simply because of species co-occurrence. This suggests that further constrains determined the rate of rewiring, as also observed in chapter 4 of this thesis. As matter of fact, during some stages of plant removal, morphology matching predicted the rewiring. This could be a result of the higher specialism emerged during the plant removal at network level, because the morphological matching predicted also other more specialized plant-pollinator networks [46,63].

In conclusion, the core generalist species should be preserved because otherwise the network will change structure and more randomness could take place. This fact might have some degree of impact also on the functionality of the system [1,64,65], although it deserves further exploration in the field. However, in the case of species rich assemblages as in the manipulated sites of this study, it must be acknowledged that the inner dynamism of the species and their asymmetrical responses allowed re-organization of interactions, and this compensated for the roles of the removed species [18]. Therefore, species-rich communities have an inner ability to reorganize, which may temporarily buffer them against external pressures and potentially prevent the expected network collapse from taking place.

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Supplementary information

Figure S1. Interaction turnover indexes detection by probability models, part 1 (NU= null, P= plant abund., I= Insect abund., A= abund., M= morphological match, S= sugar amount). The vertical lines represent the observed values (solid line for Site 1, dashed for Site 2, loosely dashed for Site3). The horizontal segments are the ranges from the simulations and are vertically distributed (Site 1, under Site 2 that are under Site 3).

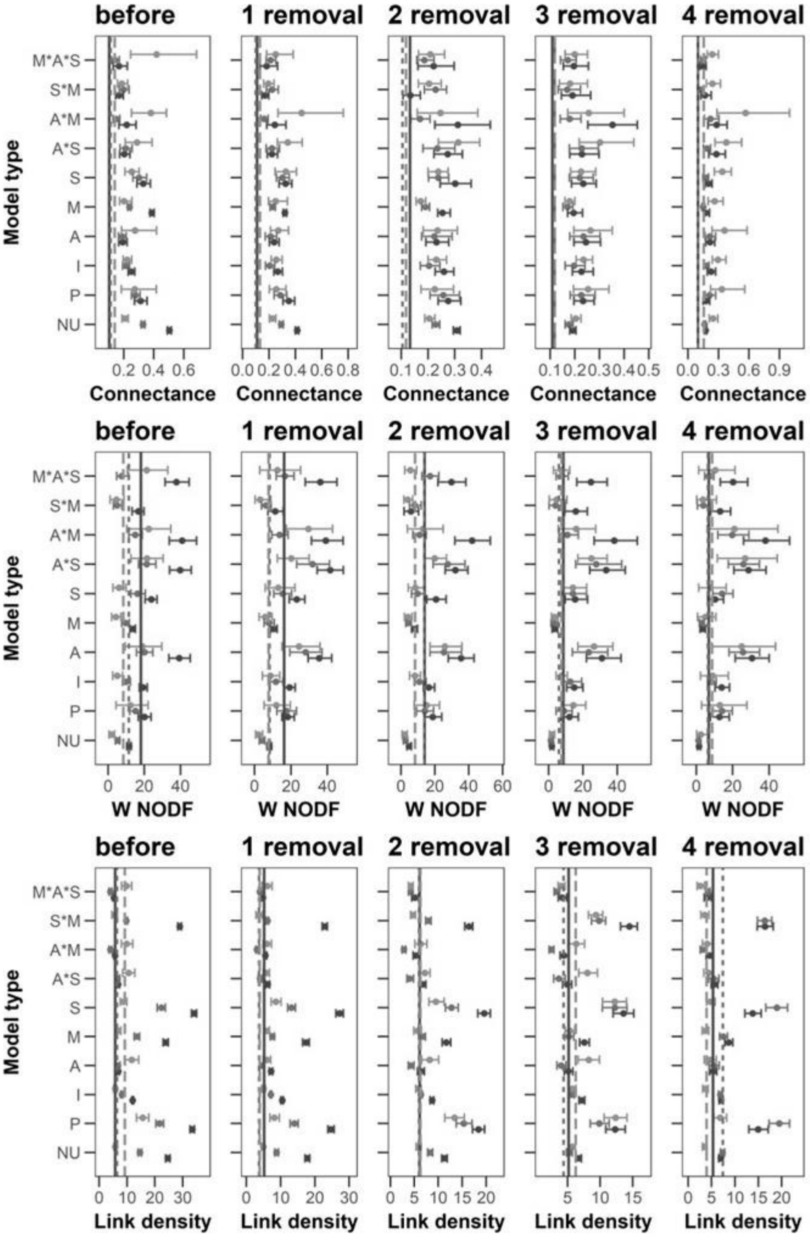


Figure S2. Network indexes detection by probability models, part 2 (NU= null, P= plant abund., I= Insect abund., A= abundances, M= morphological match, S= sugar amount). The vertical lines represent the observed values (solid line for Site 1, dashed for Site 2, loosely dashed for Site3). The horizontal segments are the ranges from the simulations and are vertically distributed (Site 1, under Site 2 that are under Site 3).

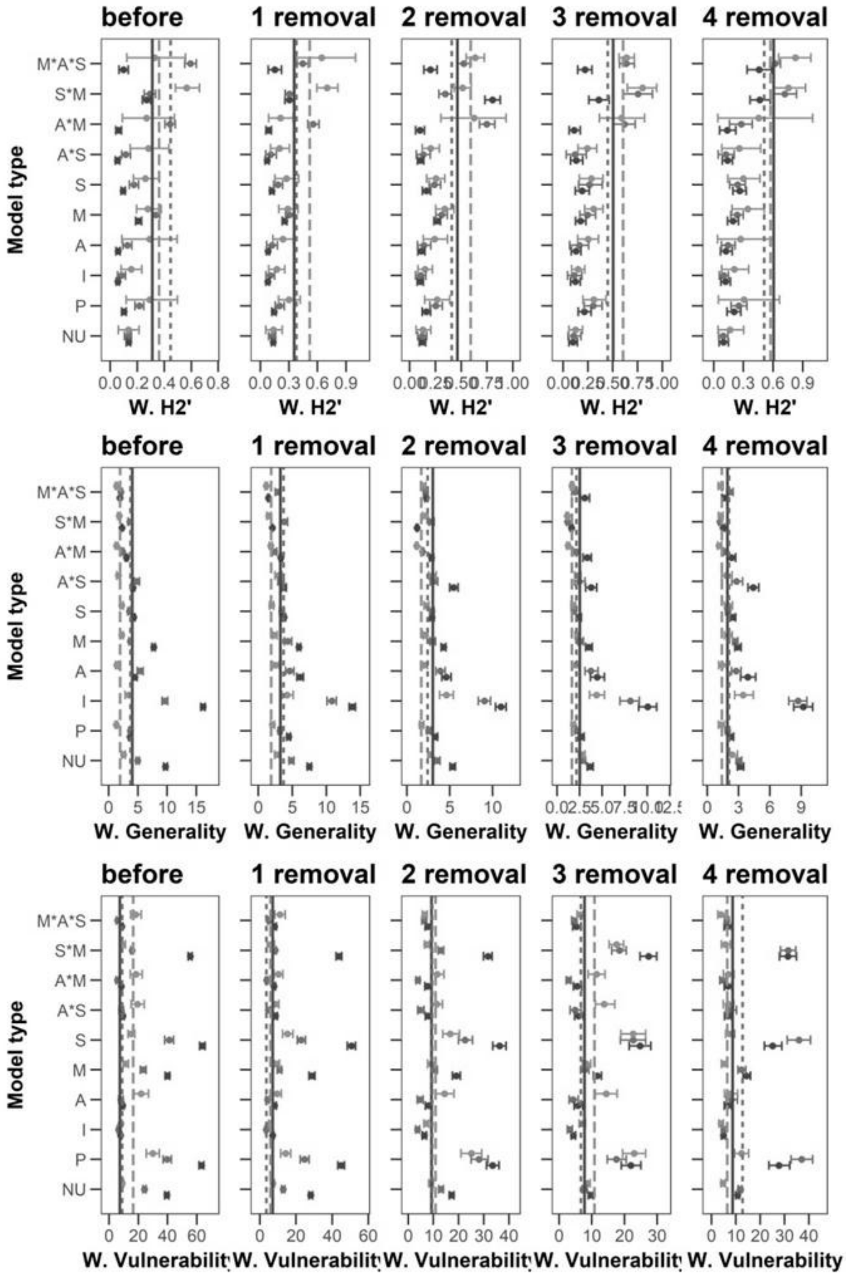


Figure S3. Interaction turnover indexes detection by probability models, part 3 (NU= null, P= plant abund., I= Insect abund., A= abundances, M= morphological match, S= sugar amount). The vertical lines represent the observed values (solid line for Site 1, dashed for Site 2, loosely dashed for Site3). The horizontal segments are the ranges from the simulations and are vertically distributed (Site 1, under Site 2 that are under Site 3).

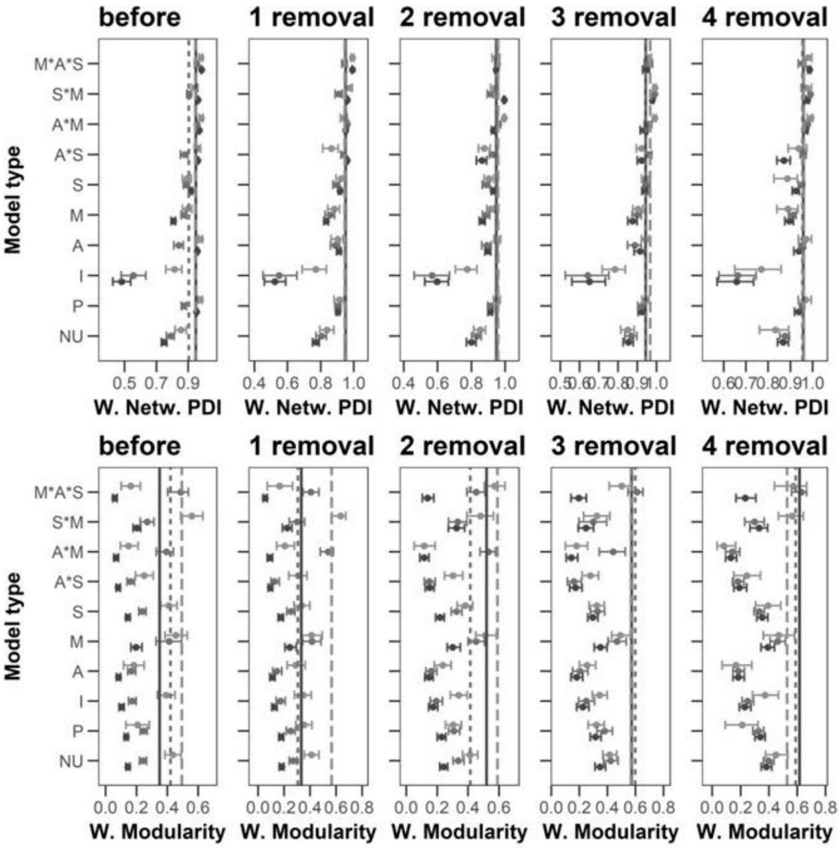


Figure S4. Interaction turnover indexes detection by probability models, part 1 (NU= null, P= plant abund., I= Insect abund., A= abund., M= morphological match, S= sugar amount). The vertical lines represent the observed values (solid line for Site 1, dashed for Site 2, loosely dashed for Site3). The horizontal segments are the ranges from the simulations and are vertically distributed (Site 1, under Site 2 that are under Site 3).

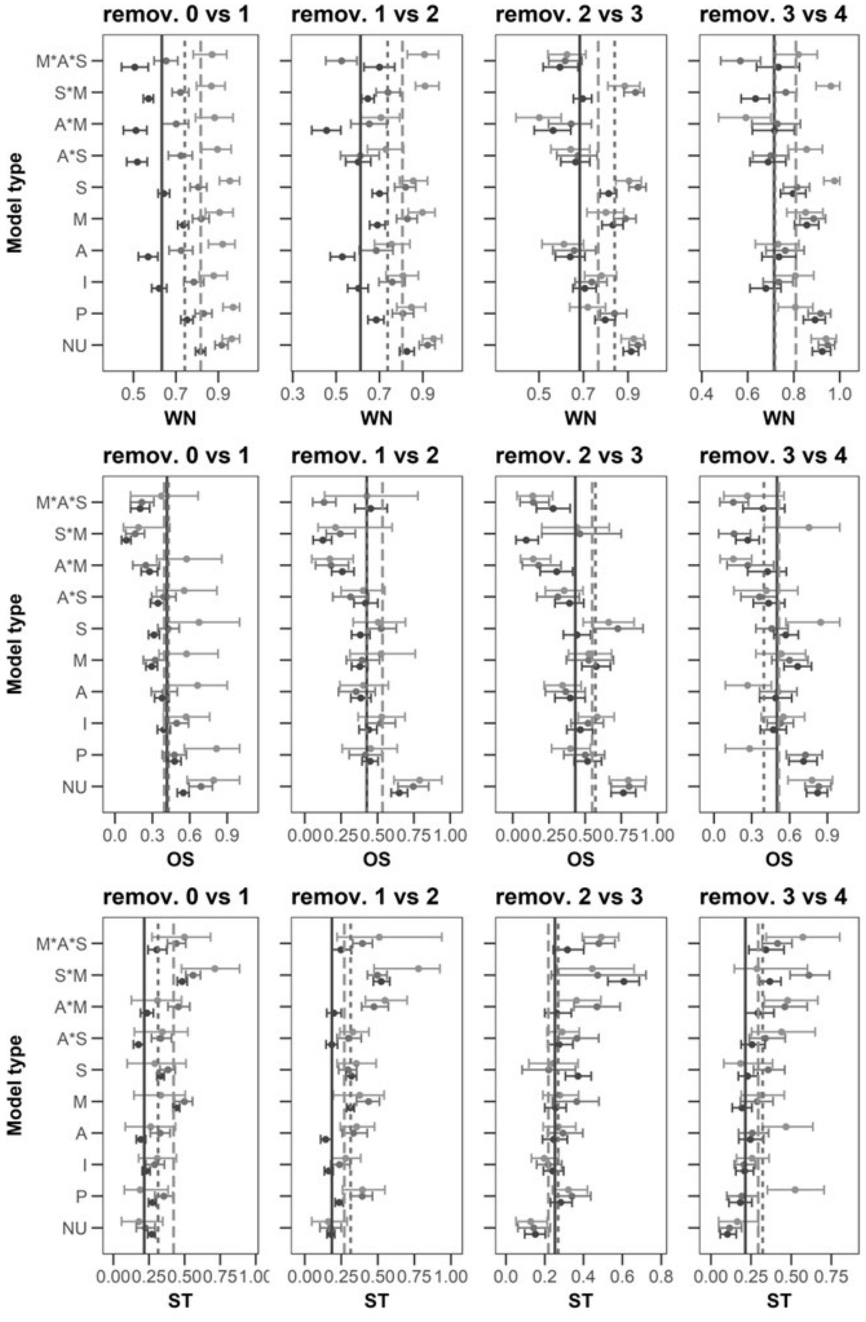
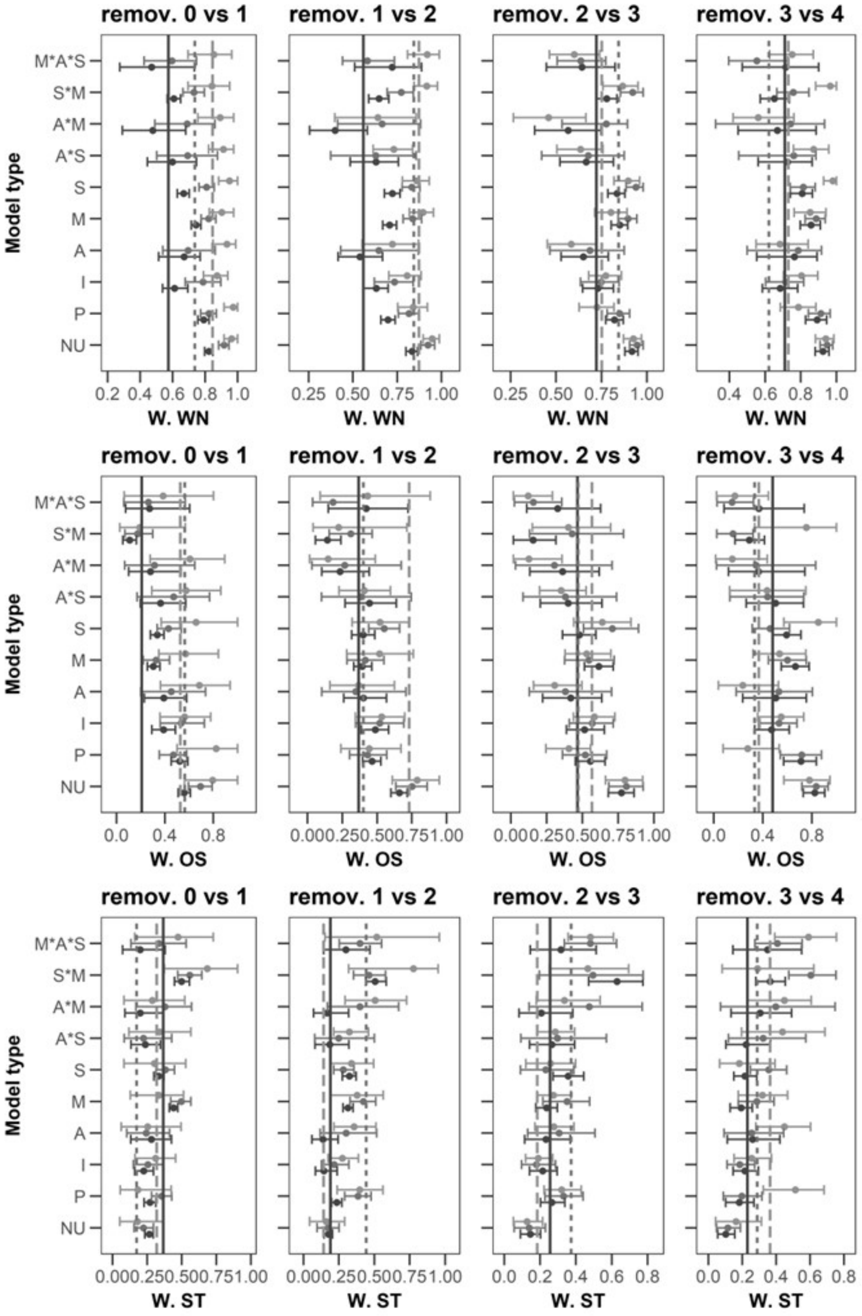


Figure S5. Interaction turnover indexes detection by probability models, part 2 (NU= null, P= plant abund., I= Insect abund., A= abund., M= morphological match, S= sugar amount). The vertical lines represent the observed values (solid line for Site 1, dashed for Site 2, loosely dashed for Site3). The horizontal segments are the ranges from the simulations and are vertically distributed (Site 1, under Site 2 that are under Site 3).



Summary

This thesis researched several aspects of plant and pollinator interactions and it focused on a conservation perspective. Chapter 1 dealt with ecosystem functioning through species interactions, something not normally considered from the conservation perspective. To inform conservation planning, I analyzed two datasets of plant-pollinator assemblages of the entire summer season and from unaltered areas. The phenology was accounted for by defining units of time of co-blooming plants. My results indicated that it is possible to pinpoint the key species ("hubs") with a network analysis of the interactions. Hub identity changed during the season: some were top-ranking for the structural importance and others were of biogeographical interest. Furthermore, the hubs were rich in links and in partner diversity. Thus, I concluded that conservation could also focus on the hubs, given that they interact with a large subset of species and support the greatest number of interactions.

In chapter 2, I focused on the problem of detecting and, consequently, of studying rare species, which often deserve high conservation effort. An elusive alpine bumblebee was studied and I built a database of occurrences ex-novo. The aims were to describe the ecological niche of this bumblebee and also to investigate what plants it visits. The analyses indicated that this bumblebee is restricted to the upper altitudes, has a narrow niche related to the glaciers but is also affected by drought and temperature variability. However, the records list this taxon visiting almost 40 plant species for gathering resources. Further, a strong altitudinal shift is also taking place which poses concerns on the future extent of this taxon's populations.

In chapter 3, I investigated the effect of population decline on the way pollinators gather resources. This aim was tested by focusing on the pollen collected by bumblebees before and after an experimental halving of the workers' population size, in the field. I expected to find either density-dependent responses, such as a higher specialism for the low competitor density, or "diet" expansion in the remaining workers for compensation of the resources of missing workforce. The collected pollen was identified with DNA metabarcoding and foraging was investigated with network analyses. The results indicate that foraging changed only minimally, both as diet breadth and as feeding network after removal. Therefore, the constancy in feeding strategy indicates that foragers of this social pollinator may not compensate for the lack of resources incoming to the colony when the population size is halved and this could impact colony growth.

The last two chapters dealt with the manipulation of plant-pollinator assemblages in the field. In chapter 4, the way a removal of generalist plants will affect flower visitors' abundances, pollinator effectiveness, and insect foraging was studied. I expected that pollinators will either shift interactions to the remaining plants or stop foraging at the sites. The results indicated exponential decrease of visitation, but also large variations among transects and among plant species. Further, flower visitors did not switch among flower shapes but they also favoured specific plant traits after plant removal (higher sugar content in nectar, smaller inflorescences). This suggests that foragers shifted interactions but also that plant traits constrained the resource utilization by flower visitors. In addition, the pollinator effectiveness fluctuated but was not linked to changes of flower visitation. I concluded that the loss of highly generalist plants triggers declines in local pollinator abundances and has implications for pollination and insect foraging. Thus, the stability of the system could depend on the subset of the core generalist species.

In chapter 5, I explored other aspects from the field manipulation of removing generalist plants, namely how plant-pollinator network structure and interaction reorganization responded to core species loss. I expected to find strong changes in network structures, given their dependency of the core species. This manipulation caused a linear negative trend in nestedness, and a positive one in modularity and in specialization, but it did not cause significant trends in robustness. These changes were mainly driven by models incorporating plant and insect abundances. I also focused on network's and species' rewiring which showed a constantly high rate of change in interactions, again driven by abundances and also by plant-pollinator morphological matching. Surprisingly, only the plant species increased their centralities within the networks after core plant removal, indicating asymmetrical responses among network levels. This study also showed that unpredictability and randomness of the interactions also increased during the experiment. I concluded that conservation of core generalists can prevent plant pollinator networks from changing sharply but it should also be acknowledged that asymmetrical topological reorganization in species interactions could temporarily buffer the species loss.

In conclusion, my thesis contributed to understanding how conservation can deal with hard-to-detect rare species and also how it could interplay with complex species interactions and the mechanisms that rule them. It was shown that rare species can be studied with a detailed analytical approach and this can hamper the knowledge on the habitat and on interactions of elusive species, which can further allow the planning of specific conservation actions. From the other analyses and also from the experimental manipulations presented in this thesis, several additional aspects on the conservation of plant-pollinator interactions emerged. Firstly, pollinators showed to forage in the field with less adaptability to the environment than what expected, since a social pollinator changed the foraging strategy only slightly after population reduction. This will have implications for resource acquisition and ultimately for population growth. In addition, it is now clearer that community-level plant-pollinators interactions are dependent on important species that interact with many others. The results of an experimental removal of several core generalist species proved increased instability of network structures, decreased flower visitor abundances and an emerging randomness of interactions. Nevertheless, the network robustness did not change and species were constantly able to rewire the interactions (i.e. shifting partners) and only plants re-organized within the network by increasing centralization. Overall, these results will possibly provide indications on a way to conserve and restore the interacting communities, such as by focusing on the core generalists plants that can prevent plant-pollinator networks from changing sharply.

Appendix

CURRICULUM VITAE

MSc. Paolo Biella

Born in Calcinate (BG, Italy), 20.08.1989

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Research interest

I am interested in plant - pollinator interactions and pollination ecology, network analysis and also taxonomy of Hymenoptera (Apoidea) and identifications of European pollinating Diptera. I'm studying the structure of plant-pollinator networks with a focus on conservation and on the mechanisms structuring them, by using field experiments. I am investigating the pollination ecology and genetic structure of rare endemic plants. I'm also exploring the habitat requirements and biogegraphy of Bumblebees, especially for conservation.

Education

Since 2014: PhD Student In Entomology, University of South Bohemia, České Budějovice, CZ. PhD Thesis: A conservation perspective on the mechanisms that influence plant-pollinator interactions

2012 – 2013: Master degree, University of Pavia (Italy) in "Scienze della natura, gestione del patrimonio naturalistico" (= Applied environmental sciences in nature management).

2008 – 2011: Bachelor (1st cycle) degree, University of Pavia (Italy) in "Scienze e tecnologie per la natura" degree (= Environmental sciences and technologies).

Specialist courses

2016 4-8 April: “Introduction to bayesian statistics applied to ecology”, by Wildlife Science snc at University of Pavia, Italy.

2014 21-25 July: “Taxonomy of Italian Coleoptera”, by ASNM at Natural History Museum, Verona.

2013 4-5 February: “Bee identification workshop”, by dr. Mike Edwards at LBRG of the University of Northampton, UK.

Employment

Since 2014: Employee at Biology Centre, CAS, České Budějovice, CZ.

2014: Research assistant, University of Pavia, Department of Earth and Environment Sciences

2014: Voluntarily, arrangement of the entomological collections and the re-classification of bees. Museum of Natural History of the University of Pavia.

2012 and 2013: Tutoring classes for Bachelor students and practice activities, University of Pavia, Department of Earth and Environment Sciences

Teaching and popularizing science

2018: Talk and excursion at the “In the field with researchers” talk series for the Natural History Society of the Verbano Cusio Ossola (Italy). Talk title: “The Apoidea (Hymenoptera Anthophila) and their role in pollination; the bumblebees. Sampling methods” (ca. 10h, 28-29 April).

2018, 2017: Lecture/seminar to students at the course “Animals biological interactions”, University of Milano Bicocca, BtBs (ca. 2h, 16 October 2018, ca. 2h, 24 November 2017)

2017: Co-supervision of the thesis of a postgraduate student of the Unversity of Milan.

2016: Practice activities classes at the course “Interactions between plants and animals”, University of South Bohemia (ca. 30h).

2012 and 2013: Tutoring classes for Bachelor students and practice activities in Botany, University of Pavia, Department of Earth and Environment Sciences (ca. 200 hrs).

Additional relevant activity

Main organizer of the conferences “ABIM, Alpine Bombus International Meeting” that took place in 2016 and 2018.

Writing a grant proposal for the funding agency of the University of South Bohemia in 2015.

I reviewed 6 research manuscripts for IF journals.

Professional memberships

Since 2017: IUCN Bumblebee Specialist Group.

Since 2018: SEI, Società Entomologica Italiana (Ital. Entom. Soc.).

Since 2018: FAB, Flora Alpina Bergamasca (Bergamasque Alpine Flora).

Skills

Languages: Italian (native speaker); English (fluent, 643/677 Toefl ITP with Listening B2, Writing C1, Reading B2); Spanish(fluent); Latin (read only); Czech, (A1).

Taxonomic identification: Hymenoptera: Anthophila; Diptera: Muscidae, Calliphoridae, Tachinidae, Sepsidae, Chloropidae, partly Empididae, Syrphidae, Conopidae. Plants.

Statistical and other computer-related skills: mainly for multivariate analyses, regressions, network analyses, spatial analyses, data visualization, morphometic and image analyses CRAN R (advanced), STATISTICA, PAST and SPSS (intermediate), QGIS (beginner), Microsoft Office (advanced), Roxas (macro for ImageJ, intermediate), tps (intermediate).

Technical skills: morphometrics, DNA extraction, PCR, integrated insect and plant identifications, pollination ecology lab. techniques, scientific writing, insect rearing.

Field samplings: especially in Botany and Entomology, flower visitors, nectar, pollen, pistils.

Scholarships

2014-2018 Scholarship as PhD student at Faculty of Science, University of South Bohemia

2013, Erasmus Placement Program Scholarship – Northampton (UK), University of Northampton (UK) LBRG lab, prof. Ollerton J. – 4 months.

2009, Erasmus Student Exchange Network grant – Granada (Spain) – 10 months.

Academic placements

2018, 1 month, with dr. A. Nielsen, at the University of Oslo, CEES (Norway)

2016, 3 months, with prof. M. Labra, prof. M. Casiraghi and dr. A. Galimberti , at the University of Milano Bicocca, BtBs (Italy).

2013, 4 months, with prof. J. Ollerton, at the University of Northampton (UK).

2010, 10 months, with Prof. J.M. Gomez Reyes, at the University of Granada (Spain).

Posters and presentations at conferences

Talks:

Scandinavian Association for Pollination Ecology SCAPE 18-21 October 2018 –Blessington, Dublin, Ireland; ABIM Alpine Bombus International Meeting 29 - 31 of July 2018 – Ecrins National Park, France. EcoFlor 1-3 of February 2018 - Palma de Mallorca, Spain; SCAPE 26-29 of October 2017, Drobak, Norway. Zoodny 9-10 February 2017 – Brno, CZ. ABIM 20-22 July 2016 - University of Turin and Gran Paradiso National Park, Italy. 1st International Meeting on Plant Reproduction 15-16 September 2014 - University of Bologna, Italy.

Posters (first name author):

XI European Congress of Entomology, ECE2018 20-22 July 2018 - Naples, Italy. SCAPE 13-16 October 2016 – Abisko National Park, Sweden. Ecological Networks: Theory, Empiricism and Practice in a Changing World 7-8 September 2015, University of Bristol, UK. 109° Congress of the Società Botanica Italiana 2-5 September 2014 - Firenze, Italy. UK and Ireland Bumblebees working group 11 April 2013 – Northampton, UK.

Publications

In IF journals

Martinet B., Brasero N., Lecocq T., Biella P., …, Rasmont P. (2018). Adding attractive semio-chemical trait refines the taxonomy of Alpinobombus (Hymenoptera: Apidae). Apidologie, in press

Klecka J, Hadrava J, Biella P., Akter A. (2018). Flower visitation by hoverflies (Diptera: Syrphidae) in a temperate plant-pollinator network. PeerJ Preprints 6, e26516v1 in press

Brasero N., Martinet B., Lecocq T., Lhomme P., Biella P., ... Rasmont P. (2018). The cephalic labial gland secretions of two socially parasitic bumblebees Bombus hyperboreus (Alpinobombus) and Bombus inexspectatus (Thoracobombus) question their inquiline strategy. Insect science, 25, 75-86.

Muñoz-Pajares A. J., Perfectti F., Loureiro J., Abdelaziz M., Biella P., ... Gómez J.M. (2018). Niche differences may explain the geographic distribution of cytotypes in Erysimum mediohispanicum. Plant Biology, 20, 139-147.

Martinet B., Lecocq T. , Brasero N. , Biella P., ... Rasmont P. (2018) Following the Cold: Geographic Differentiation between Interglacial Refugia and Speciation in Arcto-Alpine Species Complex Bombus monticola (Hymenoptera: Apidae). Systematic Entomology

Biella P., Bogliani G., ... Milanesi P. (2017). Distribution patterns of the cold adapted bumblebee Bombus alpinus in the Alps and hints of an uphill shift (Insecta: Hymenoptera: Apidae). Journal of Insect Conservation, 2, 357-366.

Biella P., Ollerton, … (2017). Network analysis of phenological units to detect important species in plant-pollinator assemblages: can it inform conservation strategies?. Community Ecology, 18, 1-10.

Akter A., Biella P., Klecka J. (2017). Effects Of Small-Scale Clustering Of Flowers On Pollinator Foraging Behaviour And Flower Visitation Rate. PloS one 12, e0187976.

Volf M., Redmont C., Albert Á.J., Le Bagousse-Pinguet Y., Biella P., ... de Bello F. (2016). Effects of long- and short-term management on functional structure of meadows due to species turnover or intraspecific trait variability. Oecologia, 180, 941-950

Monograph

Quaranta M., Cornalba M., Biella P., … (2018). Red list IUCN of the Italian threathened bees (in Italian, Lista Rossa IUCN delle api italiane minacciate). Comitato Italiano IUCN e Ministero dell'Ambiente e della Tutela del Territorio e del Mare, Roma, in press

Not in IF journals

Biella P., Avesani D., Pont A. C. (2016). New records of flower-visiting Muscidae (Diptera) from the Ligurian Apennines of Italy. Studia Dipterologica, 22(1), 51-55.

Biella P. (2015) Bombus (Alpinobombus) alpinus in the Italian Central Alps (Hymenoptera: Apidae: Bombinae). Il Naturalista Valtellinese 25, 69-72.

Biella P, Groppali R. (2013) Campylomorphus homalisinus (Elateridae): a new species for Lombardy (Italy), with notes on its ecology, distribution and biogeography. Biodiversity Data Journal

Under rewiew

Biella P., Akter A, … Klecka J. (2018). Experimental loss of generalist plants reveals alterations in plant-pollinator interactions and a constrained flexibility of foraging. bioRxiv, 279430 Resubmitted after major revision

Biella P., Tommasi N., … Galimberti A. (2018). Integrative approach reveals a constancy in the foraging strategy of a social pollinator after a strong reduction of the colony's workforce. Under review