School of Doctoral Studies in Biological Sciences

University of South Bohemia in České Budějovice

Faculty of Sciences

From resource variation to habitat loss: how changing environment shapes the plant-pollinator interactions

Ph.D. Thesis

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České Budějovice, 2020

This thesis should be cited as:

Akter, A. 2020. From resource variation to habitat loss: how changing environment shapes the plant-pollinator interactions. Ph.D. Thesis. University of South Bohemia, Faculty of Science, School of Doctoral Studies in Biological Sciences, České Budějovice, Czech Republic. 2020. 179 pp.

Annotation:

Akter, A. and Klecka, J. 2020. Interactive effects of temperature, water, and nitrogen availability on the growth, floral traits, and pollination of white mustard, *Sinapis alba.* This was an experimental approach where *Sinapis alba* (white mustard), an economically beneficial crop was grown under different environmental conditions. Here, we assess how different growing conditions affected the growth of *S. alba* and the impact of different climate changing drivers on pollination and seed production.

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(11): e0187976. In this part of research, we experimentally changed the spatial structure of flower in small patches and assessed the impact on the foraging behaviour of pollinators, particularly, *A. mellifera*.

Akter, A., Biella, P., Klecka, J. 2020. Introduction of new floral resources into plant-pollinator communities: impact on flower visitation mediated by species traits. This field-based experiment showed the impact of experimentally introduced floral resources in a plant community and how the response of the pollinator communities towards the new resources were mediated by the floral traits.

Biella, P., Akter, A., Pajares, A.J.M., Federici, G., Galimberti, A., Jersáková, J., Labra, M., Mangili, F., Tommasi N. and Mangili, L. 2020. Pollination strategies of a plant in an unstable habitat: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae). In this field-based research we assessed the pollination and reproduction strategies of an endemic plant, *Linaria tonzigii,* and the relation between the genetic variation and distribution.

Akter, A., Biella, P., Batáry, P., Klecka, J. 2020. Changing pollinator communities along a disturbance gradient in the Sundarbans mangrove forest: a case study on *Acanthus ilicifolius* and *Avicennia officinalis. Global Ecology and Conservation* 24: e01282. This study was carried out in the Sundarbans mangrove forest, where we assessed the impact of human disturbances and forest loss on the plant-pollinator communities.

Declaration:

I hereby declare that my Ph.D. thesis is my work alone and that I have used only those sources and literature detailed in the list of references.

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This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and the Institute of Entomology, Biology Centre of the CAS, supporting the study of Entomology.

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This work was supported by the Grant agency of the University of South Bohemia (065/2017/P), Czech Science Foundation (GAČR, grant no. GP14-10035P and 17-24795Y), Early Career Grant (GR- 000039683) from National Geographic Society and Flora Alpine Bergamasca, Italy.

Acknowledgements:

I would like to express my gratefulness to my supervisor, Dr. Jan Klečka, for his guidance and encouragement throughout my Ph.D. study. He has provided me the platform to become an independent researcher, always welcoming to discuss ideas and opportunities, and supported me all along my studies in every aspect. Without his continuous help and support, I would never be able to accomplish any of my achievements. I am also grateful to Dr. Paolo Biella, colleague and friend, who’s contribution and encouragement in my study is beyond my expression. I would also like to thank Dr. Jana Jersáková, who taught me many practical things in my field of study and has been always my guide whenever I had any trouble.

I would like to thank my parents, who are always proud of me, believed in me and I would have been nothing if they were never there whenever I needed them. I am also grateful to my only son Aleef Mahmood and his father, Sazzad Mahmood, for their understanding and continuous support and I apologize for not being the person that they deserve. Also, thanks to my dearest friend Marjita Chakma, who never stopped encouraging me and supporting me, even in the most difficult time. I am thankful to my little two sisters Urmi and Tasmin, who’s love, and friendship always made me feel happy and special.

I am deeply gratified to my all colleagues and friends in the Czech Republic, in Bangladesh and anywhere in the world, for enriching my life with support and happiness. This was a long journey and I embrace every part of it, and I am grateful to everyone in my life for playing their unique role in the most positive way.

List of works and contributions:

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(11): e0187976. (IF=2.74).

Asma Akter conceived the project, performed the field work and lab experiments and prepared the manuscript.

Akter, A., Biella, P., Batáry, P., Klecka, J. 2020. Changing pollinator communities along a disturbance gradient in the Sundarbans mangrove forest: a case study on *Acanthus ilicifolius* and *Avicennia officinalis*. *Global Ecology and Conservation* 24: e01282. (IF= 3.0).

Asma Akter conceived the project, performed the field work and lab experiments and prepared the manuscript.

Akter, A. and Klecka, J. 2020. Interactive effects of temperature, water, and nitrogen availability on the growth, floral traits, and pollination of white mustard, *Sinapis alba.* (manuscript prepared for submission)

Asma Akter conceived the project, performed the field work and lab experiments and prepared the manuscript.

Biella, P., **Akter, A.,** Pajares, A.J.M., Federici, G., Galimberti, A., Jersáková, J., Labra, M., Mangili, F., Tommasi N. and Mangili, L. 2020. Pollination strategies of a plant in an unstable habitat: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae). (manuscript under review in Plant Ecology)

Asma Akter contributed in the field works, laboratory analysis and in manuscript preparation.

Akter, A., Biella, P., Klecka, J. 2020. Introduction of new floral resources into plant-pollinator communities: impact on flower visitation mediated by species traits. (manuscript under preparation).

Asma Akter conceived the project, performed the field work and lab experiments and prepared the manuscript.

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1. General Introduction

Photo by Asma Akter

1.1 Plant-pollinator interactions: under the pressure of global change

Pollination, the mutualistic interaction between plants and animals, is one of the most important ecosystem functions on which both biodiversity and human welfare depend (Bos *et al.*, 2007). It is estimated that about 90% of flowering plants rely on animal-mediated pollination (Ollerton *et al*., 2011), whereas 75% are major crops which show increased fruit or seed set with animal pollination (Klein *et al*., 2007). Plant-pollinator interaction is not only essential for successful reproduction of plants but also fundamental to the ecosystem adaptability where plant and insect communities are connected (Bartomeus *et al*., 2013; Bascompte and Olesen, 2015). Without pollinators, many plants would not be able to reproduce and in turn without the reward from plants such as pollen, nectar, seeds and fruits, many animal populations would decline (Kearns *et al*., 1998). Thus, plant-pollinator interaction is not only considered as a unique, ecologically and economically important relationship, but also a key factor in maintaining the functional integrity of most terrestrial ecosystem and plays an important role in the organization and persistence of biodiversity (Bascompte, 2009, Ollerton *et al*., 2011). Impact of pollinator loss presented in a schematic chart in Fig.1.1.

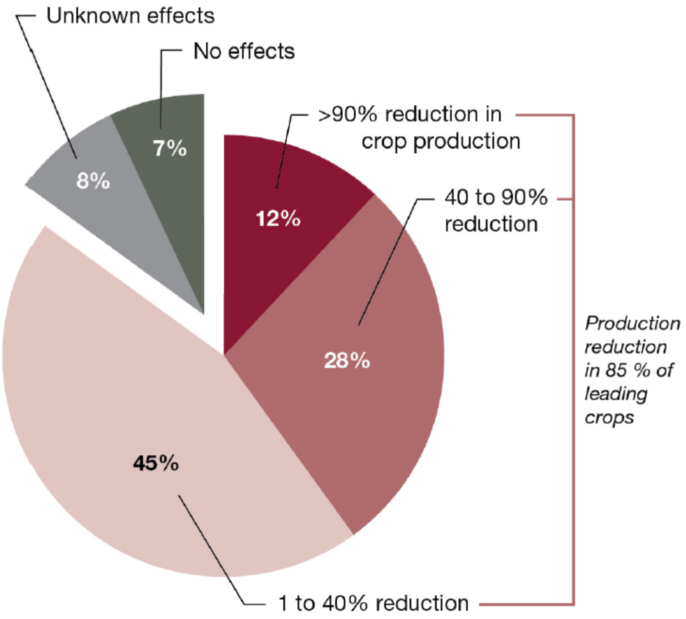

Figure 1.1: Percentage of production loss due to pollinator loss in leading global crops (Source: IPBES, 2019).

The relationship between plant and pollinators are shaped by many factors, from species level to community level and particularly susceptible to the ongoing changes in the environment (Burkle and Alarcón, 2011, Burkle *et al*. 2013). Plant-pollinator interactions is facing an on-going threat due to the multiple global changing factors including changing climate, species invasion, habitat loss and fragmentation. Given rapid environmental changes, it is also essential to consider the impact of natural spatio-temporal variation and resource variability on the plant–pollinator interactions. A global pollination crisis has been recognized recently (Kevan, 1999; Bartomeus *et al*., 2018) and understanding the fundamental component of plant-pollinator community structures, interactions and impact of changing environment on the plant-pollinator interactions is essential for developing solid conservation and management preferences for terrestrial ecosystems (Burkle and Alarcón, 2011; Potts *et al*, 2003; Biesmeijer *et al*., 2006).

1.2. Intra-species and flower resource variability due to changing climatic factors: a less considered issue

Plants are sedimentary inhabitants of environments that are capable to adapt with changing environmental conditions. Many of the environmental variables regulate the plant performance, for example, temperature, season and resource availability, show a correlated impact on the plant growth and phenology (Kudo and Molau, 1999). Evidently, different environmental conditions have affected the physiology of flowering plants in terms of pollen, nectar and flower production (Scaven and Rafferty, 2013) and these events are connected to the associations of pollinators or flower visitors. Global climate change, primarily caused by increased emissions of greenhouse gases and accelerated by human exploitation, is likely to affect ecosystems in many ways, but the consequences depend on the combine effects of climate and other global change components (Bazzaz, 1990). The timing of flowering in plants is temperature-sensitive and the rise of global temperature, which is one of the major key driver of global climate change, is inducing variation in this important determinant of reproductive success in plants (Fitter and Fitter 2002). Changing climatic conditions can alter the timing of species life cycle events and their geographic distributions, which may radically change the trophic networks and severely impair the ecosystem functioning. Researchers also showed that with increasing temperature, there is also altered precipitation and rising carbon dioxide (CO_2) concentration in the atmosphere which affect the timing of the species and ecosystem level phenology (Cleland *et al*., 2007). Although, shifting in flowering time in the plants are most pointed out response to climatic changes, but there is also influences of this event on other important traits of many species (Kudo, 1993, Inouye *et al*., 2002, Perfors *et al*., 2003). Critical response of plants to these changes also include the changes in the duration and abundance of flowering which have potentiality to disrupt the ecological relationship among plants, pollinators and other related species (Memmott et.al., 2007).

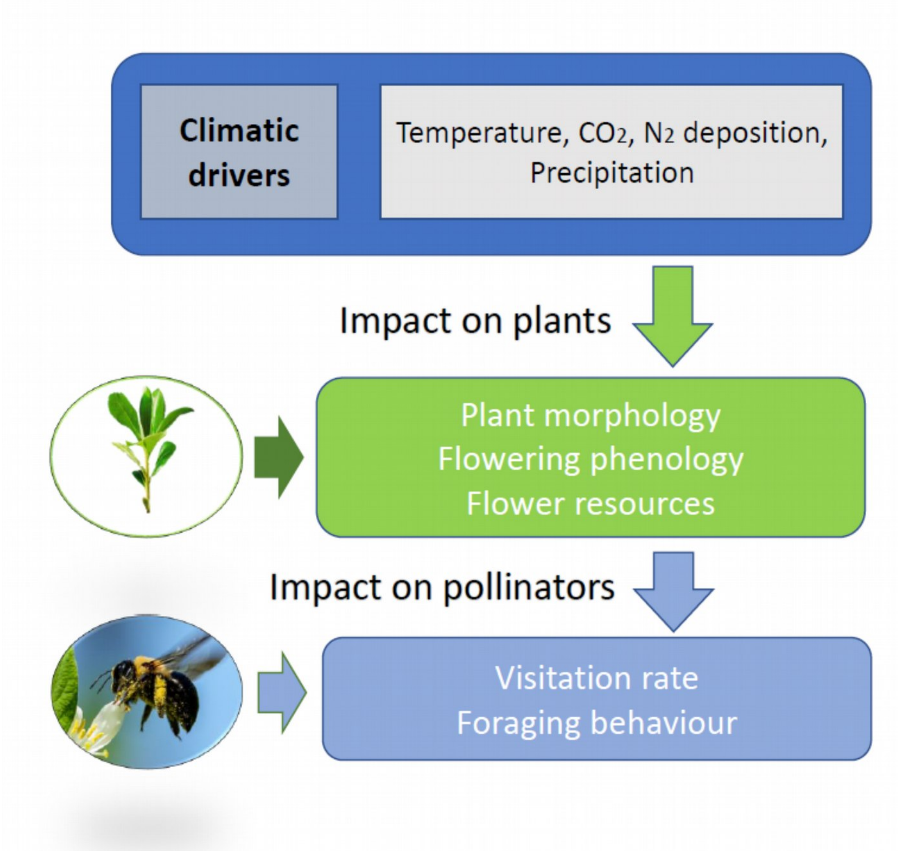

Figure 1.2: Impact of global environmental change drivers (temperature, nitrogen deposition, CO_2, precipitation) and their impact on plants and pollinators.

Other climatic changing drivers such as deposition of fixed N_2, Phosphorus enrichment, solar radiation, and scarcity of water or increased precipitation (Cleland *et al*., 2006, IPCC, 2007, Hoover et al., 2012, Wang et al., 2020) are associated parallel events which also have great impact on the plant growth, species abundance, pollinator sensitivity and plant-pollinator interactions along with increasing temperature and atmospheric CO_2 levels (Fig. 2). Many times, these drivers reported having positive impacts, for instance, increasing temperature lead to earlier blooming of many plants (Permesan and Yohy, 2003), elevated CO_2 and N_2 play an important role in improving productivity of plant (Hirel *et al*., 2007; Sala *et al*., 2000). Pollination success in insect-pollinated plant species is often correlated to the floral resources and pollinator activities and changing climatic conditions are causing the spatial variation in the plants and flower resources, thus changing the interactions between the pollinator and plants. The relative importance of these diverse impacts of climate modification on plant community and pollinator diversity is challenging to assess. Furthermore, it is important to uncover the mechanisms behind the responses of interacting plants and pollinators to those changes.

1.2.1. Effect of different environmental conditions on plant phenology

Phenology is an important and easily measured indicator of the impact of climate changes on the plant growth from the scale of individuals to whole ecosystem over a sustained period of time (Cleland *et al*., 2007). Differentiation in the phenology of species is a remarkable way to maintain species co-existence in a diverse plant community by reducing the competition for pollinators and other essential resources (Rathcke and Lacey, 1985). It is likely that global change induces alteration in the phenology of different plant species, but the question is to what extent. Increasing atmospheric CO_2 and other greenhouse gases which are considered the drivers of increasing temperatures have impact on the plant growth and their phenological changes but surprisingly less attention was given to the role of other co-occurring environmental changes. According to data obtained from thermometer records, average global surface temperature is increasing approximately 0.3°C per decade for over the last 30 years (Hansen *et al.,* 2006). Alongside, species have begun to adapt to climatic changes via altered species ranges. A recent study showed that spring has advanced globally at a rate of 5.1 days per decade (Walther *et al*., 2002) and a significant number of plant species have accelerated their phenology in 21 European countries within last few decades (Menzel *et al*. 2006_a). The variation among the phenology of different species varies between the agricultural and wild species (Menzel *et al*., 2006_b), pollination types; different stages of reproductive growth (Farnsworth *et al*., 1995); different distinct ecosystem (Suzuki and Kudo, 1997) and also time of the year. Although, the longer length of the growing season influences the primary production of ecosystem (Randerson *et al.,* 1999), but a drier and warmer summer year appears to be suppressing production that is not expected with longer growing seasons, which indicates phenology also related to the adequate precipitation. Changes in temperature are also linked with the water availability of plant via changes in transpiration (Korner, 2006) and may likely affect the plant growth so as accelerated the flower production. Altered temperature may also affect the nutrient availability as mineralization rates increase with the temperature.

Considering that elevated CO_2 generally stimulates leaf-photosynthesis rate, which can translate into faster vegetative growth (Korner, 2006), researchers also found that it could cause delayed budburst in some trees and annual grasses (Calfapietra *et al*., 2003; Cleland *et al.,* 2006), whereas flowering in nitrogen-fixing clover was accelerated (Wagner *et al*., 2001). Lake and Hughes (1999) found that elevated CO_2 significantly nectar secretion rate in *Tropaeolum majus* L. but did not have any effect on the time of flowering or pollen to ovule ration or in the concentration of amino acids. These studies indicate that stimulation varies species to species and vegetative response may not have direct relation to the phenological growth (Lewis *et al*., 2003). In some studies, no effect of the availability of nutrition and CO_2 together found on the phenology of plant (Franzaring *et al*., 2008). However, when the elevated CO_2 initiates an early flowering in forbs and delayed flowering in grasses, it might result in a decreased phenological complementarity and make an open phenological niche (Sherry *et al.,* 2007).

Another component of climate change and threat to the ecosystems is the high level of N_2 deposition or N_2 enrichment (Sala *et al*., 2000). It has been estimated that total N_2 deposition in the Mediterranean area ranges from 10–38 kg N $ha^{-1}year^{-1}$ (Rodà *et al*., 2002). Gordo and Sanz (2009) found that experimental N_2 supplementation increased the crude protein concentration in plant, therefore, enriched the nutrition quality of *Trifolium subterraneum* without affecting the fibre concentration but they did not find any phenological differences between the treated and non-treated plants. On the other hand, N_2 enrichment can enhance plant growth, increase flower abundance, duration and size (Burkle & Irwin 2009) and effect the concentration and composition of amino acids in nectar (Gardener and Gillman, 2001). However, it is understandable that the consequences of climatic and environmental changes cannot be determined by investigating a single driver of climate change on an isolated single species. For example, N_2 deposition and temperature both affect plant physiological responses to elevated CO_2 with potential cascading effects on species while individually they may not have any significant impact (Tylianakis *et al*., 2008).

Water availability is likely to change as many regions of the world are facing water scarcity and other regions are facing increased precipitation (Christensen *et al.,* 2007). In general, decreased water availability causes early phenological growth although this in not universally applicable to every species when reproduction is size and age dependant (Nord and Lynch, 2009). Effect of the water availability also related to the stages of phenological growth. For example, severe water deficiency in maize delays silking but not anthesis (Blum, 1996).

Although the impacts of climatic changes on plant are well documented but little is known about the impacts on insect pollinator's life cycle from winter hibernation through foraging to reproduction. Several bird species have advanced their nesting and timing of spring migration in response to climatic changes (Crick *et al*., 1997; Jonzen, 2006). Few studies demonstrated the importance of temperature on insect phenology with those species with a wintering larval stage and observed early emergence of adult in warmer years (Gordo and Sanz, 2006). Studies on hoverflies and butterflies showed phenological shift as a consequence of global warming (Kuhlmann *et al.*, 2012) and butterflies proved as a valuable organism to study the effects of environmental changes (Roy and Sparks, 2000; Rosin et al., 2012). Bumble bees are ectothermic pollinators and they can control their body temperatures which allow them some independence from the environmental conditions (Heinrich, 1993). However, but there is clearly lacking of data on the bee species (Brown and Paxton, 2009). To my knowledge, only a single study focused on *Apis mellifera* (L.) and the small white *Pieris rapae* (L.) to examine the climate-associated phenological shifts and found a negatively related appearance time with the mean temperature and demonstrated that insect phenology would be a sensitive bioindicator of climate change (Gordo and Sanz, 2006). But it would be critical to use honey bee as an example as it is highly domesticated and one of the minority of perennial bee species whose adult remains active in the winter and regulate hive temperature in temperate latitude (Bartomeus *et al.*, 2011).

These examples showed that the effect of climatic change and variation of the environment on species and ecosystem function has become increasingly apparent. It has also been documented that species within the same community often showed variable phenological responses to the climate change (Both *et al*., 2009). In general, while many plant species take the advantage of the changing environmental conditions and have shown phenological advancement, others have shown no distinguishable differences; and others have displayed delayed phenological changes (Gordo & Sanz 2005; Both *et al*., 2009). To understand the significance of climate change on the phenology will require a full understanding of how all the drivers affect the plant and insect growth, phenology and the species interactions together.

1.2.2. Effect of different environmental conditions on insect foraging

Nectar is the most important reward offered by the plants to pollinators in exchange for pollination. However, plants vary in their nectar quality (composition and concentration) and quantity (volume), which contributes to the variation of the flower visitation by the visitors (Canto *et al*., 2010). Nectar contains a wide variety of chemical constituents where three sugars (glucose, fructose and sucrose) dominate the solutes (Baker and Baker, 1983) along with regular presence of free amino acids which play a significant role for pollinators (Rusterholz and Erhardt, 1998). Factors that contributed to the variation of nectar volume, concentration and chemical composition have been in central focused for long time in the field of plant-pollinator interactions (Jürgens, 2004). However, most studies have focused at the level of species populations, cultivars or sub-species (Herrera *et al*., 2006), but not at the level of intraspecific variation within populations. Notwithstanding, intraspecific variation among the same plant can be extensive and mostly because of the response towards different environmental conditions (e.g. light, water, fertilizer, atmospheric CO_2 and temperature) (Canto *et al*., 2010). Significant differences can also be found among the different parts of flower in the same plant, different nectars and different phases of flowering in the single flower although this intra-variation did get even lower attention than the intraspecific variations. Additionally, variation in the nectar can be greater in the field than those plants from the greenhouse (Canto *et al.*, 2007).

Like the phenological growth, nectar production and quality in the same species can also greatly vary between the field and greenhouse populations (Canto *et al*., 2007). Many studies investigated the impact of environmental changes on the physiology and biomass of plants but relatively few have examined the impact on nectar production and their quality. Generally, elevated CO_2 can induce higher nectar production and/or high sugar concentration but may decrease the amino acid concentration (Lake and Hughes, 1999). Although, Osborne *et al.*, (1997) found that an elevated CO_2 increased flower production rate but there were no differences in floral nectar volume, total nectar sugar per flower or in nectar solute concentration between ambient and elevated CO_2 treatments. Another experiment showed that nectar concentrations of sugar and amino acids or nectar carbohydrates composition did not differ with the elevated CO_2 (Davis, 2003). However, Rusterholz and Erhardt (1998) found that presence of elevated CO_2 could even cause reduction in nectar volume, total sugar and total amino acids per flower in three investigated non-leguminous forbs. They concluded that the variation among the species might be one important factor for differences. Along with elevated CO_2 in the atmosphere, N_2 deposition can change the flower abundance, duration and size through the nutrition enrichment of plants (Burkle and Irwin, 2009). N_2 enrichment can also affect the nectar quality by changing the concentration and composition and can potentially alter the pollinator preferences (Hoover *et al*., 2012). In some cases floral production strongly affected by the flower morph type, flower age and the amount of light received (Cawoy *et al*., 2008). Another influential factor towards the amino acid complement of nectar would be soil conditions. Analysis showed that the total concentration of amino acid increased with increasing fertilizer treatment, whereas N_2 played the principal role (Gardener and Gillman, 2001). On the other hand, nutrient supply does not always enhance nectar secretion if the soil is in poor condition. However, beside these three important factors, there are other factors which also have impact on the pollen quality. In fact, studies showed that the volume of nectar contained in a flower may also be affected by evaporation or dilution by rainwater as well as resorption (Cruden and Hermann, 1983; Pleasants, 1983). Importantly, sometimes irrigation plays more important role than fertilizer (Petanidou *et al*., 1999). Most of these factors are known to be the key ingredients in the alteration of the flowering phenology along with nectar quality which have been assessed or explained through different research approaches.

1.2.3. Spatial variation in flower resources and effect of intraspecific plant traits on the foraging behaviour of pollinators

Intraspecific trait variation induced by environmental change, described above, may have an important on foraging behaviour and fitness of many pollinators. Estimated over 275000 species of flowering plant have been attributed to their specialization with different animal pollinators (Stebbins, 1970). Studying the quality, quantity, and distribution of floral traits and rewards is an approach that develops the understanding of the links between environmental variation, plant growth, and fitness. Trait variation, which is an ultimate consequences of changing climatic condition attracted few investigation thorough the history of pollination research until recently. It is almost recognised that these climatic drivers can alter the plant growth, phenology of flower and nectar quality, so as the response of pollinators towards the affected plant and different phenological responses alter the competitive ability of different species resulting in unpredictable impacts on community structure. Pollinators can differentiate between conspecific plants based on the quantity and quality of these reward and thus reward production strongly influences pollinator visitation rate, flower handling time, and the distance and direction of movement within and between individuals (Gomez et al, 2008). Rewards can also act as an underlying factor promoting selection for certain floral traits related to the display and this can be possible where floral traits are an indicators of the quality and quantity of floral rewards (Fenster *et al.*, 2006).

Different groups of pollinators are attracted to different source of food provided by the flower and flower nectar and pollen appear to be specialized for different groups of pollinators. For example, hummingbirds prefer flowers which have high sucrose/hexose ratios in nectars (Baker and Baker, 1983). Different studies showed how the changing environmental conditions altered the composition and the quality of nectar and still pollinators attracted to their preferable food sources. Experimental studies showed that many pollinators were able to detect the differences in the composition of sugar component as well as the composition of the amino acid complement while using artificial nectar (Hill and Pierce, 1989). Differences in the volume of nectar have also been shown to affect foraging behaviour (Pyke, 1981). Mevi-Schütz et al., (2003) demonstrated that several female butterfly species have shown a clear preference for nectars with high amino acid contents while they were raised on low quality food. Female butterflies raised on high quality food did not show any significant preferences. A recent study showed that nectar amino acid also enhanced the reproduction of male butterflies (Cahenzli and Erhardt, 2013). These investigations indicated that the quality of nectar influences foraging behaviour of pollinators.

Generally, increased CO_2 provides accelerated carbohydrate level of nectar in many plants and this sweeter nectar may favour some pollinators (Jablonski *et al*., 2002) but not all pollinators welcome this opportunity. Beside the differentiation of nectars, enhanced nutrition can increase the number and size of flower at an individual floral level and result into increased floral display that might attract more pollinators than usual (Muñoz *et al*., 2005). Like floral display, increased floral density may alter the pollinator attraction within a given plant species (Nattero *et al*., 2011). An addition of high level N_2 can facilitate grasses and at the same time addition of low level N_2 can positively affect forbs, resulting in higher floral production, flower size and nectar production and consequently affected the pollinator attractions (Eckhart, 1991; Burkle and Irwin, 2010). Another important advertisement of flower is a variety of signals to attract the pollinators to their rewards. Changing climate can alter this floral chemistry within the species which may reshape signalling in flower colour or scent and these intraspecific trait variations may change the visitation rate of pollinators in the same plant species (Byers *et al.*, 2014). Understanding of the impact of climate change becomes more complicated when elevated CO_2, precipitation and temperature interact together on the plant-pollinator interactions. For example, elevated CO_2 accelerates flowering time for C3 plant species but increased temperatures may reduce nectar production by limiting water or by reducing flower longevity (Wolkovich *et al.,* 2012). The impacts of such interactions of these aspects on pollinator foraging behaviour and reproduction remains unexplored.

Many studied examined the relationship between the floral density and floral display size with the pollinator where these traits evidently affected the foraging behaviour of pollinators (Glaettli and Barrett 2008). For example, bumble bees prefers visiting inflorescences with larger floral displays (Ishii et al. 2008) and honey bees tend to visit more flowers but a smaller proportion of flowers in large inflorescence (Malerba and Nattero 2011). Theory of “energetics” has been applied recently to study foraging behaviour of pollinators towards the floral resources where function of the distance between flowers and amount of the food acquired are the component of quantifying the rewards (Heinrich and Raven, 1972; Abrol, 2006). Therefore, success of the foraging activities depend on the rate of food acquisition and floral structure, density and floral display and all together play the role to make foraging decisions. In case of the individual plants pollinator prefers to visit plants with large number of flowers or flowers with larger display (Salces-Castellano et al., 2016) to assure their higher amount of rewards and also these resources are easily detectable from the distance (). How many visitors a flower will receive depends on the amount of resources and the visiting patterns by the insects follow the optimal foraging theory, where foragers feed in such a way to maximise their rate of energy intake (MacArthur & Planka, 1966). Whether the variation induced the growing environmental condition or for competition or simply for genetic variation, all divergence in the flower rewards reflect in the foraging behaviour of the visiting insects and in turn affect the reproductive success of plants.

1.3. Changes in the structure of the community

Besides the direct impacts of global climate change, other major drivers such as habitat loss, changing land use and intentional and/or unintentional introduction of new species into a given ecosystem, can have effect on the interactions of plants and pollinators, including pollination (Memmott and Waser, 2002). While climate change can bring out the consequences of intra-species plant and flower variation, other components of global change have more dramatic impact on the plant-pollinator interactions. For instance, climatic variation can induce changes in plant communities and alter their distribution, which is predicted to affect 5-20% of Earth’s terrestrial ecosystems, particularly in temperate regions (Sala *et al*. 2000). Plants have the flexibility to adapt and move towards higher latitude and altitude, which lead to an expansion of population at the leading edge and reduction of population size at the contracting age (Bellard *et al*., 2012). Because of changing climatic situations, few species may no longer be adapted to the set of environmental conditions in a given range, and some new species might get the advantage to adapt to these changed climatic conditions. Whether due to the changing climatic conditions or human induced, a non-indigenous species can integrate into native communities and have profound effects on ecosystem functioning, biodiversity and evolutionary processes (Prentis *et al*., 2008).

1.3.1. Arrival of new flower resource into the community

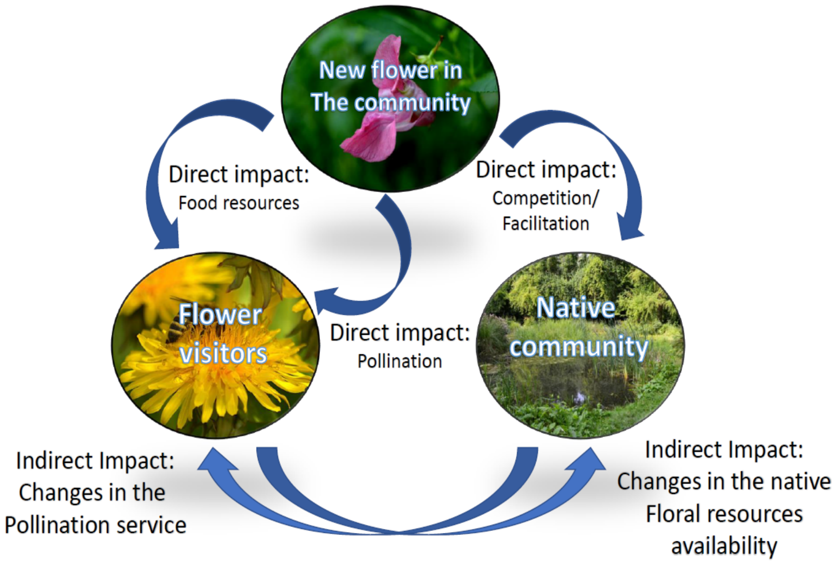

Figure 1.3: Arrival of a new flower resources in a novel community and expected outcome among plants and pollinators.

Characterizing and documenting of non-native species started as early as from the mid-nineteenth century (Baker, 1965), but unfortunately, little evidence is available about the mechanisms how new species integrate into the community and how the pollinator communitiy response. Importance has been given mostly to the agriculturally significant weed species while very little has been published on the relative importance of different pollination syndromes which might be able to explain how an alien plant become established in a novel community and in many cases, become invasive. Some studies indicated that well adapted or invasive species were facultatively self-fertile (Richardson *et al*., 2000), where others stated that approximately 56% of the successful invaders are out-crossers (Reichard, 1994) and in many cases these invasive plants were insect pollinated and relied on a wide range of native pollinators (Carr, 1993). Simple documentation of community structure in invaded and uninvaded areas reveals a little about the underlying mechanism and several studies tested the mechanisms behind the impact of an alien species on a native plant community. A new species in a novel community starts with competition. Competition within the plants and impact of new species on ecosystem function both are responsible for the changes in the structure of a native community. Competition can be for physical resources, e.g. sunlight (Woods, 1993; Martin, 1999; Lavergne *et al*., 1999), water availability (Melgoza *et al*., 1990), nutrients and ecological resources, e.g.availability of the pollinators. Competition is by far the most common expected affect when an alien plant enter a new community but can be beneficial impact like, positive impact of alien plants on the native community through the alteration of the soil structure, shading and allelopathy (Levine *et al*., 2003). When the alien plant overcomes the possible ecological barriers, they get the opportunity become an invasive species. Although, an alien species usually carrys low density population at the early stage of integration. For a pollinator dependant plant species, succession into a new community depends on the ability to attract pollinators (Moeller, 2004). In the very beginning, competition for resources and pollination can negligible (Dietzsch *et al*., 2011) but over the time, population density increases, which makes an alien species more relevant as an ecological competitor. In this very process, the impact of the introduced plant likely to be changed in the pollinator-mediated interactions, both ecological and evolutionary although this topic has rarely captured attention by the researchers (Melbourne *et al*., 2007). The fundamental question, how a pollinator-dependant new plant integrate in a novel community is the key to measure the impact of new flowering plant on the native plant-pollinator community (Goodell and Parker, 2017). Few studies listed the impact of the presence of a non-native plant species in a native plant-pollinator community. It’s been predicted that arrival of new species may affect the interactions between the native plant and pollinators thorough different mechanisms and depends on the influence of the new species on the pollinator abundance, behaviour and community structure (Connell and Slatyer, 1977). But this interactions are also complex and closely related to the floral diversity, floral characteristics and outcome of the competition/facilitation in pollinator reception among the existing species and new species (Ghazoul 2006; Bartomeus et al., 2008, Schlüter et al., 2009; Gibson et al., 2012; Goodell and Parker, 2017). If a new flower resource increases the flower diversity and flower richness, this may provide more foraging resources for the existing pollinator communities. This consequence can result into lower flower density for the native flowers and they may lose the dominance in providing the foraging resources for pollinators (Muñoz and Cavieres, 2008). On the other hand, if the newly introduced plant provides ample amount of flower which may attract more pollinators from different origin of places and that may facilitate the existing flowering communities by providing more flower visitors (Molina-Montenegro et al., 2008). Flower diversity and abundance may play another important role; for example, less abundant flower may face more threat than more abundant flowers from the newly introduced flowers, but this possibility is widely ignored (Rathcke 1983). Beside the impact on the whole community, individual plant species can also be affected by the intrusion of a new species. In general, flowering plant species which bring the similarity with the introduced species may face these changes than other species, as traits play an important role. Such similarity of floral traits like petal or bract colour, flower shape and flower arrangement will reduce the discrimination with native and new flower resources and result into increase the competition between these plant species (Waser 1986, Chittka et al. 1999, Bjerknes et al. 2007).

1.3.2. Pollinator behaviour towards the new resources

Studies are available about the impacts of non-native plant species on pollinator behaviour (Totland *et al*., 2006), although very few studies focused on the impact of flower or pollinator population densities of pollinators have been accounted. Most of these studies focused on the intrusion of an alien plant species into a single plant community on small spatial scales and based on this emphasized the impact on the behaviour of pollinators. As discussed, presence of a new species in such small spatial scales may affect the community in two ways; direct effects on the pollination of natives by attracting more pollinators and increasing the visitation of native plants (Moragues and Traveset, 2005), or pollinators will prefer new flower resources over the natives (Chittka and Schürkens, 2001). This leads to the possibilities of having either increased or decreased visitation by the pollinators on native flowering plants. In the case of insect poulation, it is possible that a new flower resource can promote the size of the population by increasing the availability of floral resources (nectar, pollen) and providing a wide range of foraging period by extending the seasonal availability of resources (Davidson *et al.*, 2011). Another positive impact of the introduction of the new resource might be possible when new plants have different flowering seasons or extended flowering time comparing to the natives, it may facilitate the pollinators to maintain a large population. But so far, there is little evidence that such positive effects are common, on the contrary, most of the studies reported that pollinator populations can be negatively affected by the presence of the new plant species by reducing the resource availability for specialized pollinators exclusively dependant on native plant species (Cox and Elmqvist, 2000; Thijs *et al.*, 2012).

1.3.3. Insect visitation and pollen deposition

Competition for pollination is visible when a plant species suffers pollen limitation due to the presence of another plant species resulting in pollinator sharing and produces less fruit and/or seeds than it would make in the absence of the second plant (Ghazoul, 2006). Presence of this new flower resources therefore can result into decreased flower visitation of native plants and increases heterospecific pollen deposition and/or reducing conspecific pollen deposition on the native flowering plants (Campbell, 1985; Morales & Traveset, 2008).

In addition to the changed pollination preferences, this introduction of new species can precipitate a change in the fidelity of pollinator movements among the conspecific natives (Brown *et al.* 2002). A reduction of pollinator fidelity (measured as the proportion of intraspecific pollinator movements) can result into decreased fitness through increased levels of interspecific pollen deposition, in addition to the loss of conspecific pollen deposition (Flanagan *et al.*, 2009; Harder and Routley, 2006). The effect of a new introduced plant species on pollen movement patterns in a community usually depend largely on the degree of constancy exhibited by the pollinator community. Therefore, if, for example, pollinator constancy is uninfluenced by the new species, the impact on native plants should be minimal. On the other hand, if the new species is highly rewarding, and an important pollinator of native species switches to the new flower resources, this can devastate the reproductive success of any native plants that rely on it (Chittka & Schürkens, 2001).

Introduction of a new flowering resouces can be devastating in other ways. For example, pollen from the new flowers can make its way on the native flowers and mechanically block the stigmas (Brown & Mitchell 2001; Jakobsson, Padrón, & Traveset 2008) or styles by clogging or chemically interfering with fertilization (Larson *et al*., 2006) and producing reduced seed set or hybrids (Brown and Mitchell, 2001). On the other hand, facilitation is also possible, if the new flower is highly showy and can attract more flower visitors that will increase the rate of pollination and a rewarding capability of new flower and eventually, can make the alien plant a ‘magnet’ species (Lammi and Kuitunen, 1995, (Lopezaraiza-Mikel *et al*., 2007). It is not clear whether the pollination change occurs in the native community due to the presence of exotic pollen, or, a change in the visitors of native plants, or, a change in the visitation rates in the native plants, or a combination of all of these (Larson *et al*., 2006). This issue is not only about the pollen limitation of the plant but also related to the declining of the pollinator populations (Spira, 2001), which might be the co-occurring results of plant invasions. Some recent studies showed that the floral abundance or difference of floral traits were also important to determine the visitation rate or the composition of pollinator visitors at the community level (Stang *et al*., 2006). Generally, species create large and dense populations than the natives (Bjerkness, *et al*., 2007) and this might be another important reason to create the differences in flower visitation between the invasive plant and natives.

1.4. Impact of environmental limitation on pollination and reproduction of plant:

While many of the species (both plant and animal) are taking the advantage of the changing weather, some species are bound to stay in their geological origin and do not have much of a possibility to distribute further. This species faces a wide range of geological, physical or environmental barriers and limit their distribution in a certain range, e.g. endemic species. The most recognizable physical and geological barriers are the mountain ranges, deserts, oceans, rivers and most recently, human development. These obstacles directly prevent a species from dispersal and alternatively shape their life cycle (Mott, 2010). However, the range limits of species are not imposed directly through physical barriers, but rather through spatial gradients in climatic variables. Even a species can be capable of breaking the physical barriers, by means of using other resources for travelling, but a different distance ecosystem with different, temperature, soil and water chemistry, seasonal variation, and precipitation can limit their succession. For example, most aquatic organisms are unable to travel between freshwater and marine habitats, not because of physical barriers, but because of negative physiological responses to changes in salt content. In case of endemic plants, along with all these mention factors, their distribution can also be limited by the reproduction strategies that they may adapt in a particular geographical range. This range of conditions that species can tolerate, and how their morphological and biological responses impact their geographic distributions, are commonly described in terms of their ecological niche (Hutchinson 1958).

1.4.1 Nature of Environmental limitation

An endemic species often exhibits an abundant center distribution, where the highest population densities can be observed in the core of the range, but increasingly declines towards its margin of range (Andrewartha & Birch 1954; Brown *et al.* 1995; Sagarin & Gaines 2002). Plant population of this type of species can show reduced densities and may be even reduced fitness in their peripheral region and can experience greater physiological and biological stress due to suboptimal conditions. In case of pollinators, this may result into either abundant but low quality of foraging resources, or higher quality patches in great distances. According to optimal foraging theory, this relates the costs of moving among different habitat patches and flowering plants in the peripheral populations may be unable to get adequate pollinator visitation due to the energetic costs involved. Alternatively, peripheral populations may adapt entirely different strategies than those of core populations due to the unavailability of optimal pollinator communities at range margins.

Reduced densities due to the geographical limitation can also influence the reproductive fitness and local adaptation, which further reduce the ability of species to expand beyond their current range limits. Due to the increased physiological stresses of inhabiting areas, individual species in peripheral populations may exhibit major differences in morphological and life history traits relative to individuals in core populations, such as different sizes, shorter or longer lifespan, and reduced or changed reproductive fitness. In case of a pollinator dependant flowering plant species, it may also face difficulties to find a suitable pollinator due to the decreased population densities and increased distance between the patches. These factors mat collectively contribute to reduced genetic diversity among peripheral populations as well (Howes & Lougheed 2008). However, recent studies have also concluded that some species may actually exhibit increased genetic variation at their distribution range edges (Budd & Pandolfi 2010) and facilitating range expansions. Climatic factors and their influences on the population density, habitat suitability and reproductive fitness within the species strongly strengthen the range limits, and, may help to maintain abundant center distributions.

1.4.2. Plant strategies for pollination and reproduction

Plant adapt their reproduction system with the existing environment by means of vegetative or self- and cross- pollination, although by alteration of different mechanisms (Igic, et al, 2008; Yang & Kim, 2016). Self-pollination, in many cases, assures the establishment of successful phenotypes, on the other hand, cross-reproduction increases genetic diversity and phenotypic plasticity (Busch & Delph, 2011). In other words, self-pollination limits gene flow among individuals and preserves gene combinations that maintains high fitness of a plant to adapt in a local environment (Schmitt & Gamble, 1990; Massol & Cheptou, 2011). In some exceptional cases of modulating the reproduction, plants that can iteratively switch from out-crossing to selfing. It has been also observed that some plants can activate one mode and deactivate other mode in relation to the level of habitat stressors (Yang & Kim, 2016). Specifically, when a plant grows with a limited and stressful condition, self-pollination can assure reproduction without the need for the plant of allocating extra resources in pollinator attraction (Schemske, 1978). The relationship between growing environment and pollination mode is still unclear and evolutionary history of populations may play a role in such groups of related *taxa* with monophyletic selfing traits (Miller & Tanksley, 1990). Thus, understanding how the reproductive strategies of endemic plants relate to both the growing environment and the species evolutionary dynamics is a key question of the reproductive biology of plants.

1.5 Habitat alteration and forest destruction: long-term changes in the terrestrial ecosystem

Another highly widespread changes and alarming for the biodiversity in current world is habitat fragmentation and forest degradation (Aizen and Feinsinger, 1994). A net amount of 5.2 million hectares of earth’s forest underwent to be lost every year between 2000 and 2010 and the greatest loss is occurring in the tropical and subtropical woodlands (FAO, 2010). Although some forest lost occurred due to natural causes (Harrod *et al.*, 1999), but most of the current forest loss is the results of human land use (FAO, 2010). This alteration of landscape geometry is likely to affect the species interaction in different stages of their life history and subtle changes on plant-pollinator interactions may be amplified into long-term effects on the integrity and stability of forest ecosystem (Dirzo and Miranda, 1991, Fortuna and Bascompte, 2006). Habitat loss has a consistent and strong negative impact on biodiversity, species richness, population abundance, and genetic diversity (Laurance *et al.*, 2002, 2008; Aguilar *et al.*, 2008). Additionally, habitat loss can also reduce the trophic interaction chain length; alter species interactions, reduce foraging, breeding and dispersal (Fahrig, 2003). Pollinators are critical components of forest ecosystems where the existence of both plants and pollinators are highly dependent on each other. In any ecosystem, this complex relationship plays an important role in the organization and persistence of biodiversity (Bascompte, 2009) and the loss of plant-pollinator interactions may lead to failure of ecosystem function.

1.5.1. Consequences of habitat loss and forest degradation on pollinator diversity

The relationship between a habitat size and the quality of habitat is almost always correlated and difficult to distinguish whether the impact is due to the habitat loss or habitat modification (Jackson and Fahrig, 2013). While habitat loss is highly associated with greater extinction and reduced species richness, habitat fragmentation, on the other hand, can have both positive and negative impact on the biodiversity. Hypothetically, small fragmentation tend to loose specialised species but does not influence the number of generalized species (kremen and Ricketts, 2000), specially, when the specialized pollinators depend on one or few floral resources. These species with special requirements or limited ability to move and dispersal can be particularly vulnerable to habitat loss and habitat degradation, and have experienced greater relative declines (Biesmeijer *et al.*, 2006), and this will lead to and increasing dominance by generalist species But, loosing nesting habitat might be more stronger than reduced floral resources in response of pollinators to the habitat fragmentation. This is much more alarming for the ground nesting pollinators where they are likely to be disappear from the small fragments or face reduced abundance, while cavity nesting pollinators do not get affected by the habitat fragmentation, in general (kremen and Ricketts, 2000). While habitat fragmentation often accompanies with habitat loss but it has its own ecological impacts on pollinating communities. Depending on the type of pollinators, therefore, habitat loss can affect at both small, local scales, and at larger, landscape scales and can affect the pollination services to both wild and domestic plant species (Potts et al. 2010, Ricketts et al. 2008).

1.5.2. Consequences of habitat loss and forest degradation on the plant-pollinator interactions

Habitat loss and fragmentation can affect plat-pollinator interactions in different ways. Primarily, pollinators can be affected by lack of suitable habitat and resources, which may affect their performance (Ward and Johnson, 2005). On the other hand, decreased plant abundance, density and health will result into lower pollen availability, resulting to reduced seed set for plants (Ward and Johnson, 2005). Secondly, fragmentation can alter the foraging behaviour of pollinators according to the optimal foraging theory (Charnov, 1976) and to maintain their energetic gain, pollinators may change their foraging strategies. Increased flight distance among the fragments can cause less effective pollen transfer (Aizen and Harder 2007) and pollinator abundance can decrease due to the lower attractiveness of isolated fragments or small population size or density of flowering plants (Cheptou and Avendano, 2006). Small plant populations due to the fragmentation also face increasing genetic drift and inbreeding depression (58,228). This may be due to increased geitonogamy, as pollinators may visit a higher proportion of flowers on individual plants, resulting in more self-fertilization (Klinkhamer et al., 1989). Overall, the stability of plant-pollinator interactions tends to be altered when native habitat is changed or removed. Even small disturbances may cause plant–pollinator interaction disruption within the remaining habitat patches in fragmented landscapes (Keitt, 2009). Increasing loss of habitat and disturbances not only ecologically harmful for the species, lack of wild pollinators lead to wide spread yield gap in fruit and crop production, which is negatively affecting the agricultural production, economy and rural livelihoods (Garibaldi et al., 2016).

1.6. Conclusion

Approaches to understand the interactions are entitled as small-scale researches with a focus on one or a few ‘focal’ species and large-scale landscape based research (Cardinale et al., 2011), but both provide knowledge of ongoing ecological functions with an understanding of the stability and maintenance of an entire ecosystem (Winfree, 2013). Therefore, understanding the relationship between the environment, biodiversity and ecosystem functions and studying impact of environmental changes on the plant-pollinator interactions have a greater application value, both in research and in conservation. This study focused on several aspects of environmental changes, including the impact of different climate changing factors on the plant morphological growth and floral resources and consequences on pollination and seed production. This also included the variation of floral resources on small spatial scale and on community level and interrelated the flower visitors behaviour and foraging preferences. Study also included the natural communities and discussed the pollination strategies, pollinator communitied and plant-pollinator interactions. These elaborated study indicated the dynamics and resilience of plant-pollinator interactions, both experimentally and naturally and showed how different environment changing factors affect the relation between plants and pollinators. This study answered few fundamental questions related to foraging behaviour of pollinators and pollinator preferences of plants in different conditions as well as adapted pollination strategies of plant under environmental pressure. Therefore, we expect this study to contribute significantly both in general research question in pollination ecology and, also in conservation in broader aspect. Finally, this work also brought opportunities in future research related to plant-pollinator interactions and plant conservation.

1.7 General research objectives

In this thesis, I aimed to resolve multiple aspect of environmental and ecological impact on the plant-pollinator interactions, from a single-focus plant to forest loss. The main underlying question being addressed in different chapters are as following:

(i) How different climatic drivers and conditions affect the morphological growth of a plant and how this relates to the pollination and reproduction of a plant?

(ii) Does changes in the spatial flower arrangement changes the foraging behaviour of pollinators?

(iii) Does forage preferences of pollinators can be affected by the introduction of new flower resources into the community?

(iv) What are the strategies that plant follows in pollination and reproduction to overcome a geographical and ecological limitation?

(v) Can pollinator diversity cope up with the large-scale habitat loss and human disturbances?

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2. Interactive effects of temperature, water, and nitrogen availability on the growth, floral traits, and pollination of white mustard, *Sinapis alba*

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Content of this chapter is prepared for submission as:

Akter, A. and Klecka, J. 2020. Interactive effects of temperature, water, and nitrogen availability on the growth, floral traits, and pollination of white mustard, *Sinapis alba*

2.1. Interactive effects of temperature, water, and nitrogen availability on the growth, floral traits, and pollination of white mustard, *Sinapis alba*

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Abstract
Multiple drivers associated with climate change play a crucial role for the growth of plants, their phenology, plant-pollinator interactions and plant reproductive success. Investigation of the impact of these changing climatic factors on plant growth, phenology and pollination will give us a better understanding of the impact of climate change on plant-pollinator interactions. In our study we tested the impact of temperature, water and nitrogen supply variability on the plant growth, floral traits, flower visitation by insect pollinators and seed production. Our study showed that water stress impairs vegetative growth, decreases flower production, reduces visitation by pollinators and negatively affects plant reproduction., where nutrition played an important role in the nectar production. Temperature and season also played an interactive role in the plant growth and flower production. Our study resulted into a highly variable plant and flower traits with different functional diversity. These functional traits lead to the variation of flower visitation, which explain how plant pollinator interactions can be affected by the impact of changing climatic factors.

Key words: Temperature, Water, N_2, *Sinapis alba*, climate change, pollination

2.2. Introduction

Plants require an optimal condition for their morphological growth and changes in any of the resources will cause the interruption both in their morphological growth and consequently their reproduction. For a pollinator dependant plant this change may also directly impact their relationship with pollinators and the success of pollination, hence the reproduction of a plant strongly dependant on both growing conditions and successful pollination (Scaven and Rafferty, 2013; Gérard *et al.*, 2020). But these resources are not independent and act interactively and can directly and indirectly contribute to the reproductive success through the changes in morphological and floral traits. Fitness of animal pollinated plants are generally influenced by the floral traits and floral traits act as an advertisement of reward to the pollinators (Hegland and Totland, 2005, Basnett *et al.*, 2019). Typically, pollinator prefers large, showy flower with high nectar rewards along with other preferable morphological characters (Hodges, 1995; Galen, 1999). Despite the strong selection from pollinators, plant population naturally shows significant variation in their morphological, phenological and floral characters. Some of these variations are result of heritable genetical differences among the individuals, while rest of the variation are caused by the local environmental factors (Galen, 1996, Holtsford and Ellstrand, 1992, Gray & Brady, 2016). Through the response to the variable environmental factors, plant may not only change their morphological and phenological characteristics, but with this may also alter the pollination fitness and pollinator-mediated selection of flower traits (Carrol *et al.*, 2001). Climate change results into shifting temperatures, precipitation and changes in the atmospheric composition are the most triggering factors for the development of plant and its reproduction. The ecosystem worldwide is facing an unprecedented change due to the different climate changing factors, including high atmospheric carbon, which directly resulting into increasing temperature, N_2 deposition, biological invasions and habitat loss (Hoover *et al.*, 2012). Recent increase of world temperatures allows many plants to emerge earlier, especially in the temperate region but also may experience the spring freezing, while tropical region is facing hotter temperatures than the past with shorter winter. Temperature is a major determinant of the timing of key developmental phases including morphology and phenology (Bahuguna & Jagadish, 2015). Increasing temperature can directly affect the physiological and phenological growth of plant (Schweiger *et al.*, 2010) and interactive impact of environmental factors in the presence of high temperature can potentially affect the physiological response of plants and disrupt other species interaction with plants. Plant flowering phenology is the onset of time and duration of flowering events and it determines the species fitness and existence of plant and sensitive to the variable environmental components (Burkle and Irwin, 2010; Parachnowitsch and Kessler, 2010). While most of the study focus on how changing environmental factors affecting the phenonlogical cycle of plants and their interaction with their perspective pollinators, there still a breach of lacking how different environmental factors play interactive role in plant morphology, phenology, pollination and in seed production.

Single diver studies indicate that impact of water availability on the plant flower resource is ambiguous. While most of the studies showed that supplementary watering result into higher nectar production (Zimmerman and Pyke, 1988) and increased nectar sucrose content (Wyatt, Broyles, and Derda, 1992), one study claimed the lack of a water status index to determine the exact nature of these responses (Lee and Felker, 1992). On the other hand, lack of water affects the plant growth and reduces the floral resources in many ways, e.g. decreased nectar volume (Carroll *et al*., 2001) and pollen and seed development (Barnabás *et al.,* 2008; Hedhly, 2011; Snider & Oosterhuis, 2011). (Burkle thesis). Similarly, increasing temperature evidently responsible for early flowering in many temperate plants and insect-pollinated plant seemed to response stronger than wind-pollinated plant (Fitter & Fitter 2002, Miller-Rushing *et al.,* 2007; Heglad 2009). In addition to dry and hot condition, water availability directly influence the flowering time and duration along with the morphological growth as water plays important role in nutrient acquisition (Aronson *et al.,* 1992; Bernal *et al.,* 2011; Lasky *et al.,* 2016). Plant growth and development directly related to the surrounding temperature of a specific plant and each plant has a species range of temperature tolerance represented by minimum, maximum and optimum. With the average increasing global temperature by 2-3 °C over the next 30-50 years predicted (IPCC, 2007), temperature events are going to be more frequent, extreme and long lasting (Meehl *et al*., 2007). These events would have dramatic consequences on the plant growth and development, so as in pollination and seed production. Barlow *et al*., 2015, showed that extreme heat conditions can reduce the grain production, with decreasing the grain maturity period. Because of the specificity of plant growth and reproduction to the temperature, temperature changing events will eventually affect the overall growth and other biological and ecosystem function related to this (Hatfield and Prueger, 2015).

Another important environmental component is Nitrogen (N_2), an essential plant nutrient and plant community diversity is highly related to the N_2 availability in the soil (Bobbink *et al.,* 2010). With the current N_2 emission rate in global scale, most of the region will have increased atmospheric N_2, and Europe and North America might be shifted to the plant composition favourable to higher N_2 availability in soil (Bobbink *et al.,* 1998, Bobbink *et al.,* 2010). In a plant community, N_2 heterogeneity can occur within few meters and this can directly affect the plant growth and reproduction (Scott-Wendt *et al.,* 1988), so as their mutualistic relationship with the respective pollinators. Higher N_2 level can enhance the plant growth and enable plant to produce better floral resources (Gardener & Gillman 2001, Burkle & Irwin 2009) and can potentially alter the pollinator preferences (Gardener & Gillman,2002).

Given the importance of changing resources on the plant growth and phenology, it is importance to investigate how these factors act interactively on the pant morphology and phenology of plant and affect the impact of this important factors on the pollination. In this study we examined the interactive impact of three important environmental factors (water availability, N_2 deposition and temperature) on the plant morphological and phenological growth, impact on the flower rewards, pollination and seed production. We selected *Sinapis alba* as our experimental model because; 1) can easily be grown in the greenhouse and easy to carry for field observations, 2) produce enough flower reward to be measured, 3) Presence of self-incompatibility and 4) an economically important crops, cultivated over a wide geographic range for oil and fodder. In our study we aimed to answer the following question: a) What are the interactive effects of water availability, N_2 enrichment and temperatures on the morphological and phenological growth of *S. alba*; b) how flower rewards vary among the experimental plant groups; c) how this interspecific variation caused by the environmental factors affect the pollinator reception, pollination efficacy and seed production.

2.3. Methods

2.3.1 Growing *Sinapis alba* under variable temperature, water and N_2 conditions in the greenhouse

S. alba (white mustard) is a rapidly growing annual plant from Brassicaceae family with short vegetation period and widely cultivated for seed, oil, fodder or as a catch crop. Flowers are yellow, produces in elongated raceme, four petals, four sepals and 6 stamens, of which four long and two shorts. Fruit is pod with usually four seeds but can be up to six seeds. A wide range of pollinating insect visits this plant but *Apis mellifera* and solitary bees are the main pollinators.

This experiment was conducted in the greenhouse, where *S. alba* seedlings were grown in winter, early spring and in early summer from 2017-2018, under variable environmental conditions. Plants were grown in three temperature ranges, in average 21°C, 25°C and 29°C. A combination of garden soil: compost soil: sand= 2:2:1were used in same size pots (11cm x 11cm x 11cm). Seeds were germinated in germination trays and transferred to individual pot after four days. Two global change components were applied in each batch to create four combinations with two water levels and two level of N_2 application. Based on the average ~700cm precipitation rate in the Central European

Region, we determined the lower level of water as 20 ml and higher level of water as 40 ml. N_2 application rate was determined by the estimated rate of N_2 deposition within the Europe and ideal N_2 fertilizer Urea application in the agricultural field for *S. alba*. N_2 was applied in each week for 8 weeks as 0.242g/pot as higher level of application and 0.121g/pot as lower application. Day/night range was controlled for all the experiments and varied between 10 to 16 hours. Before conducting the real experiment, a preliminary trial was performed to determine the described water level, N_2 application and soil combination to optimize the growth condition for the *S. alba*. Soil remnant were checked after the experiment to determine the use of soil mineral by the plants of each treatment. Position of plants were altered regularly to avoid any impact from other possible environmental impact.

2.3.2 Assessment of plant morphology, phenology and nectar production

Growth stages, plant height, and leaf number of each plant were measured regularly for every treatment. Plant height and leaf number were measured several times throughout the growing period and final height was taken after the end of flowering and number of leaves were counted for the main shoot only. Diameter of each plant was taken at the point of 20 cm height. The number of days of onset of flowering was determined from the day of the seedling transfer to the opening of first flower and total number of flowers bloomed were counted till the end of flowering. Nectar was collected in each treatment after one day of flowering by using calibrated 0.5µl Capillary tubes Drummond Microcaps®. The volume of nectar was determined by the following formula:

$X=a/a*b$, where, X= nectar volume in µl, a = final volume of the capillary tube; b= nectar volume acquired.

An additional data was collected on the plant fresh weight and dry weight from the plants grown under 25°C to determine the biomass of the plants due to the different growth conditions. Soil analysis were done to verify the nutrition uptake by individual groups.

2.3.3. Pollination efficacy treatment and field pollination observation

To determine the impact of the treatments on the pollination efficacy of *S. alba*, hand pollinated self and cross pollination was carried out in the greenhouse on the plants grown in average 22°C and fruit and seed production were recorded. Plants grown in average 25°C and 29°C were brought outside and placed in a sunny location nearby the Institute to assess the pollinator response and natural pollination efficacy rate. First pollinator observation was carried out in spring for the plants grown under 25°C and second observation carried out in summer for the plants grown in 29°C. Plants were observed for 30 minutes, from 9:00 to 14:00. Altogether, forty-four observations were done for four types of plants from two temperature groups, which resulted into a total 22 hours of observation for 8 types of plants and each treatment received in average total 165 minutes of observation. Pollinators were observed, collected and identified in the field and documented immediately. Later, plants were brought to the greenhouse and flowers were marked to determine the seed production through the natural pollination by measuring the seed production rate after fruiting.

2.3.4. *Statistical analysis*

We used factorial ANOVA and stepwise regression to assess the individual and mixed impact of water, N_2, temperature and day length on the plant's height, diameter, number if leaf, flowering time, flower and nectar production. Differences among the morphological growth, fruit, seed and seed/fruit production variation of the treated plant groups were tested by analysis of variance (ANOVA) and TukeyHSD was used for post-hoc analysis. Two-way ANOVA was used to analyse the impact of treatments and number of flowers on the pollinator visitation in total and individual flower visiting insect groups. All Analysis were done using R programming environment (Version 3.2.1) and graphs and plots were created using R programming environment and GraphPad Prism (Version 6.01).

2.4. Results

2.4.1. The effects of environmental factors on vegetative traits

We observed a complex response of the selected vegetative traits of *Sinapis alba* to the three environmental variables. Plant height was affected by a three-way interaction of water availability, nitrogen, and temperature, i.e. the effect of each variable was dependent on the values of the other two variables (Table 2.1, Fig. 2.1). While higher water availability made the plants taller (especially at higher temperatures), increasing nitrogen availability made the plants shorter. We also observed a negative effect of temperature – plants grown at the highest temperature were almost 50% shorter than those grown at the lowest temperature (Fig. 2.1a). Stem diameter was affected by water and nitrogen, in both cases in interaction with temperature (Table 2.1). The plants had larger stem diameter under the conditions of higher water availability and lower temperature. Interestingly, higher nitrogen availability increased stem diameter at the highest and lower temperature but had a weak opposite effect at the intermediate temperature (Fig. 2.1b). On the other hand, the number of leaves was affected only to a limited degree by an interaction of water and temperature – the plants had a higher number of leaves under higher water availability and intermediate temperature, while the effect was negligible under the lowest and highest temperatures (Fig. 2.1c). Finally. dry weight, which was measured only under the intermediate temperature, was more than 2.5x higher under high water availability, with a weak effect of nitrogen (Fig. 2.1d, fresh weight showed a very similar pattern).

Table 2.1: The effects of water, nitrogen deposition, and temperature on several vegetative traits describing plant growth. We report F and P values for individual variables and their interactions estimated by generalised linear models (see Methods). Dry weight was measured only during spring 2017 at intermediate temperature, so the effect of temperature on dry weight was not tested. The data are visualised in Fig. 2.1.

Variable	**Plant height**		**Stem diameter**		**Leaf number**		**Dry weight**	
	F	**P**	**F**	**P**	**F**	**P**	**F**	**P**
water x nitrogen x temperature	**5.0**	**0.008**	1.2	0.306	0.1	0.951	NA	NA
water x nitrogen	2.1	0.147	2.1	0.154	1.9	0.167	**5.5**	**0.023**
water x temperature	2.2	0.113	**3.1**	**0.047**	**3.3**	**0.039**	NA	NA
nitrogen x temperature	0.5	0.636	**14.4**	**0.000**	0.2	0.817	NA	NA
water	23.2	<0.001	32.9	<0.001	8.8	0.004	105.7	<0.001
nitrogen	22.3	<0.001	24.1	<0.001	0.1	0.787	0.2	0.643
temperature	60.7	<0.001	41.9	<0.001	9.0	0.000	NA	NA

Impacts on morphology

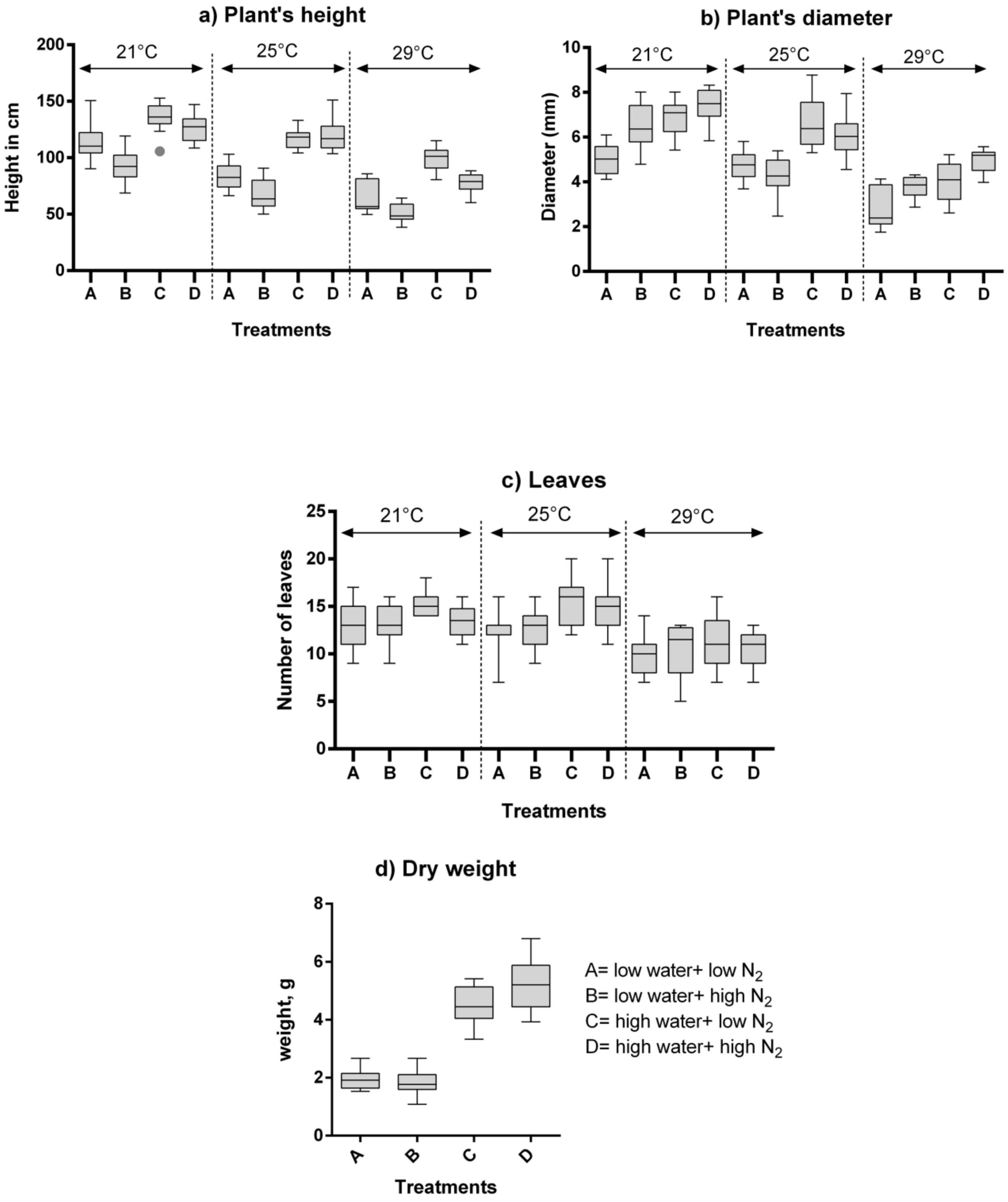


Figure 2.1: Impact of temperature, water and N_2 supplementation on vegetative traits of the plants; a) plant height; b) Plant diameter; c) Number of leaves; d) dry weight. Results of statistical tests are summarised in Table 2.1.

2.4.2. Phenology and flower resource variation

All three environmental variables had a significant effect on some of the floral traits we measured. First, the number of flowers produced over the plants' flowering period was affected by an interaction of water and nitrogen availability and an interaction of water and temperature (Table 2.2, Fig. 2.3). The plants generally produced a higher number of flowers under high water availability, although the strength of this effect varied with temperature, while there was a week trend of reduced number of flowers by high nitrogen availability when water availability was low, although the effect was not entirely convincing (Table 2.2, Fig. 2.2a) Second, the onset of flowering was delayed by low temperature and slightly also by high nitrogen availability (Fig. 2.2b). Finally, nectar volume showed a complex dependence on the interaction of all three environmental variables. Higher water availability increased nectar volume under low temperature, but not under intermediate and high temperature, while higher nitrogen availability increased nectar volume under low water availability and intermediate or high temperature (Fig. 2.2c). Nectar volume was also generally maximised under intermediate temperature.

Table 2.2: The effects of water, nitrogen deposition, and temperature on floral traits. We report F and P values for individual variables and their interactions estimated by generalised linear models (see Methods). The data are visualised in Fig. 2.2

Variable	**Flower number**		**Onset of flowering**		**Nectar volume**	
	F	**P**	**F**	**P**	**F**	**P**
water x nitrogen x temperature	1.3	0.288	0.1	0.950	**3.6**	**0.030**
water x nitrogen	**6.1**	**0.015**	0.0	0.930	0.2	0.648
water x temperature	**3.5**	**0.033**	0.5	0.595	1.2	0.318
nitrogen x temperature	1.3	0.265	0.6	0.564	8.8	<0.001
water	11.6	<0.001	2.9	0.089	4.9	0.028
nitrogen	8.4	0.004	**11.3**	**0.001**	0.2	0.622
temperature	25.1	<0.001	**177.9**	**<2e-16**	10.7	<0.001

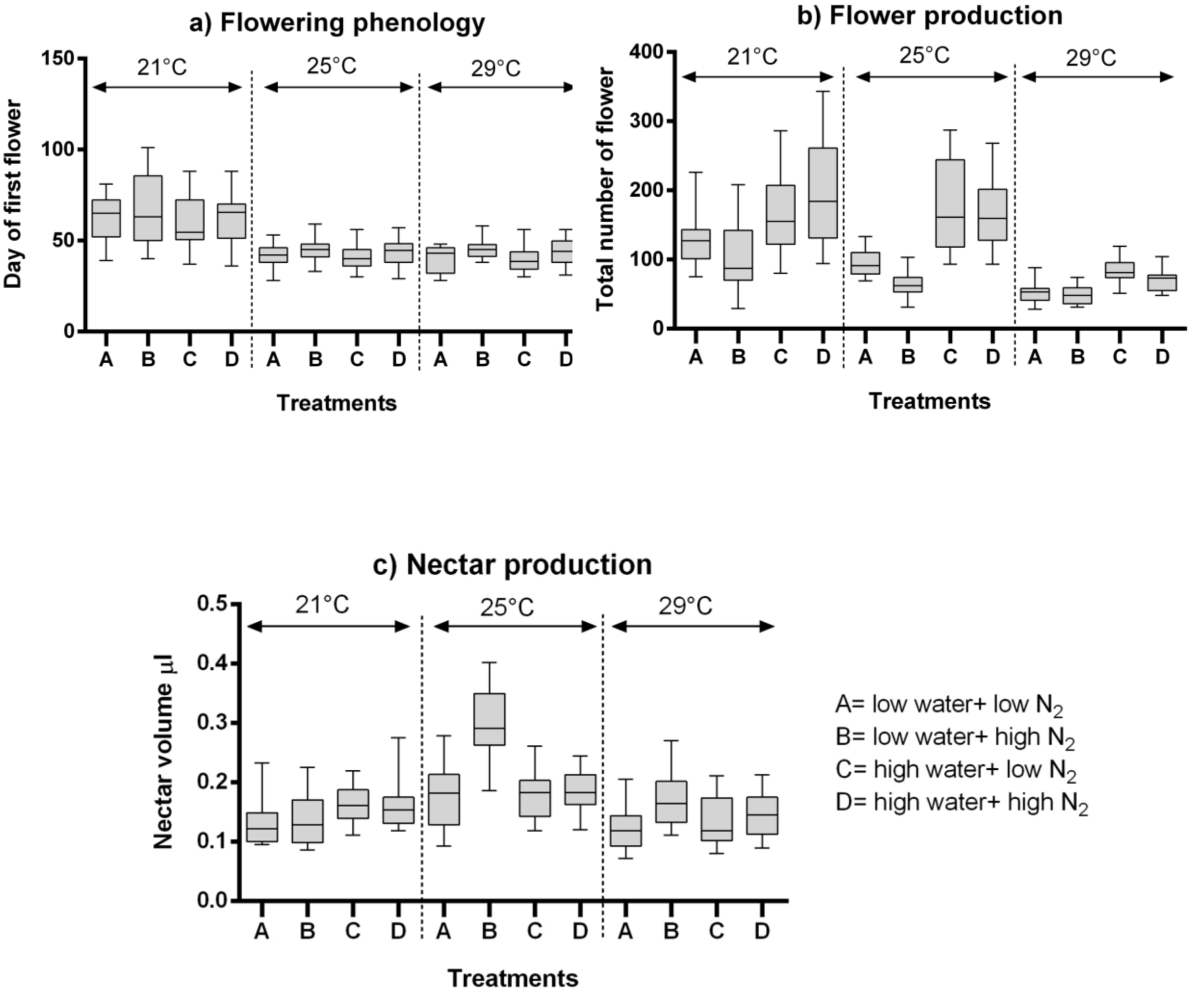


Figure 2.2: Impact of water, temperature and N_2 on the flowering time, flower and nectar production of *S. alba*: a) Day of first flowering, b) Number of flowers produced per plant and c) Nectar production.

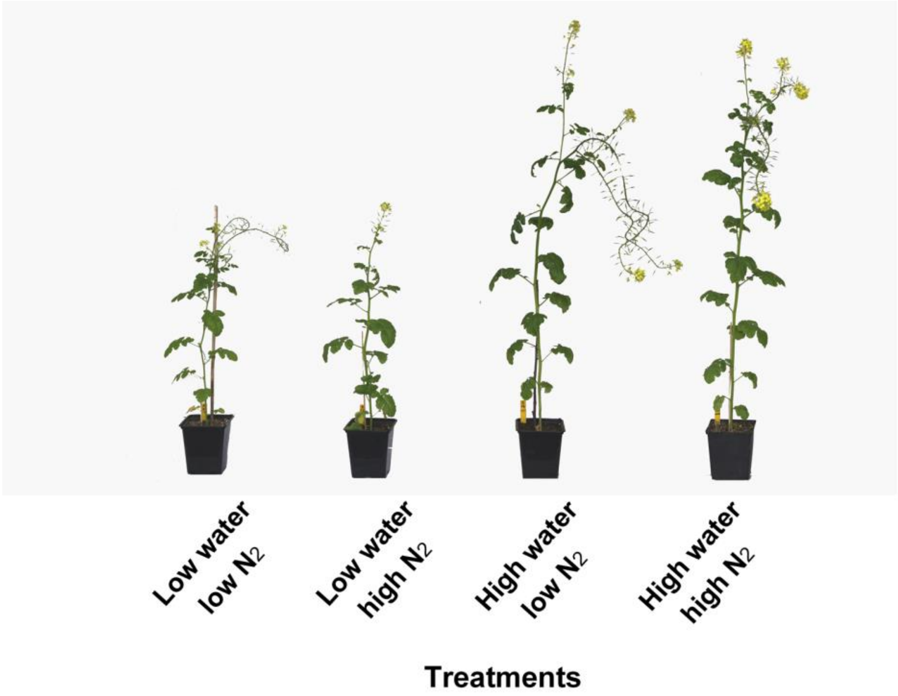


Figure 2.3: The example of plant individuals grown under varying levels of water and nitrogen availability under the intermediate temperate (25°C).

2.4.3. Response of pollinators

We observed flower visitation by eight major types of flower-visiting insects in the spring of 2017 and summer 2018 which we distinguished as: the honeybee *Apis mellifera*, solitary bees, small wasps, bumblebees, rapeseed beetles *Brassicogethes* (=*Meligethes*) sp., other beetles, hoverflies, and other flies. Rapeseed beetles were the most abundant flower visitor in the spring 2017, followed by honeybees, while solitary bees were dominant in the summer 2018, followed by hoverflies.

Plants grown with high amount of water were visited more frequently than plants grown with low amount of water (GLM, F=23.6, P<0.001) in both spring 2017 and summer 2018 and the total number of flower visitors was higher in the spring of 2017 (GLM, F=14.2, P<0.001) (Fig. 2.4). Nitrogen availability under which the plants were grown did not consistently affect their flower visitation (GLM, F=0.26, P=0.612). Flower visitation was also affected by the number of open flowers (GLM, F=33.1, P<0.001, Fig. 2.4c, but the effect of water availability and year remained significant after accounting for the variation in flower number, i.e. the differences in flower visitation between plants grown under different conditions were driven by a more complex set of differences between the plants. In addition to differences in total flower visitation, we detected differences in the composition of the flower visitors observed on plants grown under different water availability according to a redundancy analysis (RDA) performed separately for observations from the spring 2017 (F=4.0, P=0.004) and summer 2018 (F=3.1, P=0.028), while nitrogen availability under which the plants were grown did not affect the composition of flower visitors (F=0.37, P=0.869 for spring 2017 and F=0.57, P=0.669 for summer 2018). Some flower visitors visited plants grown under high water availability more frequently (e.g. solitary bees and rapeseed beetles), others apparently did not discriminate based on that-see Fig. 2.5.

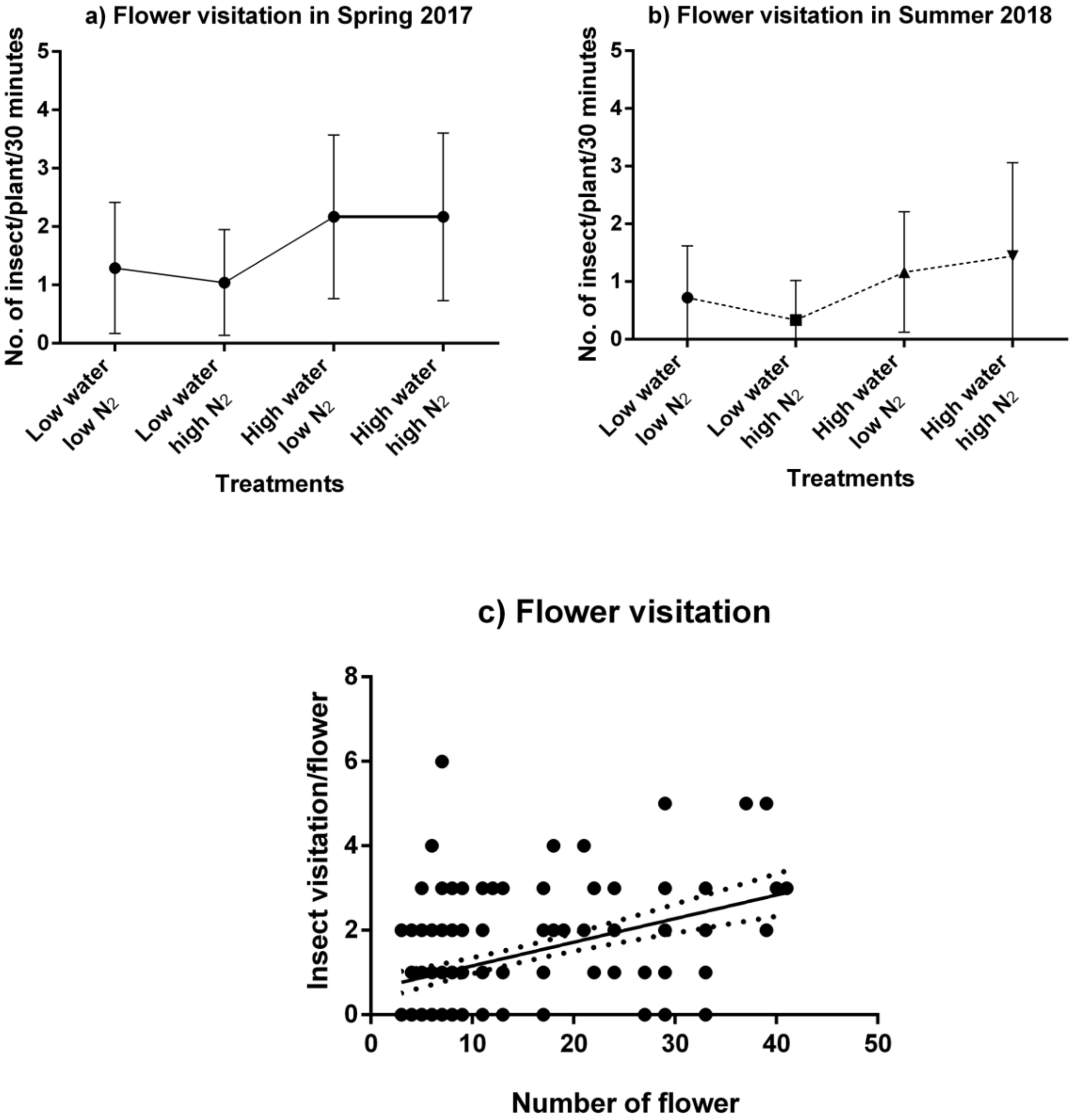
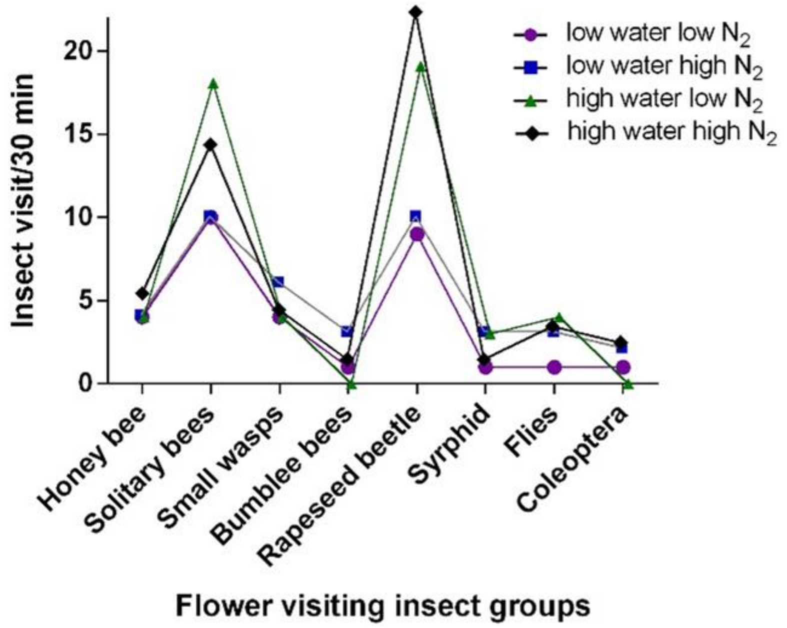


Figure 2.4: Flower visitation of plants grown under varying water and nitrogen availability. The number of flower visitors per plant per 30 minutes during two observation periods are shown: a) spring 2017 (plants grown in the temperature of 25°C) and b) summer 2018 (plants grown in 29°C). Flower visitation also varied depending on the number of open flowers (c).

a) Pollinator's response in spring



b) Pollinator's response in summer

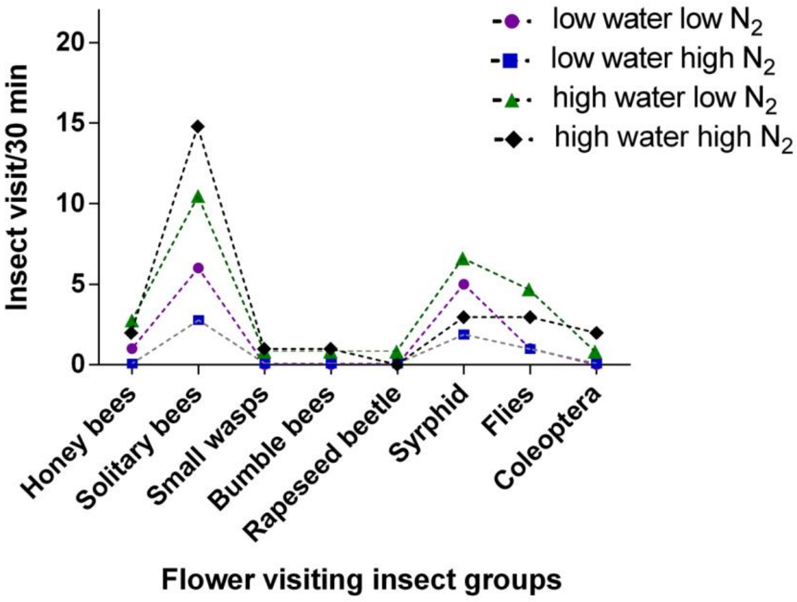


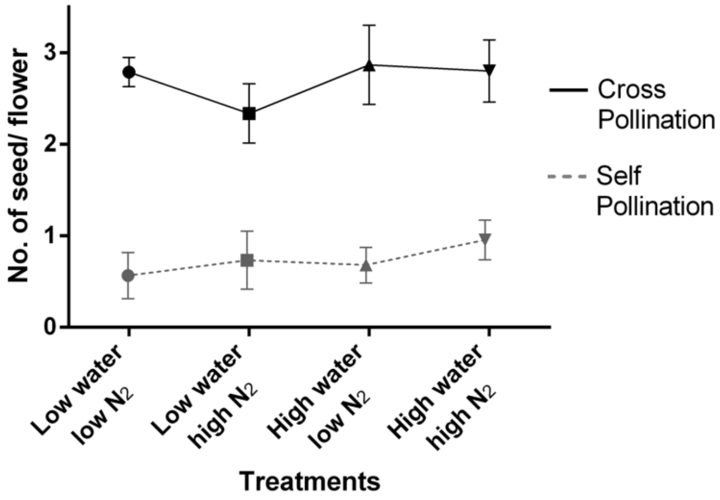
Figure 2.5: Pollinator responses towards the plants grown under 25°C and 29°C in spring (a) and summer (b) respectively.

2.4.4. Pollination efficacy and seed production

Sinapis alba shows partial self-incompatibility in pollination and our hand pollination result also confirmed it. Plants cross-pollinated by hand using a brush produced significantly higher number of seeds per flower than the self-pollinated ones, but the seed set depended not just on the mode of pollination (self-pollinated compared to cross-pollinated) but on its interaction with nitrogen availability (GLM, F=10.6, P=0.0023). Specifically, higher nitrogen availability increased seed set in self-pollinated plants, but decreased seed set in cross-pollinated plants. In addition, higher water availability increased seed set in both self-pollination and cross-pollination irrespective of the nitrogen level (GLM, F=5.2, P=0.028) (Fig. 2.6a).

Plants exposed to natural pollination in two periods, spring 2017 and summer 2018, produced a variable number of seeds per flower depending on the interaction of water availability and year (GLM, F=9.0, P=0.004) and on the nitrogen availability (GLM, F= 5.1, P=0.028). We observed a slightly higher number of seeds per flower in plants grown under high water availability in the spring 2017, but lower in the summer 2018. In addition, plants grown under higher nitrogen availability produced a lower number of seeds per flower (Fig. 2.7a). As we showed above, plants grown under different combinations of water and nitrogen availability varied in their total production of flowers. Combined with the variation in the number of seeds produced per flower, this led to differences in the total seed set per plant. Specifically, total seed set was higher in plants grown under high water availability, but the effect was stronger in the summer 2018 than in the spring 2017 (the interaction between water availability and year, GLM, F= 5.1,P= 0.028), see Fig. 2.7a and 2.7b.

a) Seed production per flower



b) Seed production per fruit

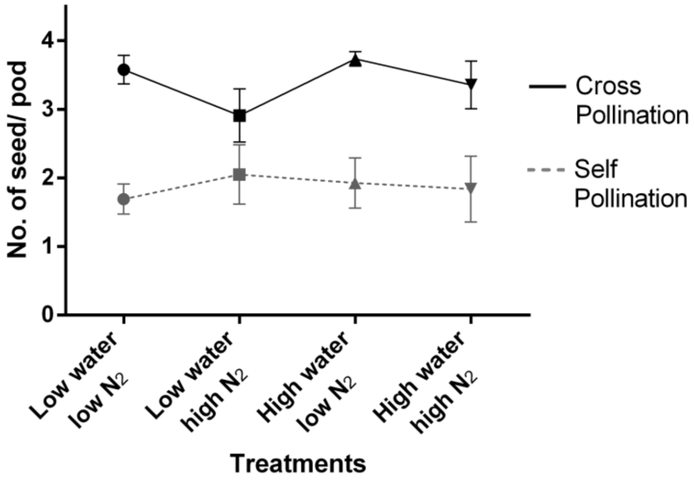


Figure 2.6: Seed production after the hand pollination of *S. alba* grown under different growing conditions: a) seed production per flower and b) seed production per fruit.

Seed production assessment from the natural pollination

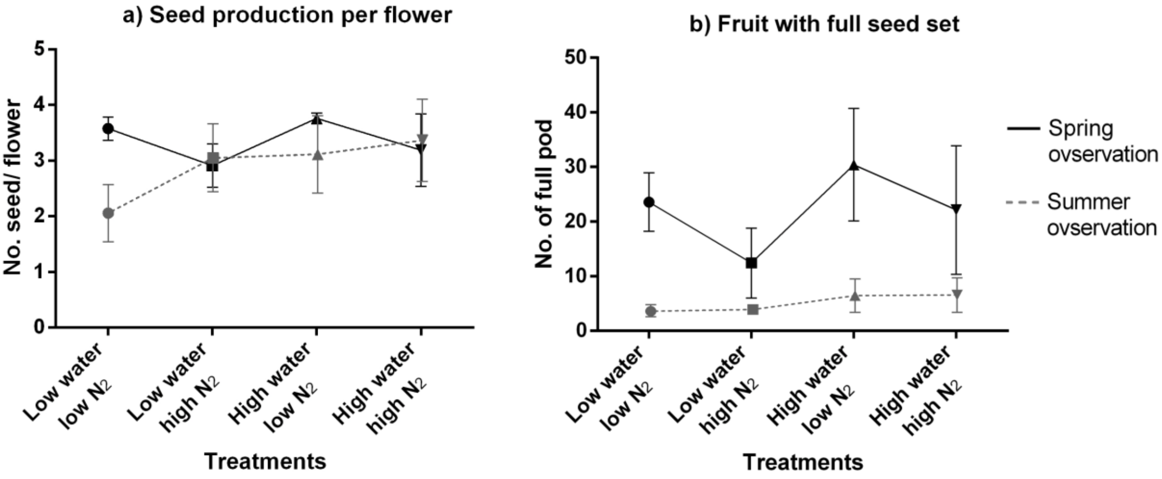


Figure 2.7: Seed production of *S. alba* for treatment groups after natural pollination in two seasons. a) the number of seeds per flower in the spring 2017 and the summer 2018 and, (b) and the total seed set per plant in the spring 2017 and the summer 2018 in plants exposed to natural pollination.

2.5. Discussion

2.5.1. Response of plant growth and floral traits to the changing environmental factors

Our experiments provide evidence of interactive effects of three crucial environmental variables, temperature, water, and nitrogen availability, on the life cycle of a plant, from morphology to plant reproduction. Generally, plant’s growth and fitness increase with the increasing temperature, reach a transition peak at an optimal temperature and rapidly decrease above this optimal range (Vasseur *et al*., 2014, Hatfield & Prueger, 2015). We found a similar trend also in our experiment, where *S. alba* grew better in 21°C and 25°C temperatures compared to a higher temperature (29°C). Overall height, stem diameter and number of leaves dropped with increasing temperature, but this phenomenon is also highly dependent on the water availability. We found that higher amount of water positively affected the plants and increased the plant growth, especially in higher temperatures. In nature, these responses can vary species to species and depend on the water amount and aridity index of a region (Zeppel *et al*., 2014; Chen *et al*., 2018).

Nitrogen supplementation played a complex role on the morphological growth of *S. alba*. Nitrogen is an essential component of plant life cycle and plant biomass production is related to co-function of N_2 and water availability (Chen *et al.*, 2018). These are crucial for increasing productivity of plants while mitigating other environmental impacts (Quemada & Gabriel, 2016). Water deficiency leads to reduced biomass production and diminished N_2 uptake in plants (Cossani *et al*., 2012). In contrast, sufficient nitrogen supply may enhance drought tolerance and increase water use efficiency in plants (Quemada and Gabriel, 2016). In our all temperature conditions, additional N_2 supplementation to *S. alba* decreased the plant height regardless the amount of water supplementation. However, stem diameter increased in 21°C and 29°C temperatures and decreased in 25°C temperature with the addition of higher amount of N_2. This indicated that N_2 use efficiency by the *S. alba* largely depend on the water availability and temperature and plant growth has a complex relationship to the amount of water, temperature, and N_2 supplementation. Results of measurements of fresh and dry weight of the plants grown in 25°C temperature also showed that higher availability of N_2 had positive impact on plant biomass when higher amount of water was given, but not when low amount of water was available.

In contrast to the morphological growth, climatic drivers had more complex impact on the flowering time and flower-nectar production of *S. alba*. First flower emergence was significantly delayed by ca. 21 days at the lowest temperature (21°C) compared to 25°C and 29°C. In addition, the onset of flowering was also delayed by high nitrogen availability across all temperature and water levels, on average by 3.2 days. Molecular mechanisms of metabolism involved in controlling flowering time have been observed in plants to be slowed down in lower temperature (Amasino, 2010; Song *et al*., 2013, Jeong *et al*., 2015; Lee and An, 2015). Generally, plant phenological shifts are usually more visible in the early-flowering plants and less strong in the late flowering species and closely related to temperature: most plants tend to flower early in response to the warmer weather (Jagadish *et al*., 2016, Takkis *et al.*, 2018, Kehrberger & Holzschuh, 2019). A previous study also showed that increasing temperature can cause flower bud abortion and significantly reduce the total number of flowers produced by *Borago officinalis* (Descamps *et al.*, 2018). We found the same pattern in *S. alba*, where the total number of flowers significantly dropped with increasing temperature. However, water also played an important role in flower production, with plants producing more flowers with high amount of water, especially at intermediate and high temperature, which suggests that thermal stress can be reduced in plants by water supplementation up to a certain limit (see also Mahan *et al*., 1995, Li *et al*., 2020).

An optimal temperature is also required for the maximum nectar secretion and nectar production can be decreased in lower or higher temperatures (Pacini & Nepi, 2007, Lu *et al*., 2015). In our case, *S. alba* produced comparatively higher amount of nectar under 25°C which appeared to be the optimum condition for this species in terms of nectar production. However, our results show that nectar production was affected by a complex interaction off all three variables with water increasing nectar production at low temperature, but not at intermediate and high temperature. Moreover, higher levels of nitrogen increased nectar production at intermediate and, also at high temperature, but only under low water availability. Mechanistic explanation of this complex relationship is not straightforward. Few studies showed that nectar production can be reduced with water reduction and increased temperature (Keasar *et al*., 2008; Scaven and Rafferty, 2013; Takkis *et al*., 2015). However, in our case, nectar production did not decrease under these conditions. We report an opposite result to Hoover *et al.,* (2012), where nectar production of *Cucurbita maxima* was positively affected by increasing temperatures and low N_2 supplementation. On the other hand, Villarreal and Freeman (1990) showed that nectar production of *Ipomopsis longiflora* was affected by water supplementation and not by the temperature. In conclusion, different plant species have different strategies to cope with environmental stress (Zimmerman, 1998; Lu *et al*., 2015), which leads to contrasting species-specific effects of variation in temperature, water, and nitrogen availability on nectar production.

A caveat in the interpretation of the effects of temperature is that plants in different temperature levels were not grown simultaneously, so we cannot completely rule out a possible role of other confounding effects. Specifically, the plants were grown in the greenhouse in the end of winter (average T = 21°C), spring (T = 25°C), and summer (T = 29°C). Hence, apart from different temperature, the plants could have been affected also by differences in day length and light intensity, although we tried to limit these differences by the use of artificial lights in the greenhouse. We believe that the possible role of light intensity was at best minor. In particular, we would expect that lower light intensity would lead to taller and thinner plants, but this is not what we observed in plants grown in the winter (T = 21°C). In addition, a positive aspect of our modest experimental greenhouse setting was that the plants were exposed to natural day-night variation of temperature, rather than being grown under constant temperature.

2.5.2. Impact on pollination and seed production

Our results from two different seasons showed that growing conditions of the plants, in particular water availability, affected flower visitation, likely mediated by changes in plant vegetative and floral traits. Pollinator’s attraction to a plant depends on visual cues indicating high floral reward such as the number of open flowers (Akter *et al.,* 2017) and the size of floral display (Biella et al. 2019), and on the amount and quality of nectar and pollen (Cresswell, 1999; Grindeland *et al*., 2005; Makino *et al*., 2007) along with general conspicuousness of the plant which increases its detection by potential pollinators, such as plant height (Klecka *et al*. 2018a, Biella *et al.*, 2019, Hernández-Villa *et al*., 2020), local plant clustering (Elliot and Irwin, 2009, Akter *et al.*, 2017), and flower colour (Klecka *et al.*, 2018b). We have shown above that multiple of these traits were affected by differences in temperature, water stress, and nitrogen availability. These changes had a cascading effect on the number and identity of flower visitors of *S. alba* and on its reproduction.

Plants grown with higher amount of water had a significantly higher number of flower visitors compared to plants grown under low amount of water irrespective of the nitrogen availability in both seasons (spring 2017 and summer 2018). This is likely a consequence of differences in vegetative and floral traits induces by differences in water availability. As we showed above, plants grown with high amount of water were taller and produced more flowers. Indeed, the number of open flowers had a positive effect on the visitation of individual plants as reported in other plant species (e.g. Akter *et al.*, 2017). However, other modifications of plant traits induced by water stress decreased the visitation of plants grown with low amount of water, because the effect of water availability on the number of flower visitors per plant was significant even after accounting for differences in the number of open flowers. We did not do analyses of nectar chemistry, such as concentration of sugars and aminoacids, which could also affect flower visitation (Petanidou *et al.*, 2006, Hoover *et al.*, 2012), but the fact that there was no effect of the nitrogen availability under which the plants were grown on flower visitation (in contrast to Hoover *et al.*, 2012), previous studies showing limited variation in sugar concentration in response to water stress (Descamps *et al.* 2018), and previous studies on plant floral traits (e.g. Akter *et al.*, 2017, Klecka *et al.*, 2018a), it seems that reduced flower visitation of plants grown with low amount of water was caused mainly by lower number of open flowers and smaller plant height induced by water stress.

Plants grown under low amount of water had not only lower total flower visitation, but also different composition of flower visitors compared to plants grown with high amount of water. In the spring 2017, rapeseed beetles, solitary bees, as well as hoverflies visited flowers of plants grown with high amount of water more frequently than plants grown under low amount of water, while the other flower visitors, including honeybees and bumblebees did not discriminate among the plants. The results were similar in the summer 2018, when rapeseed beetles were almost absent. Differences in the composition of flower visitors on plants grown with low compared to high water availability may be mediated by the effect of flower number and plant height, which are both known to have species-specific effects on flower visitation by different species of pollinators (Klecka *et al.*, 2018). Differences in the composition of flower visitors may either amplify or counteract the consequences of changes in total flower visitation on plant seed set, depending on the pollination efficiency of individual species of flower visitors (King *et al.*, 2013).

In contrast to measurements of vegetative and floral traits, we conducted the measurements of flower visitation only with plants grown at 25°C in the spring 2017 and plants grown at 29°C in the summer 2018. While we observed lower flower visitation in the summer 2018, this may be caused by differences in overall insect abundance and not necessarily by the growing conditions of the plants compared to spring 2017. In reality, increasing temperature may affect flower visitation by a number of mechanisms, from differences in plant traits caused by high temperature stress (Descamps *et al.*, 2018), through phenological shifts of plant flowering and pollinator emergence (Hegland *et al.*, 2009, Bartomeus *et al.*, 2011), to changes in pollinator foraging activity caused by their responses to temperature (Corbet *et al.*, 1993) and direct and indirect effects of temperature on the fitness and mortality of pollinating insects.

Seed production of *S. alba* was affected by water and nitrogen availability, apparently both directly through physiological mechanisms and indirectly through changes in insect pollination. Our hand pollination assessment using plants grown in 25°C clearly showed that *S. alba* is a partial self-incompatible plant as number of seed produced per flower and per fruit were significantly lower for the self-pollinated plants than cross pollinated ones. Although the rate of self-compatibility for *S. alba* is highly dependent on the cultivars (Fan *et al*., 2007), our selected cultivar evidently indicated partial self-incompatibility in pollination. Seed production in self-pollination was ca. 3.9 times lower than in cross pollination; on average 2.9 compared to 0.7 seeds per flower, depending also on water and nutrient availability. Low water availability reduced seed production per flower in both self-pollinated and cross-pollinated plants, which is consistent with previous studies suggesting that water stress may lead to seed or pod abortion (e.g. New *et al.*, 1994, Behboudian *et al.*, 2001). However, we also observed an intriguing effect of nitrogen availability on seed set: increased nitrogen availability increased seed set in self-pollinated plants, but decreased seed set in cross-pollinated plants. While the positive effect of nitrogen availability on seed set in self-pollinated plants is not surprising because seed production is energetically costly, the cause of the negative effect on seed set in cross-pollinated plants in not clear. We are not aware of any studies which would show that high N_2 can cause seed abortion. The effect was stronger under low water availability, so this may be related to physiological changes induced by water stress.

Seed count per flower from the naturally pollinated plants in the spring 2017 (grown under 25°C) also showed similar trend as cross-pollinated plants by hand pollination, where the number of seeds per flower increased in plants grown with high water availability, but decreased with high nitrogen availability. In contrast, in experiments done in the summer 2018 (plants grown under 29°C), the number of seeds per flower was not affected by nitrogen availability and decreased in plants grown with high amount of water. Total seed set per plant, which takes into account differences in the number of flowers produced by plants grown in different combinations of water and nitrogen availability, was unaffected by nitrogen availability and increased in plants grown under high water availability – moderately in the spring 2017 but much more in the summer 2018. It is not clear whether the differences between results from the spring 2017 and summer 2018 are caused by differences in the abundance and efficiency of pollinators or by physiological mechanisms responsible for seed formation, which could be affected by the interplay of temperature, water, and nitrogen availability. While data on flower visitation discussed above clearly showed higher visitation of plants grown under high water availability and no effect of nitrogen availability, the composition of the flower visitor community varied between plants grown under low and high water availability and the total visitation rate was generally higher in the spring 2017 than summer 2018. It is possible that the level of pollen limitation was thus generally higher in the summer 2018 which could explain why the number of seed per flower was lower and, why seed set was more strongly reduced in plants grown with low amount of water. Pollen limitation is considered an important condition for observing variation in seed set. However, seed set may be reduced under water stress and other unfavourable conditions also by seed abortion (e.g. New *et al.*, 1994, Behboudian *et al.*, 2001). Our results on seed production in plants cross-pollinated by hand are consistent with this possibility, but the effect was not strong enough to explain variation in seed set in plants exposed to natural pollination by flower-visiting insects.

Apart from total flower visitation, often used as a proxy for pollination, seed set may be affected by the composition of the flower visitor community because the pollination efficiency of different flower-visiting insects varies widely (King *et al.*, 2013). We did not measure the efficiency of individual pollinator species, but we note that the difference in the strength of the effect of water availability on seed production per plant between spring 2017 and summer 2018 may be also related to changes in flower visitor community. In particular, rapeseed beetles were the most frequent flower visitors in the spring 2017, when they visited preferentially flowers of plants grown with high water availability, but they were almost absent in the summer 2018. These beetles feed on pollen and are known to cause damage to flowers, so it is possible they had a negative effect on seed set in the spring 2017, especially in plants grown with high water availability. Our data do not allow us to confidently quantify these effects, but we can conclude that seed set, and by extension fitness, of plants exposed to water stress, increased temperature, and variable nitrogen supply, is likely affected by these global change factors through multiple mechanisms, both direct effects on plant physiology, and indirectly through interactions with pollinators.

2.6. Conclusions

In this paper we conclude that multiple drivers of environmental change have a complex and interactive impact on plant traits, visitation by pollinators, and seed production. Our model species, *S. alba*, is an important crop itself and a close relative to many other economically important crops and vegetables from the Brassicaceae family, so this experiment shows how different climatic drivers may affect both vegetative growth and crop yield in many plant species form this family in the upcoming extreme climatic events. In addition to increasing temperature, many regions are experiencing overall decrease in precipitations or irregular rainfall leading to periods of drought. Our results suggests that the resulting water stress has a strong potential effect not only on plant growth, but also on floral traits and pollination, which may lead to decreased yield of agricultural crops or population decline in wild plants. While nitrogen availability had a generally weaker effect in our experiment, all three variables often affected the measured plant traits interactively, which demonstrates that various drivers of global change may have complex effects on plant reproduction and plant-pollinator interactions and multiple drivers need to be studied simultaneously to make informed predictions of the likely effects of climate change and nutrient deposition on communities of plants and pollinators.

2.7. References

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3. Effects of small-scale clustering of flowers on pollinator foraging behaviour and flower visitation rate

Photo: Asma Akter

Content of this chapter was published as:

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(11): e0187976.

3.1. Effects of small-scale clustering of flowers on pollinator foraging behaviour and flower visitation rate

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Abstract

Plants often grow in clusters of various sizes and have a variable number of flowers per inflorescence. This small-scale spatial clustering affects insect foraging strategies and plant reproductive success. In our study, we aimed to determine how visitation rate and foraging behaviour of pollinators depend on the number of flowers per plant and on the size of clusters of multiple plants using *Dracocephalum moldavica* (Lamiaceae) as a target species. We measured flower visitation rate by observations of insects visiting single plants and clusters of plants with different numbers of flowers. Detailed data on foraging behaviour within clusters of different sizes were gathered for honeybees, *Apis mellifera*, the most abundant visitor of *Dracocephalum* in the experiments. We found that the total number of flower visitors increased with the increasing number of flowers on individual plants and in larger clusters, but less then proportionally. Although individual honeybees visited more flowers in larger clusters, they visited a smaller proportion of flowers, as has been previously observed. Consequently, visitation rate per flower and unit time peaked in clusters with an intermediate number of flowers. These patterns do not conform to expectations based on optimal foraging theory and the ideal free distribution model. We attribute this discrepancy to incomplete information about the distribution of resources. Detailed observations and video recordings of individual honeybees also showed that the number of flowers had no effect on handling time of flowers by honeybees. We evaluated the implications of these patterns for insect foraging biology and plant reproduction.

Key words: Flower cluster, foraging behaviour, visitation rate, *Apis melifera*, *Dracocephalum moldavica*.

3.2. Introduction

Plants typically vary in the number of flowers they produce and individuals often cluster together at various spatial scales. Clustered spatial distribution of flowers has implications both for plant reproduction and food intake of flower-visiting insects [1, 2]. Pollinator responses towards clustering of flowers at various spatial scales have long been studied and the outcomes are highly diverse. However, behaviour of flower visitors in relation to the number of flowers on individual plants, as well as their foraging behaviour in larger clusters of multiple plant individuals can be understood in the context of selection for behaviours maximising the efficiency of resource acquisition [3, 4]. Behavioural responses of pollinators to resource clustering at different spatial scales in turn affect reproductive success of plants [5-7]. At the scale of individual plants, pollinators often prefer to visit plants with a larger number of flowers. These provide a higher total amount of rewards (nectar and pollen); moreover, they can be detected from a larger distance [8]. Despite that, the number of visitors usually increases less than proportionally with the number of flowers [9-18]. Although pollinators generally visit more flowers in larger inflorescences, they tend to visit a smaller proportion of available flowers [10, 12-15, 17-19]. This behaviour is consistent with classic predictions of the optimal foraging theory, which assumes that foragers feed in such a way as to maximise their rate of net energy intake [3, 4]. When foraging in patches, they should leave after the rate of energy intake drops below the average level provided by other patches [20]. Because insects have a limited ability to remember which flowers they have already visited, they start to revisit empty flowers after some time [21]. The risk of revisiting empty flowers increases with increasing number of flowers per inflorescence [17, 22]. As a result, visiting a decreasing proportion off lowers in larger inflorescences is an optimal foraging strategy [21].

Each individual flower can be thought of as a small patch of food [10], where extracting nectar may become more difficult as the nectar is depleted. This could prompt the bee to move to another flower earlier in rich habitats to maximise the amount of food extracted per unit time [20]. Many invertebrates [23-25] and vertebrates [26, 27] feeding on various food sources were observed to shorten their handling time and discard partially consumed food items when food was abundant. However, this behaviour was not observed in previous studies on bees and syrphid flies [9, 22]. This suggests that these flower visiting insects may handle individual flowers in a constant manner independently of flower abundance, but more data are needed before drawing firm conclusions. From the plant's perspective, higher per-flower visitation rate should translate into higher reproductive output [28]. Most published studies found no relationship between the number of flowers in an inflorescence and per-flower visitation rate [14, 15, 17-19], although some reported an increasing [28] or decreasing [12] relationship. Moreover, the link between visitation rate and seed set is not straightforward. Percentage seed set may increase with the number of flowers when visitation rate also increases [16, 28], but it may be reduced in self-incompatible species due to geitonogamous pollination which occurs when a single pollinator visits multiple flowers on the same plant [15, 29, 30].

At the local scale, plants often grow in groups of multiple individuals, which we refer to as clusters. As in single plants, higher number of flowers in a cluster usually leads to a less than proportional increase in the number of visitors [10, 31-33], although proportional or higher increase was also reported [34]. Pollinators also tend to visit a decreasing proportion of flowers in larger clusters [10, 22, 31]. Visitation rate per flower usually stays constant because the increase in the number of visitors is counterbalanced by a decrease in the proportion of flowers visited by individual insects. This leads to the ideal free distribution of flower visitors [10, 17, 31, 33, 35]. At this spatial scale, optimal foraging theory is equally applicable for understanding flower visitor behaviour as in the case of individual plants with different sizes of inflorescences described above, and these patterns fit well to its predictions [10, 20-22]. However, the consequences for plant reproduction can be very nuanced. Percentage seed set was reported to be independent of cluster size [35], or increasing in response to higher visitation rate per flower in clusters with more flowers [34, 36]. However, seed set may also depend on the density of plants within the cluster [37], on their genetic compatibility [38], and on species-specific consequences of geitonogamous pollination whose frequency may vary with cluster size [30].

Here we report results of a field experiment conducted to test how flower visitation and foraging behaviour of pollinators depend on the number of flowers at two spatial scales: single plants and clusters of multiple plants. We conducted the experiment with potted *Dracocephalum moldavica* L. (Lamiacea). Specifically, we tested whether the number of visitors increases proportionally to the number of flowers on a single plant or in a cluster and whether plants with larger inflorescences or in larger clusters enjoy higher flower visitation rates. We then studied foraging behaviour of the most abundant flower visitor, *Apis mellifera*, in more detail to test how visit duration, number of flowers visited, and handling time per flower depend on the number of flowers. Our data show that the number of insects increased less than proportionally with the number of flowers and that honeybees visited a smaller proportion of flowers in larger clusters. Together, this led to maximal visitation rate per flower in clusters of intermediate size.

3.3. Materials and methods

3.3.1. Plant selection and site

Dracocephalum moldavica is a plant of the family Lamiaceae native to temperate zone of Asia; China, Russia, Tajikistan, and Turkmenistan. It is partly naturalised in a large part of Eurasia, introduced to the USA, and sometimes grown as an ornamental plant. It produces hermaphrodite flowers with violet colour which are oriented in whorls with 5-6 flowers in each whorl, have a semi long corolla tube with nectaries at the bottom typical for Lamiaceae. The flower has a two-lobed stigma positioned below the upper lip and four anthers slightly shorter than the stigma. Each flower can produce four seeds. Interactions with pollinators are not well known; a related species, *Dracocephalum ryushiana*, is pollinated probably mostly by bumblebees [39]. We sowed the seeds in the beginning of May to germination trays in the greenhouse. Seedlings were transplanted individually to 1 litre pots containing a mixture of compost and sand (2:1) and grown in the greenhouse with daily watering. The plants fully flowered at the end of July with an average plant height of ca. 60 cm. The first experiment (see below) was conducted in a meadow near ČeskyÂ Krumlov, 18 km southwest of ČeskeÂ BudeÏjovice (N 48Ê49.48', E 14Ê18.98'). The rest of the project was carried out in a meadow near the campus of the University of South Bohemia in ČeskeÂ BudeÏjovice (N 48Ê58.50', E 14Ê26.15'). All experiments were carried out on sunny days with no strong wind and no rain. No permits were required for this project because no protected species were collected and the study was conducted on public land.

3.3.2. Experimental setup

The first experiment was designed to study pollinator visitation on single plants with different numbers of flowers. We used plants grown individually in pots. We adjusted the number of flowers per plant by cutting some of them, which provided plants with the number of flowers ranging from 1 to 174. Eight plants in pots were placed along a 35 m long transect; i.e. five meters apart. We observed and captured all flower visitors for 30 minutes per plant. Two to three people were collecting data simultaneously, each observing a different plant. We then replaced the plants by a new set of eight plants and repeated the observations. Overall, we sampled eight transects with different plant individuals during three days (4th, 7th, and 8th August 2016), which resulted in a total of 64 observations. Sampling was conducted between 10:00 and 16:00 hours under good weather conditions (sunny, no rain). Insects were collected using an aspirator or a handnet, counted and preserved for identification. The second experiment was aimed at studying visitation of clusters of multiple plants of different sizes. In this experiment, potted plants were placed to form five clusters 20 m apart in a 60 x 20 m grid (one position in the grid remained empty). Each cluster contained a different number of plants varying from 1 to 37. We also counted the number of open flowers at each plant. The number of flowers in a cluster ranged between 42 to 2476.

Each cluster was observed for 30 minutes during which all insects visiting *Dracocephalum* flowers in the cluster were captured and preserved. We completed seven sampling periods on 16th and 17th August 2016, which yielded in total 35 observations of cluster visitation. The numbers of flowers in each cluster were counted every day after finishing the experiments. We also conducted detailed observations of foraging behaviour of *Apis mellifera* at the site of the second experiment. The total number of flower visitors in the experiments was dominated by *Apis mellifera*, which was thus selected for additional measurements. We measured the duration of visits, the number of flowers exploited, and handling time per flower of *A. mellifera* in clusters of different sizes. Potted plants were placed in the same grid as in the second experiment to form five clusters on 25th and 26th August 2016. The number of plants per cluster ranged from 1 to 22, and the number of flowers was 2 to 643 per cluster. In these observations, a single *A. mellifera* was followed from its entry into the cluster until its last visit to a flower in the same cluster. Data collection included both the time spent in one cluster measured by a stopwatch and the number of flowers visited by each individual *A. mellifera*.

To test the hypothesis of partial consumption in honeybees, we measured the average number of flowers per minute over individual foraging bouts based on direct observations and then measured time spent on individual flowers using video recordings. Video recordings were taken at the same time as observations of honeybee foraging, but in those clusters which were not observed at the time to minimise disturbance of the recordings. We distinguished: i) total time from landing at a flower until leaving and ii) actual feeding time (head inserted deep inside the flower). We tested whether these two measures of handling time depended on the number of flowers in a cluster.

3.3.3. Data analyses

For the experiments on flower visitation, we conducted the analyses at the level of the total number of insect visitors per plant or per cluster. We tested how the number of flower visitors and other measures of visitation varied with the number of flowers using generalised linear models (GLM), or generalised additive models (GAM) implemented in a package mgcv 1.8-17 [40] when the relationship was nonlinear. Analyses were done in R 3.2.4 [41]. The number of flowers, used as an explanatory variable, was log-transformed before the analyses. We fitted the GLMs and GAMs using overdispersed Poisson distribution (quasipoisson) or Gamma distribution with log link function depending on the response variable. Analysis of proprotion data was performed using Beta regression implemented in betareg package for R [42].

3.4. Results

We found that the number of flower visitors increased with the increasing number of flowers on a plant or in a cluster, but less than proportionally (GLM, quassipoison distribution, F1,62 = 31.5, P < 10−6; Fig 3.1A, blue line and points). The relationship was linear at the log-log scale with a slope of 0.57 (SE = 0.108). Data from larger clusters of multiple plants qualitatively showed an extension of the patterns observed in single plants. The number of insects increased with the increasing number of flowers (F1,33 = 45.6, P < 10−6; Fig 3.1A, orange line and points) with a slope of 0.58 (SE = 0.093) at the log-log scale. Also, the ratio between the number of visitors and flowers increased when the number of flowers increased from one to around 20, but decreased when the number of flowers increased further (GAM, Gamma distribution, log link function, F = 3.2, P = 0.0026; Fig 3.1B, blue line and points). The log-linearly decreasing relationship in clusters of plants was an extension of the relationship reported in single plants (F = 26.1, P = 1 × 10−5; Fig 3.1B, orange lines and points).

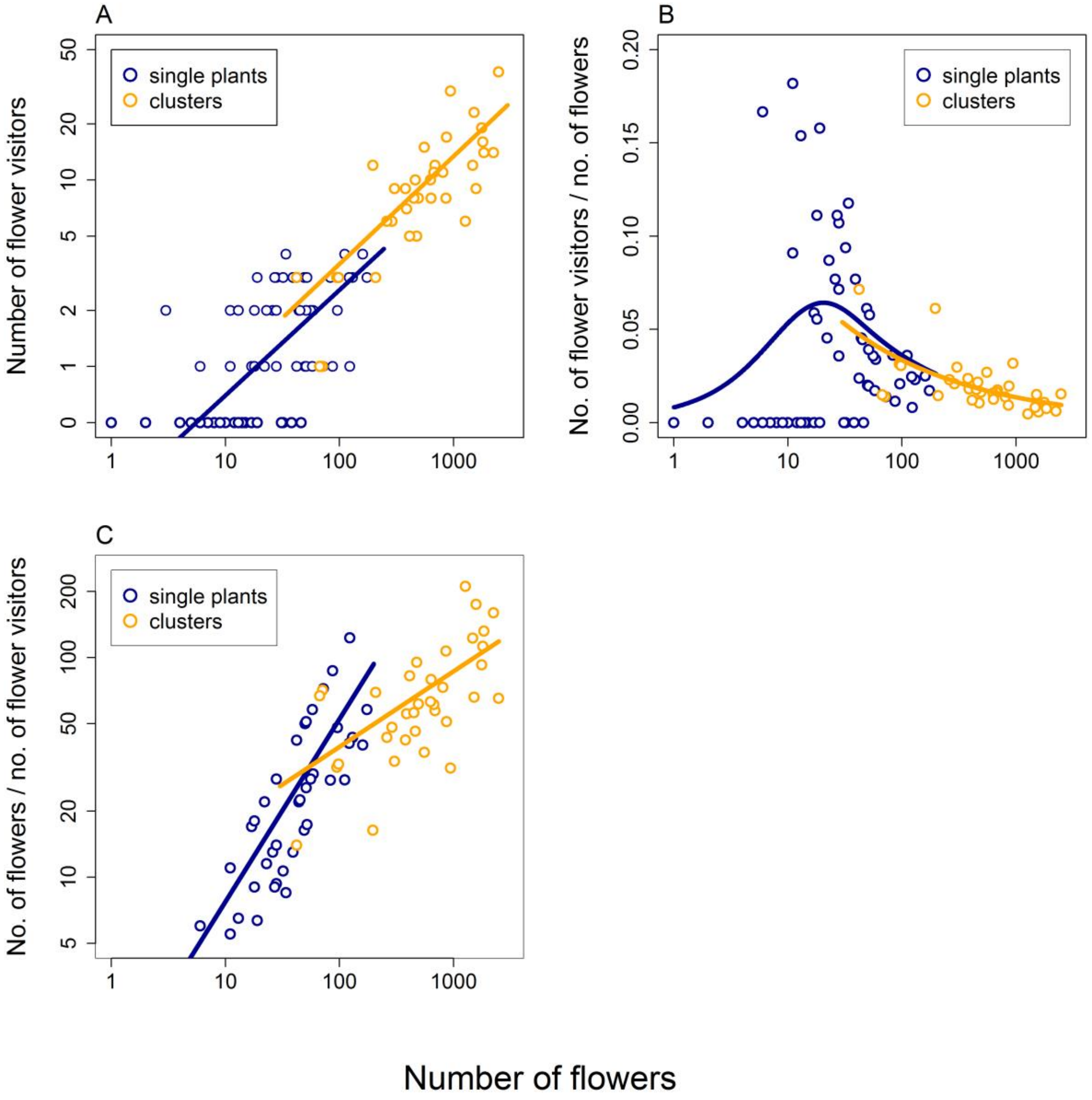

Figure 3.1. The effects of the number of flowers in single plants and larger clusters on visitation by insects. The plots combine data from two separately conducted experiments: one with single plants differing in the number of flowers (blue circles and fitted lines) and another with larger clusters of up to 36 plants (orange circles and fitted lines). Data from these two experiments were combined for the purpose of visualisation, but were analysed separately. A: The number of flower visitors observed during 30 minute observation periods on single plants and larger clusters varying in the number of flowers. B: The number of insects visiting the plants or clusters of multiple plants relative to the number of flowers available. C: The number of flowers available per visitor; i.e. the potential pay-off for the flower visitors.

We also calculated potential payoff for flower visitors defined as the mean number of flowers per visitor, assuming that already visited flowers did not renew their nectar reward during the observation time (30 minutes). The potential payoff increased with the increasing number of flowers on both single plants (GLM, Gamma distribution, log link function, $F1,39 = 67.5$, $P < 10{-}6$; Fig 3.1C, blue line and points) and clusters of plants ($F1,33 = 21.0$, $P = 6 \times 10{-}5$; Fig 3.1C, orange line and points), although with a shallower slope in the latter case; slope was 0.83 in single plants ($SE = 0.096$) and 0.34 in clusters of plants ($SE = 0.076$). This means that there were more free resources available for each visitor in larger clusters.

Detailed observations of foraging behaviour of individually tracked honeybees showed that, as expected, individual honeybees spent more time foraging in larger clusters (GLM, Gamma distribution, log link function, $F1,80 = 8.5$, $P = 0.0045$; Fig 3.2A) and visited more flowers there (GLM, quasipoisson distribution, $F1,80 = 11.3$, $P = 0.0012$; Fig 3.2B). However, the increase was only modest in both cases; significantly less than proportional. The slope was 0.34 ($SE = 0.114$) for time and 0.38 ($SE = 0.117$) for the number of flowers visited. There was also considerable variation around the fitted relationships. The proportion of available flowers visited by individual honeybees decreased significantly with the increasing number of flowers per cluster (Beta regression, $\chi2 = 8.9$, $P = 0.0029$; Fig 3.2C). In large clusters, all individuals visited only a minority of flowers, while in small clusters, the proportion of flowers visited varied widely from just a few to all flowers available (Fig 3.2C). Honeybees foraged with the same speed across the range of cluster sizes; i.e. the number of flowers visited per minute did not depend on the number of flowers available (GLM, Gamma distribution, log link function, $F1,80 = 1.87$, $P = 0.1756$, Fig 3.2D).

Neither of our two measures of handling time per flower depended on the number of flowers in a cluster (Fig 3.3). Feeding time per flower, defined as the time a bee spent with its head deep inside a flower, apparently engaged in nectar extraction, did not depend on the number of flowers in a cluster (GLM, Gamma distribution, log link function, $F1,132 = 0.75$, $P = 0.3869$; Fig 3.3A). Also, the total time spent on the flower from first contact until take-off was independent of the number of flowers (GLM, Gamma distribution, log link function, $F1,132 = 0.95$, $P = 0.3308$; Fig 3.3B). Compared to data shown in Fig 2D, these measurements exclude travelling time between flowers and estimate only the time spent handling the flowers and the actual duration of feeding per flower. There was a considerable variation in both total time spent on a flower and feeding time per flower in individual flower visits, which was quantified by generalised linear mixed models. We fitted a model including the intercept and individual identity as a random factor with logtransformed response variables to quantify the variance of handling time between individuals and within individuals. Based on this, we calculated the ratio of between-individual variance and total variance, i.e. repeatability, as a measure of differences between individuals (rptGaussian function in rptR package for R, [43]). We found that repeatability of the total time spent on a flower was 0.15 (95% confidence interval = [0, 0.321] based on bootstrap); i.e. 15% of the variance occurred at the between-individual level and 85% at the within-individual level. Repeatability of the feeding time was only slightly higher, 0.21 (95% confidence interval = [0.0346, 0.405] based on bootstrap). There were thus only small differences between individuals in both measures of their handling times.

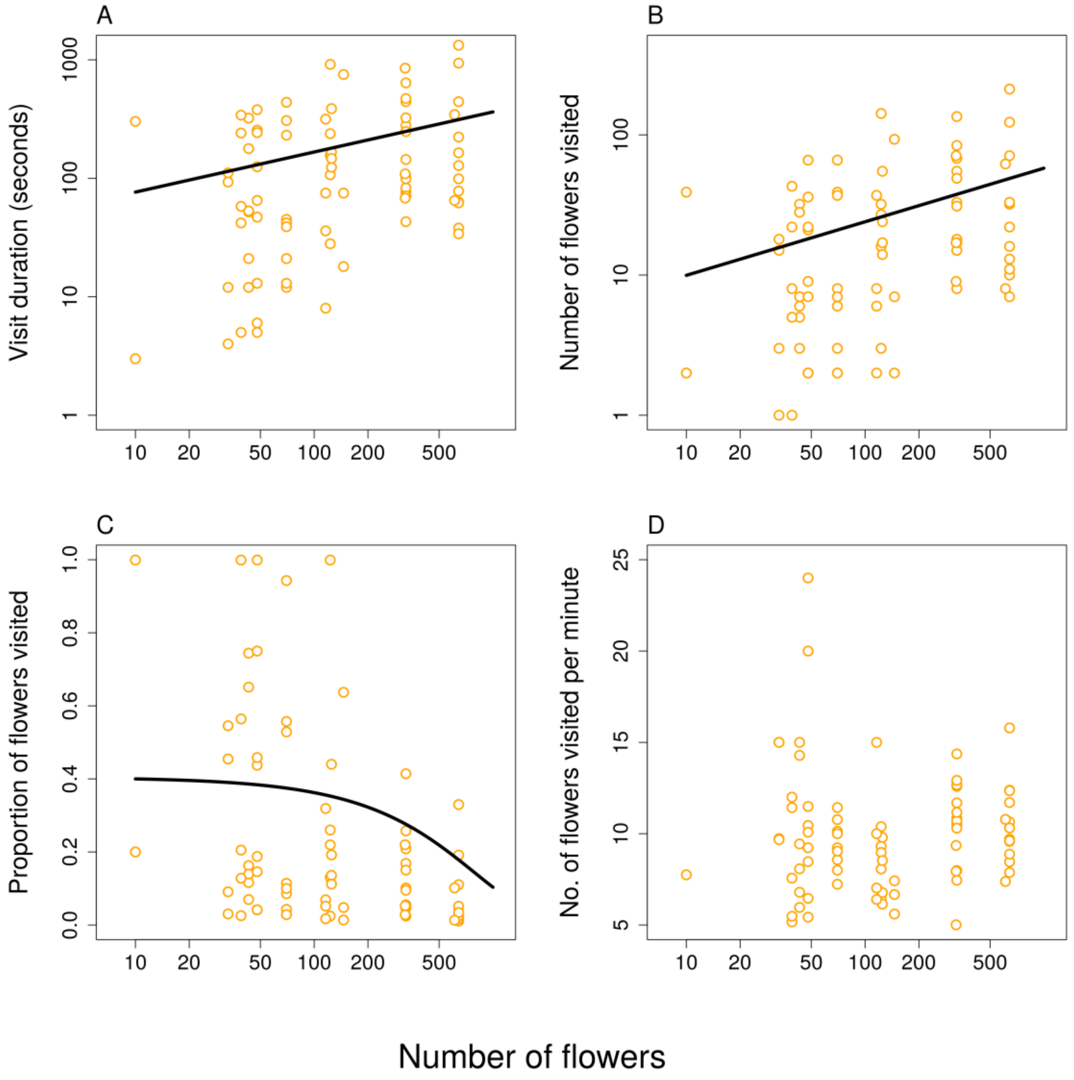

Figure 3.2. Foraging behaviour of honeybees in response to the number of flowers in clusters of multiple plants. A: The time spent foraging within the cluster by individual honeybees increased with the number of flowers available. B: The number of flowers visited increased with the total number of flowers available. C: The proportion of flowers visited by individual bees decreased with the number of flowers available. D: The number of flowers exploited per minute did not show any

significant relationship to the number of flowers available. X-axis in A. and B. and y-axis in A. and B. are on a logscale.

Finally, we calculated visitation rate per flower and unit time by multiplying the estimated dependence of the number of honeybees on the number of flowers (GLM, quassipoison distribution, $F1{,}33 = 10.009$, $P = 0.0034$, slope = 0.40, $SE = 0.1313$) and the dependence of the proportion of flowers visited on the number of flowers per cluster (Fig 3.2C). The estimated visitation rate showed a unimodal relationship peaking at the intermediate level of the number of flowers per cluster (Fig 3.4).

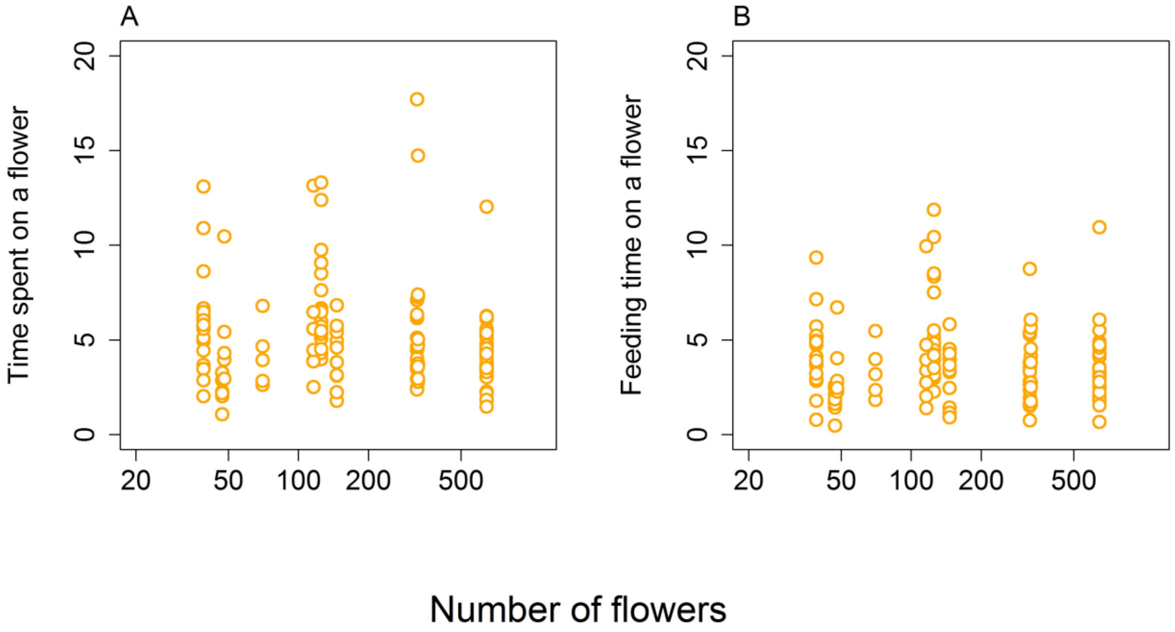

Figure 3.3. Honeybees' handling time per flower was independent of the number of flowers in clusters of different sizes. A: Total time spent on a flower measured from video recordings as the time from the first contact until take-off. B: Feeding time estimated as the time honeybees spent with their head deep inside the flower. Both are measures of handling time excluding movement between flowers.

3.5. Discussion

We observed that single plants with many flowers and large clusters were generally more attractive to flower-visiting insects than those with a smaller number of flowers. This is a classic pattern expected for optimally foraging animals who maximise net energy intake per unit time. However, our data show several departures from simple theoretical expectations. The number of flower visitors increased with the increasing number of flowers, but less than proportionally (Fig 3.1A). Optimally foraging animals should reach *ideal free distribution* (IFD) where they would possibly ignore very poor patches altogether, and they would be distributed between the rest of the patches in such a way as to equalise patch payoff [10, 44]. In the case of flower visitors, this leads to flower visitation rate independent of the number of flowers per plant or cluster [10]. However, our data show that plants with many flowers and large clusters were underutilised. The number of insects per flower decreased sharply in single plants with many flowers and in large clusters (Fig 3.1B), and the number of flowers available per visitor increased (Fig 3.1C). Detailed observations of foraging honeybees, the most numerous flower visitor species, showed that flower visitation rate peaked in clusters of plants with an intermediate number of flowers and dropped in clusters with both few flowers and many flowers (Fig 3.4). This observation is inconsistent with the prediction of a constant flower visitation rate based on optimal foraging theory [10]. Previous empirical studies generally found that i) the number of visitors increased less than proportionally with the number of flowers and ii) that an increasing number of flowers was visited per individual in larger clusters. Our results also show these patterns. In a number of previous studies these two relationships had such slopes that they resulted in constant flower visitation rate [10, 17, 31, 33, 35].

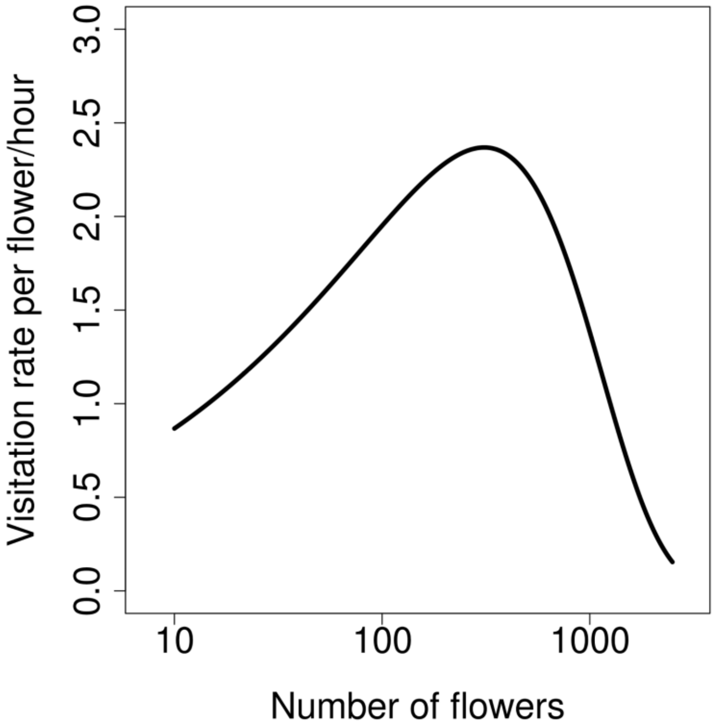

Figure 3.4. Flower visitation rate peaks at the intermediate number of flowers. Visitation rate per flower per hour was estimated as a product of the number of visitors (honeybees only) and the proportion of flowers visited by an individual honeybee.

However, in our case, these relationships had such shapes that they combined to form a unimodal pattern with the highest flower visitation rate in clusters with an intermediate number of flowers (Fig 3.4). This represents suboptimal foraging behaviour because large, most profitable clusters were underutilised. Other reported deviations from the expected pattern include a decreasing [12] as well as increasing [28] flower visitation rate in larger clusters. The lack of flower-visitors on plants with few flowers is consistent with expectations based on optimal foraging theory [20] and the IFD model [44]. It is generally not profitable to use poor resources, i.e. plants with few flowers, unless resources are very scarce [44]. Honeybees are known to adjust their selectivity for clusters of flowers based on the overall abundance of resources, so they avoid poor resources when food is plentiful [45]. However, an alternative explanation is that this is not due to choice on the part of insects but due to low detection probability of plants or clusters with few flowers. Detectability of an object increases with the visual angle subtended by the stimulus, which means that bees and other animals can see large flowers or inflorescences easier and from a larger distance [8, 46, 47]. Unfortunately, our data do not allow us to decide whether plants with few flowers were not detected or ignored.

Underutilisation of plants and clusters with a high number of flowers could be explained by a limited amount of information insects had about the quantity and spatial distribution of resources, because we placed the plants at the meadow only shortly before we started our observations [48-51]. Classic models of optimal foraging theory [20] and IFD [44] assume that foragers are omniscient, i.e. that they know the quality of all individual patches of food. This is rarely if ever the case in reality, so animals must make foraging decisions with imperfect information [48-50, 52]. They are generally thought to use information about the quality of previously visited clusters together with their perception of the quality of a new cluster to decide whether to enter the cluster or go elsewhere [48, 50]. This may provide explanation for our observation of underutilisation of the richest clusters. The meadow where the experiment was conducted had fairly low abundance of flowers, so medium-sized and larger clusters of *Dracocephalum* probably provided a richer source of food than the original plant community. At the same time, insects had a limited amount of information about the location and quality of the clusters of *Dracocephalum*. Our experimental manipulation thus represents a case of quick changes in resource availability and spatial distribution, similar to common natural situations such as when some plants start flowering and the spatial distribution of resources for flower visiting insects changes over short time-scales. In such cases, bees have a limited amount of information about their resources, so they are not able to forage optimally [53]. Our data thus support previous observations that foragers are usually over represented in poor clusters and under represented in rich clusters, leading to suboptimal food intake [48].

At the within-cluster scale, we observed that individual honeybees spent more time and visited more flowers in larger clusters, but they visited a smaller proportion of the available flowers (Fig 3.2). This pattern has already attracted considerable attention because it seems to be at odds with optimal foraging behaviour [10, 22, 31, 54]. However, due to larger numbers of insects visiting larger clusters of flowers, visiting a smaller proportion of flowers leads to an IFD and thus to an optimal use of resources [10]. For example, Goulson [22] performed experiments which showed that as the insect visits flowers in a large patch it becomes difficult to avoid revisiting already emptied flowers, so at some point it becomes advantageous to leave the patch rather than search for the remaining unvisited flowers because food intake rate is depressed [20]. Another aspect of foraging biology we studied was handling time per flower.

None of the measures we used varied with the number of flowers per cluster (Figs 3.2D and 3.3), so it appears that bees did not adjust the way they used individual flowers depending on the number of flowers in a cluster. This result is in line with several previous studies on various bees and syrphid flies [9, 22], so these insects apparently handle individual flowers in a constant manner independently of flower abundance. It is important to note that most studies of foraging behaviour focused on honeybees or bumblebees, which may behave differently from other groups of pollinators. For example, it seems that honeybees visit a higher proportion of flowers before moving to another plant compared to other pollinators [55]. Comparative studies on multiple flower visitors will be needed to shed more light on the generality of patterns discussed here, see e.g. [9]. Our current data do not allow us to evaluate the implications of flower clustering for plant reproduction. Our observations of flower visitation and foraging behaviour suggest that the number of flowers on individual plants and in clusters of plants could affect plant reproductive success. Specifically, there was a lower number of insects per flower on plants with more flowers and in larger clusters (Fig 3.1) and per-flower visitation rate peaked in clusters with an intermediate number of flowers (Fig 3.4). Variation in flower visitation rate should lead to differences in pollination and consequently percentage seed set depending on the number of flowers per plant or per cluster. However, previous studies show that the link between visitation and seed set in plants is often weak and not at all straightforward [32, 56]. Additional data from a different type of experiments will thus be needed to resolve this question in our system.

3.6. Conclusions

Our results show that flower-visiting insects preferred plants and clusters of multiple plants with larger numbers of flowers. However, visitation rate per flower and unit time peaked in clusters with an intermediate number of flowers in violation of ideal free distribution expected for optimally foraging animals. We consider imperfect information about the location and quality of plant clusters to be a likely explanation of this pattern. Detailed observations of foraging honeybees showed that they visited more flowers, but a smaller proportion of flowers in larger clusters. Finally, although handling time per flower was highly variable, it was unrelated to the number of flowers per cluster. Bees were thus not flexible in handling flowers depending on their local abundance.

Acknowledgements

We would like to thank Pavla DudovaÂ for help with field observations. We are also grateful to two anonymous reviewers who provided valuable comments on the manuscript.

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4. Introduction of new floral resources into plant-pollinator communities: impact on flower visitation mediated by species traits

Photo: Asma Akter

Manuscript in preparation:

Akter, A., Biella, P., Klecka, J. 2020. Introduction of new floral resources into plant-pollinator communities: impact on flower visitation mediated by species traits.

4.1. Introduction of new floral resources into plant-pollinator communities: impact on flower visitation mediated by species traits.

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Abstract:

Invasion of non-native plants into natural habitats is a potentially lasting and pervasive threat to ecological communities. Consequences of species introduction, especially for plantswhich are pollinator dependant for the pollination, in the new communities are complex and difficult to predict. We conducted a field experiment to manipulate the structure of flowering communities in small grassland patches. We tested the effects of introduction by single plant species, one generalized and one for long- tongue bees, and by both plants together across multiple sites. The short-term nature of our experiment sheds light on the role of shifts in pollinator behaviour in response to the introduction and changes in flowering diversity and richness composition and how different flower traits affect the pollinator richness and visitation.

Keywords: Alien plant, native community, flower resources, visitation rate, flower traits

4.2. Introduction

Ecological communities are dynamic and subject to continuous changes through species immigrationor extinction. Human activities such as introduction of new species into their environment to cover their needs (Geslin et al., 2017) and climate change, which shifts the environmental conditions and allows a new species to immigrate (Chen et al., 2011), are two major drivers behind the accession of new species into a non-native community. Species of a community exhibit complex interactions by interacting with each other in an antagonistic way (such as predator-prey) or through mutualism (plant-pollinator interactions) (Tylianakis et al., 2010). Therefore, introduction of a new species into a native community may have alternating impacts, either facilitation or competition, on these interactions, as this may disturb the existing interactions and in case of a mutualistic interaction, like pollination, this disturbance may alter the structure and strength of this relationship (Russo *et al.*, 2014; Campbell et al., 2015, Tylianakis and Morris, 2017).

Understanding the new plant species integration into a native plant-pollinator community is a key step to assess the impact of a new species on plant-reproduction of native plants and the resilience of existing plant-pollinator networks towards the altered flower resources (Ghazoul, 2004; Bartomeus et al., 2008; Chung et al., 2015; Goodell and Parker, 2017). Impact of the presence of a non-native plant species in a native plant-pollinator community are predicted to affect the interactions between the native plant and pollinators thorough different mechanisms. The magnitude and direction of the impact of new species in a native plant-pollinator community depend on the influence of the new species on the pollinator abundance, behaviour and community structure (Connell and Slatyer, 1977). But such outcomes can be complex and are closely related to the floral diversity, floral characteristics and competition/facilitation in pollinator reception among the existing species and new species (Ghazoul 2006; Bartomeus et al., 2008, Schlüter et al., 2009; Gibson et al., 2012; Goodell and Parker, 2017). First, a new floral resource may increase the flower diversity and flower richness and provide more foraging resources for the existing pollinator communities. This may result into lower flower density for the native flowers and they may lose the dominance in providing the foraging resources for pollinators (Muñoz and Cavieres, 2008). Study suggested that if the new species increases the total number of flowers in the community and the pollinator pool remains constant, that can potentially decrease the visitation rates of native flowers by reducing the attractiveness of native flowers to the pollinators (Rathcke 1983, Cox and Elmqvist 2000; Essenberg, 2012). On the other hand, new species may facilitate the pollination on native species by promoting the flower attractiveness and luring flower visitors to a patch and improve pollinator visitation of native plants (Molina-Montenegro et al., 2008). Phenotypically similar species generally share similar pollinators and similar floral traits between the new and native flowering species will lead to greater competition for pollinators (Bjerknes et al., 2007; Carvalheiro et al., 2014). Similarity of floral traits such as flower colour, flower shape and flower arrangement will reduce the discrimination with native and new floral resources and increase the competition between these plant species (Waser 1986, Chittka et al. 1999, Bjerknes et al. 2007). Flower diversity and abundance may also play an important role, and less abundant plants may be more strongly affected by the newly introduced species, although this possibility is widely ignored (Rathcke 1983). All these factors can eventually affect the pollinator visitation rate, compatible pollen transport and receipt ratio, and, finally the seed production of native plants (Aizen et al., 2008; Vanbergen et al., 2018; Hernandez-Castellano et al., 2020).

The interplay between the new floral resources and their impact on the visiting choices will determine the impact of new flower on the native flowering community. Examining these impacts will require large scale manipulation in the plant-pollinator communities and may counter many other ecological and environmental factors which contribute to the outcome (e.g. species diversity and richness, intra-specific competition, variation in associated communites etc.). Assessment of the future of an alien plant whether it would become established as a harmful or neutral or beneficial to the native community will be related to many other factors, such as nutritional or other growth factor related competition (Levine et al. 2003, Ehrenfeld 2010, Skurski et al. 2014), invasiveness of a plant including vegetative and reproductive growth (Forcella et al., 1986; Crawley et al., 1989; Thompson et al., 1995) and naturalization through the incorporating to the natural flora (Richardson et al., 2006). Answering how a new plant with new floral resource can affect the native plant-pollinator interactions without confounding effect of competition for abiotic resources can be difficult. In most cases, the effect of the presence or absence of a non-native plant of pollination of native plants was studies in the communities where alien plants were already established, in most of the cases as invasive, and the comparison was conducted by removing the invasive plant species (Lopezaraiza-Mikel et al., 2007). Other studies compared sites with similar vegetation, either invaded or uninvaded by an invasive species (Bartomeus et al., 2008; Vilà et al., 2009). The ability to predict the direction of the interactions in the floristic level without any other ecological influence therefore would be helpful to understand what drives the pollinator communities to interact with the new floral resources and will benefit the species conservation concern.

In this study we introduced two different flowering plants into several semi-natural meadows and observed the pollinator visitation on the flowers. The flowering plants were cultivated in the greenhouse and transferred to the field in pots; therefore, plant growth and development did not have any impact on the native plant community. We kept the similar density of new floral resources across our study site to avoid any impact from the difference among the new floral diversity and richness. In order to do that we introduced similar number of plants in every site.

To study the impact of the new floral resources on the native flower community, our study aimed to answer the following research questions: i) do new floral resources brings benefits or do they compete for pollinators with the native flowers? ii) what are the differences among the visiting groups and how they interact with the flowers? and, iii) which floral traits mediate the impact of the introduced species on the visitation of native plants.

4.3. Methods

4.3.1. Field sites and plant survey

Five sites; one control and four experimental sites were selected for the study in the semi-natural meadows in the Czech Republic. All the filed sites were located nearby Český Krumlov, about 25 km from České Budějovice in South Bohemia (site locations: experimental plots: 48°50´6.582´´N, 14°15´36.953´´E; 48°49´43.522´´N, 14°18´49.338´´E; 48°49´51.468´´N, 14°17´15.086´´E; 48°49´42.518´´N, 14°19´24.688´´E; control plot: 48°49´30.223´´N, 14°18´57.690´´E). This region contains a rich habitat of fauna and flora. Two of our experimental sites were in semi-open meadow with dense forest trees nearby and other two locations were mixed in vegetation with herb, shrubs and large trees. Location and description of five field sites and photos are added in the Supplementary table 4.1 and Supplementary Figures 4.1 and 4.2. Before the introduction of new plants and insect visitation sampling, initial data on the flowering communities were collected. Detail data on the plants, flowering type, arrangement of the flowers, flower colours and photos were collected. Flower surveys were conducted three times through the fieldwork to observe the flower diversity and richness. A total of 54 flowering entomophilous plant species from 21 families were listed. A complete list of flowering plants in the fields with the flower traits is added to the Supplementary Table 2.

4.3.2. Species selected for the introduction into the communities

For the experimental introduction, we chose two flowering species, which are not invasive and are grown as ornamental plants to avoid any risk of negative consequences of our experiments for the local communities. One was the *Dracocephalum moldavica*, which is well known for pollinating by long-tongue pollinators (Akter et al., 2017), specially bees and other one was *Calendula officinalis*, known as pollinating by generalist pollinators, receives a wide variety of pollinators (Carreck and Williams, 2002). (Photos in Fig. 1). Both plants were grown in the greenhouse and later brough to the filed on the respective experiment days, photo of the greenhouse plants in the Supplementary Figure 3.

Figure 4.1: Photos of chosen flowers for experiments; A) *Calendula officinalis*, B) *Dracocephalum moldavica.*

4.3.2.a. Dracocephalum moldavica

D. moldavica is partly naturalized in many locals of Eurasia, including Russia, China, Tajikistan, Turkmenistan and introduced to a diverse area of United States. It produces hermaphrodite flowers with violet colour and are oriented in whorls with 5-6 flowers in each whorl, have a semi-long corolla tube which is typical for Lamiaceae and are reported to be pollinated by long-tongued insects (Milberg & Bertilsson, 1997, 2). Plants were potted into a 1L pot containing standard potting soil and grown under greenhouse condition with regular water and fertilization. The seeds were sown in March and fully flowered at the end of June with an average plant height of 60 cm.

4.3.2.b. Calendula officinalis

C. officinalis, from the family Asteraceae is widely cultivated as a garden flower in the warm temperate region of the world where it is probably native to the Southern Europe. The plant is partly naturalized also in the northern Europe. It's a short-lived herbaceous perennial, growing up to 80 cm. The colour of the inflorescence ranges from yellow to orange, comprising a thick, 4-7 cm diameter capitulum, surrounded by two rows of hairy bracts. In the wild plant they have a single ring of ray florets surrounding the central disc florets, while the hybrid cultivars contain several layer of floret ray rings. *C. officinalis* is a generalist insect-pollinated plant where the pollinators include bees, butterflies, hover flies and other non-major pollinators. It is also a source of food for a few species of Lepidoptera larvae. For our experiment, seeds were sown in March and fully flowered at the end of June.

4.3.3. Insect visitation survey design

Insect visitation survey and sampling were carried out from the early July to beginning of August 2018. We used 'transect method' to survey the pollinators. In every site, six random transects were set up, each 10m long and 1m wide. Insect visitation surveys were conducted before the introduction of floral resources, in their presence and after their removal. Each transects were surveyed three times a day, from about 8.30 am – to 4.00 pm. One person was walking slowly along the transect and tried to capture all flower-visiting insects encountered on flowers. The duration of the transect walk varied between the transects depending on the flower density, but it was usually between 10-30 minutes per transect. We defined the flower visitors as pollinators when an insect contacting the reproductive part of the flowers or entering the flowers for tubular shaped flowers.

For the introduction of new floral resources, we brought the potted flowering plants into the field and placed them randomly along the transects either as a single plant or in groups of four pots to mimic the distribution of naturally growing plants. We introduced 36 plants per experimental plot, six per transect (one group of four pots and two single pots), either 36 individuals of *C. officinalis,* or 36 individuals of *D. moldavica,* or 18 individuals of each species. The plants were exposed in the field only for one day to evaluate an immediate behavioural response of the flower visitors to the appearance of the new resource. All treatments were applied to each experimental plot in a randomised sequence with a few days between each manipulation to provide time for the pollinator community to adjust. In each plot, we thus sampled flower visitors in the natural community, in the presence of each of the two introduced plants and in the presence of both of them at the same time. This allowed us to compare the effect of the two introduced species and also to evaluate their interactive effect when they were introduced simultaneously. We sampled in the control site every day with the sampling in the experimental sites. Days with strong wind, rain or relatively low temperatures were avoided. At end of every sampling day, we immediately preserved our insect samples in the freezer for future analysis. Complete schedule for the fieldwork has been added to the Supplementary Table 3.3.

4.3.4. Statistical analysis

Shannon's diversity index (Shannon and Weaver, 1975) and Menhinick's species richness calculating method (Whittaker, 1977) were used to calculate the flower diversity and flower richness with and without the introduced floral resources in the sites. We used generalised linear models (GLM) to test the differences between the flower diversity and richness with and without the introduced floral resources. Pollinator diversity was also calculated using Shannon diversity index method. Comparison of the pollinator diversity before and after the introduction of new floral resources was done by ANOVA. Generalized linear mixed model (GLMM) was used to test the impact of introduced plants on the overall insect visitation on all plants and native plants, with sites as a random factor and Poisson error distribution for response variables. GLMM was also used to analyse the impact of flower traits on the total visitation of insects on all and only native plants. All analysis was done by using R 4.0.2. (R Core Team, 2020).

4.4. Results

4.4.1. Flower diversity and richness

There were no significant differences among the flower diversity and flower richness in the presence and absence of the non-native flowers (F= 0.016 and P= 0.665 for diversity and F=0.37 and P= 0.775 for the richness). Although there was a large difference between flower diversity between the sites (F=6.375, P=<0.001) and between the beginning and end of field work (F= 5.795, P=<0.05, Fig. 4.2a), the flower richness did not show any differences (Fig. 4.2b).

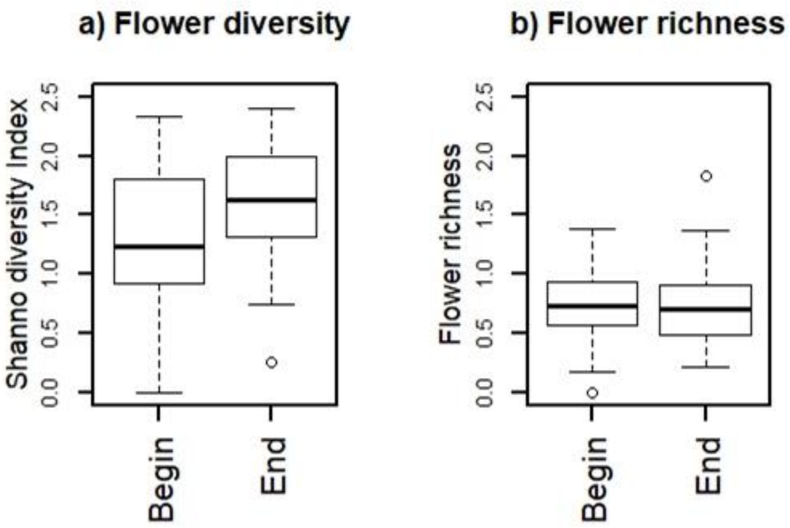

Figure 4.2: Comparison of flower diversity and richness in the communities between the beginning and end of experimental period; a) Shannon's index for flower diversity and b) Menhinick's index for flower richness.

4.4.2. Insect visitation rate before and after the introduction of new flower resources

In total we sampled 5342 insect pollinators belonged to 72 families of 5 major insect orders; Hymenoptera, Hemiptera, Coleoptera, Diptera and Lepidoptera. There was a great variation from site to site but we found that the pollinator diversity at the site-level, i.e. for all the plants together (including the introduced plants when present) did not change after the introduction of the new floral resources into the community (F= 0.046, p=0.832, figure 4.3a). However, pollinator diversity of native plants dropped after the new floral resources were introduced (F=4.055, p= <0.05, figure. 4.3b).

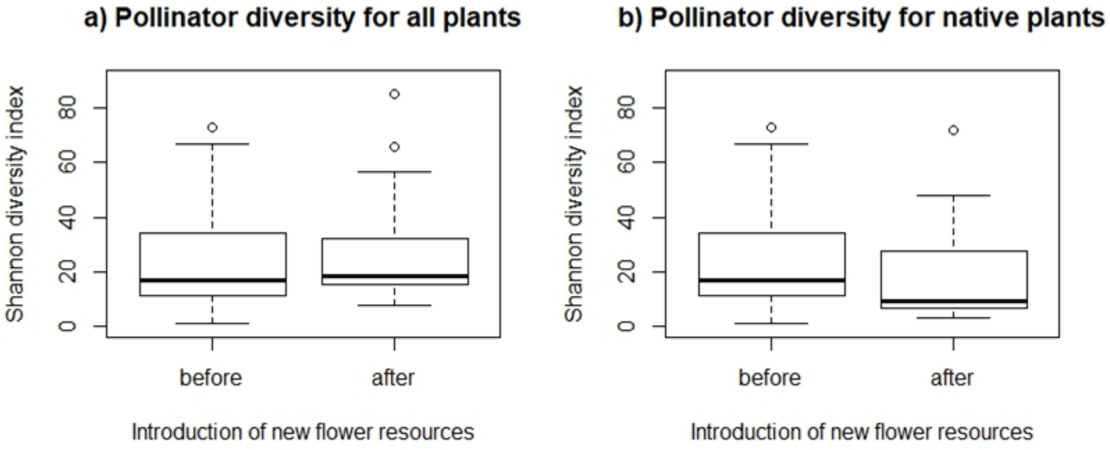

Figure 4.3: Flower visiting pollinator diversity before and after the introduction of new floral resources into the native communities; a) diversity considering all the flowers present in the communities, b) diversity only for the native flowers in the communities.

4.4.3. Response to the different flower resources from different pollinator groups

Insect visitation on all plants showed that overall total visitation per transect changed significantly after the introduction of the new floral resources into the community depending on the type of the new flower. Insect visitation rate increased when considered all native and introduced plants (F=10.878). Individually, when *C. officinalis* was introduced, both individually (p=0.02) and in association with *D. moldavica* (p=<0.001). But did not show any difference when only with *D. moldavica* (p=0.198, Fig: 4.4a). In contrast, visitation only on native flowers significantly dropped in the presence of *D. moldavica* only (p=<0.001) but did not change in case of the other two cases (Fig. 4.4b). There were significance differences among the response of pollinators from different orders. Total insect visitation per transect from the order Hymenoptera significantly increased in the presence of both new species for all the plants together, but did not show any impact when they were introduced individually. On the other hand, insect visitation in native plants significantly increased in the presence of *D. moldavica* (Table 4.1). Overall visitation by butterflies (Lepidoptera) increased in the presence of *D. moldavica* and decreased for the combination for the all plants together. But for the native plants, total visitation decreased in the presence of *C. officinalis* and when both new plant species were introduced together (Table 4.1). Coleoptera, Diptera and Hemiptera did not show any differences in visitation rate in the presence of the new floral resources, except for the

Hemiptera, which showed decreased insect visitation in native plants when both species were introduced together (Table 4.1).

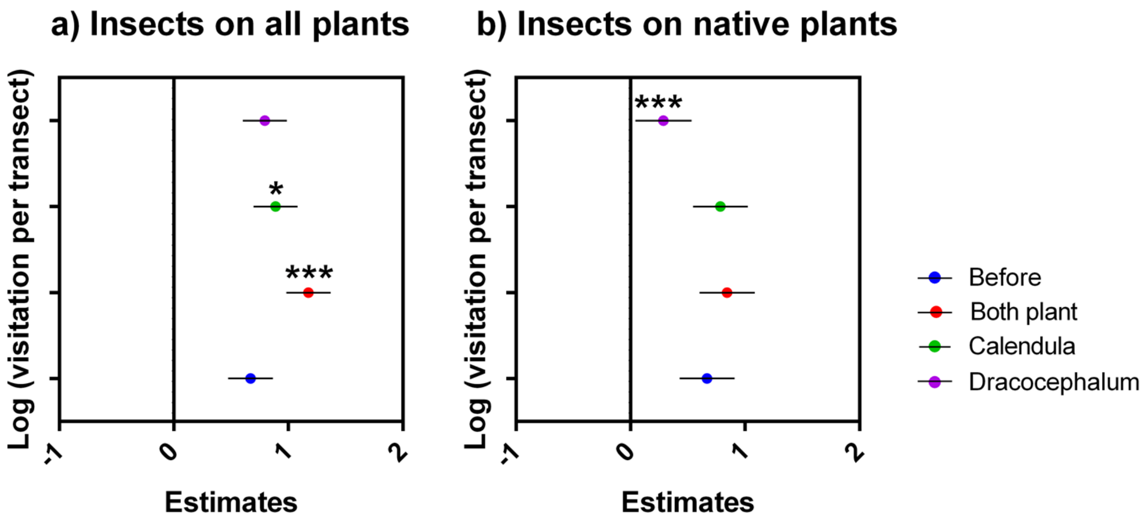

Figure 4.4: Visitation rate of insects after the introduction of new floral resources; a) for all the flowers and b) for native flowers. Asterisks refer to the significance level of the changes: * refers to p value <0.05, *** refers to p value <0.001.

Table: 4.1: Insect visitation response differentiation among the major insect orders: Results are the estimates from GLMM showing the decreased or increased log visitation per transect and the significant results are showing in bold.

Order	***Introduced plants***	***State of introduced plants***			
		Before	*Calendula*	*Dracocephalum*	Both
Hymenoptera	All plants	0.6733	0.8474	0.8051	**1.1768**
	Native plants	0.6714	0.7182	**0.3057**	0.8449
Lepidoptera	All plants	-0.37748	-0.04326	**0.3353**	**-0.0152**
	Native plants	-0.2570	**-0.7278**	-0.3112	**-0.4939**
Coleoptera	All plants	0.4869	0.2112	0.0563	-0.2101
	Native plants	0.426	-0.0584	-0.1788	-0.4527
Diptera	All plants	-2.4824	-1.8851	-2.3571	-1.766
	Native plants	-2.762	-2.345	-2.912	-2.627
Hemipteara	All plants	-1.540	-1.938	-1.277	-1.618
	Native plants	-1.446	-2.134	-1.700	**-1.932**

4.4.4. Impact of floral traits on insect visitation:

4.4.4.1. Flower shape

We used flower shape and flower colour, two most important flower traits in advertising the flower resources to the pollinators to assess the impact of flower traits on pollinator visitation. Considering both native and introduced flowers together, flower shape significantly affected the overall insect visitation rate (F= 23.45 for all plants and F= 37.308 for native flowers. However, different shape had different impact on the visitation rate, for example, ray-disc shaped flowers received more visitors in the presence of *C. officinalis* (F= 13.48, p<0.001) on all flowers, and introduction of *D. moldavica* or both flowers together did not have any impact (Fig. 4.5a). Also, introduction of new species affected the visitation in all open shaped flowers (F= 9.2248), where *C. officinalis* increased the total number of flowers visitors (p=0.0057) but decreased when introduced with *D. moldavica* (p= 0.0187), although later species did not show any significant differences when introduced individually (p=0.379 (fig. 4.5b). Total visitors for all flowers in long-corolla shaped flowers significantly decreased when both of the new flowers were present (F= 4.7144, p= <0.001), but when these flowers were individually introduced, number of visitors did not show any differences (Fig. 4.5c). No changes were observed for short-corolla shaped flowers for total flower visitation on all flowers (Fig. 4.5d). Finally, for zygormorphic flowers, total visitors for all flowers together significantly increased with the introduction of *D. moldavica* (F= 11.302, p=<0.001) but remain unchanged when introduced with *C. officinalis* individually or only in the presence of the later species (Fig. 4.5e).

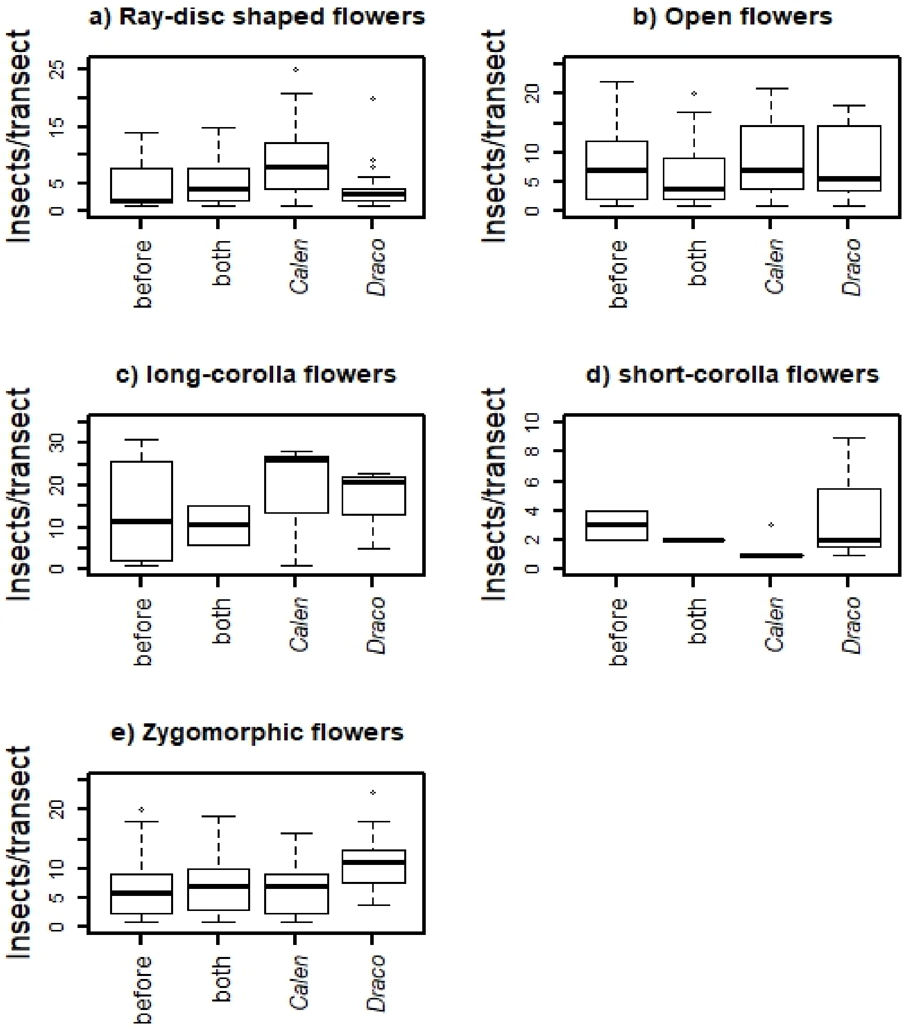

Figure: 4.5: Changes in flowers visitors on both native and introduced flowers together; a) for ray-disc shaped flowers; b) for open-shaped flowers; c) long-corolla shaped flowers; d) short-corolla shaped flowers; and e) zygomorphic flowers.

When considering only the flower visitors on native flowers, we found ray-disc shaped flower lost the flower visitors when mixed resources were introduced (F= 2.4492, p= 0.017) but did not show any impact when *C. officinalis* and *D. moldavica* were introduced individually (Fig. 4.6a). Zygomorphic native flowers showed completely opposite trend comparing the all flowers together as presence of *D. moldavica* significantly reduced the flowers visitors on native flowers (F= 13.5 p=<001), even when introduced in combination with *C. officinalis* (p= <001), but introduction of *C. officinalis* individually did not show any impact on flower visitors of zygomorphic native flowers (Fig. 4.6b). Changes in other native flower types of shape are similar to the all flowers.

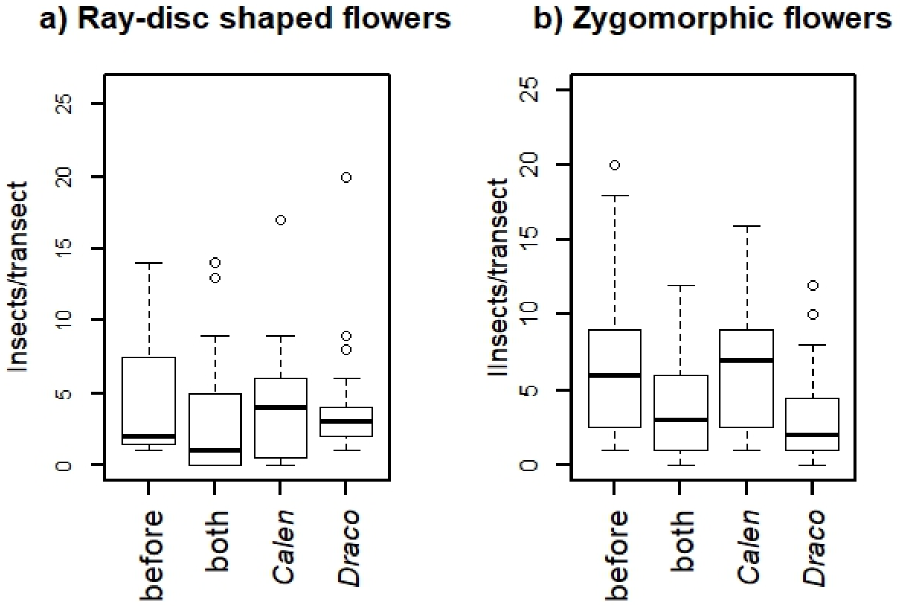

Figure 4.6: Changes in flowers visitors only on native flowers; a) for ray-disc shaped flowers; b) for open-shaped flowers; c) long-corolla shaped flowers; d) short-corolla shaped flowers; and e) zygomorphic flowers.

4.4.4.2. Flower colour

Flower colour played more significant role than flower shape in our experiments and had greater impact on insect visitation of all flowers together (F= 12.869, p= <0.001), and also on native flowers (F= 77.201, p= <0.001). Introduction of *C. officinalis* significantly increased the overall visitation of all white flowers (F= 6.7502p=<0.001), while it had no impact on blue, yellow and pink flowers (Fig 4.7a, 4.7b, 4.7c). However, mixed introduction with *D. moldavica* decreased the number of total flower visitors only on all yellow flowers (p=<0.01), whereas introduction of *D. moldavica* significantly increased the flowers visitors on all blue flowers in the community (p=<0.001) (Fig. 4.7a). Comparison of flower visitors on native flowers showed that introduction of *D. moldavica* significantly decreased the flowers visitors of blue and yellow flowers (p=<0.001, p=<0.0021, for blue and yellow flowers respectively) (Fig 8a, 8b). Number of flower visitors also decreased for blue and yellow flowers when *D. moldavica* and *C. officinalis* were both introduced in combination (p=<0.001, <0.001, for blue and yellow flower respectively) (Fig. 4.8a, 4.8b). Introduction of *C. officinalis* alone only reduced the flower visitors in yellow native flowers (p=<0.01) (Fig 4.7b). No impact of introduction of *C. officinalis* and *D. moldavica* were found on native flowers with pink colour. Basically, results of insect visitation for native flowers are only different than the all flowers for blue and yellow flowers only.

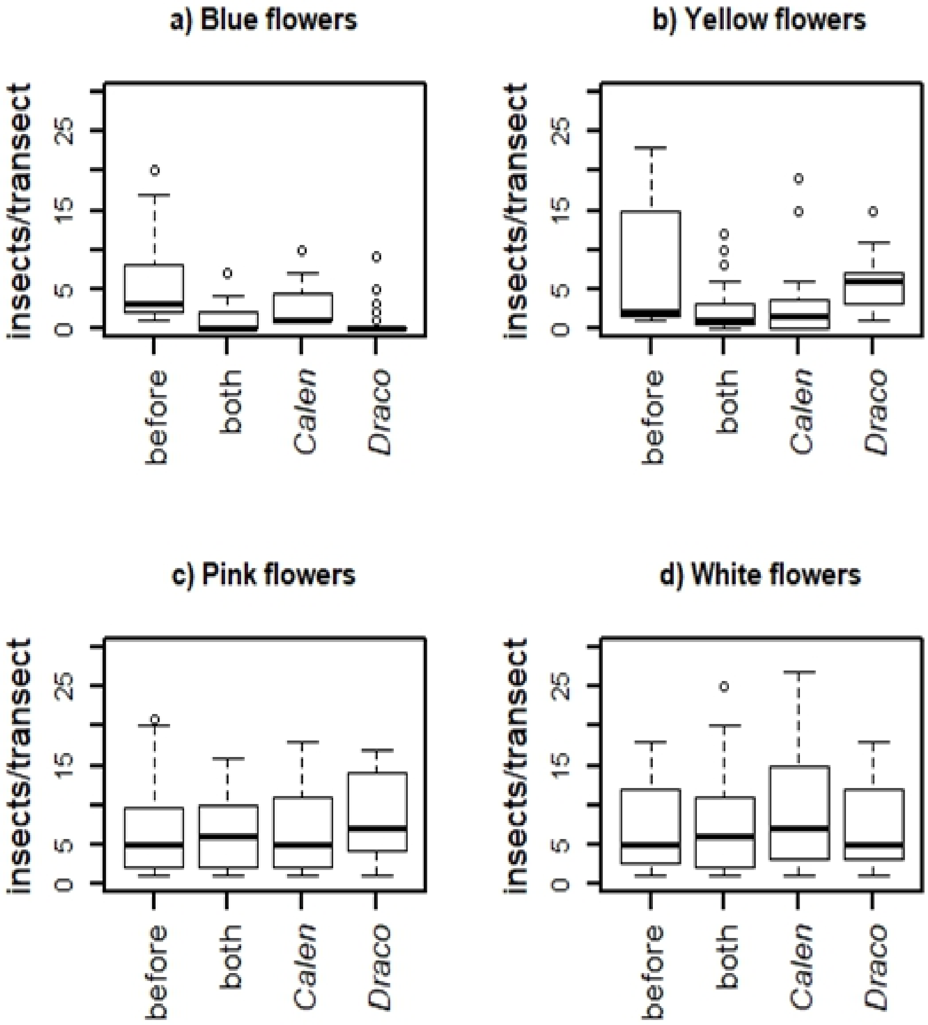

Figure 4.7: Changes in flowers visitors on all flowers; a) for blue flowers; b) for yellow flowers; c) pink flowers and d) white flowers.

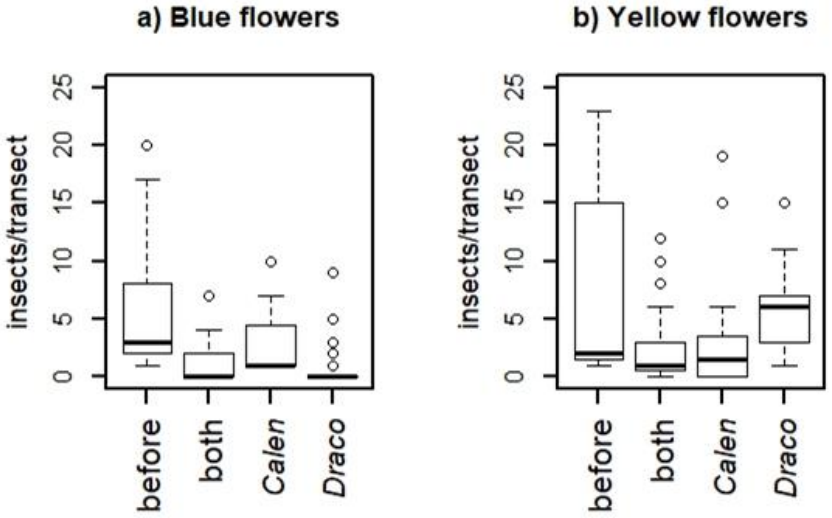

Figure 4.8: Changes in flowers visitors only on native flowers; a) for blue flowers and b) for yellow flowers.

4.5. Discussion

4.5.1. Facilitation or competition

The presence of pollinator dependant introduced plants known to affect the foraging behaviour of native flower visiting insects and the degree of the impact can be community context depended and closely related to plant-pollinator species (Montero-Castaño and Vilá, 2012; Bruckman and Campbell, 2014; Waters et al., 2014). Previous studies showed that pollinator dependant introduced plants may have both detrimental and beneficial impact on the native plant communities, where introduction of new floral resources can reduce the visitation (Morales and Traveset, 2009), or increase the pollinator population by attracting more pollinators to the local community (Bjerknes et al., 2007; Masters and Emery, 2015). In our study, we found that introduction of new floral resources did not attract other pollinators from outside, and the pollinator diversity for all flowers remain unchanged. This led to the other fallout where pollinator diversity for native flowers decreased eventually and indicated that when there is less chance of pollinator inclusion from outside, introducing new floral resources can potentially reduce the pollinator visitation on the native plants (Fig 2). In our case, the new floral resources were exposed at the sites for a limited time and this restrained the possibility of attracting pollinators from outside and directly impact the pollinator visitation on native plants. However, even over the time there was no changes in the overall pollinator diversity and pollinator richness in the communities and indicated that new floral resources were not capable of luring pollinators from outside and that left with one possibility of outcome on pollinator visitation within the new flowers and native flowers: competition. This is a common scenario in a comparative closed community and introduction of new flowering plants in these types of plant community usually led to competition for pollinators (Rathcke, 1983, Essenberg, 2012).

4.5.2. Selection of flower resources by pollinator groups

Competition for pollinators between the flowers in the communities also reflected on the visitation rate of pollinators, where overall pollinator visitation rate was reduced for all the native and non-native flowers with the introduction of new flower resources. But considering only the native flowers we found facilitation due to the introduction of *D. moldavica*, as pollinator visitation for native flowers increased after the introduction of this species. These responses suggest that although the introduction of floral resources ignited competition for pollinators between the flowers, but this can be different in response of different pollinator groups. Presence of *D. moldavica* positively affected the Hymenoptera pollinator visitation of native plants but did not affect the other groups of insects. On the other hand, presence of *C. officinalis* significantly reduced the Lepidoptera pollinator visitation of native plants but did not affect visitation of native plants by the other groups. This outcome reflects that species level response can be more variable than the overall community response and largely depend on the type of introduced flower and their interaction with specific pollinator group.

4.5.3. Relation of flower traits on insect visitation

Results from the flower traits in our study showed that native flowers with similar shape and colour of introduced flowers received reduced pollinator visitation. Similar results were also found in previous studies where the presence of alien plants significantly reduced the pollinator visitation on native plants with similar floral traits (Gibson et al., 2012a, 2012b). In our study, blue flowers and zygomorphic shaped native flowers significantly lost their visitors when *D. moldavica*, whose flowers are blue and zygomorphic, was present in the field. Similarly, in the presence of *C. officinalis*, which has yellow ray-disc inflorescences with ray flowers with short corollas, visitation of both ray-disc and short-corolla flowers and yellow flowers by pollinators was reduced. Floral traits mediated pollination related to the pollinator mediated selection, where trait variation depends on the species' ecological generalization, flower richness and dependence on pollinators (Lázaro and Totland, 2014). We had similar outcome in our study. Along with blue flowers, pink flowers also lost their pollinator visitation in the presence of *D.*

moldavica and this was due to the similar zygomorphic shape of those pink flowers. It has been already confirmed that floral shape is a good indicator in pollination system along with colour traits (Janecek *et al*., 2015), and mostly related to the size of the floral chamber (Ruchisansakun *et al.*, 2016). This result indicates that floral traits can explain the impact of non-native flowers on native flowers.

Our study indicates that both flower and pollinator diversity and traits complexly response towards the introduction of new floral resources into a native community. A trait-based approach can thus provide understanding of the extend a non-native alien plant can integrate into a native community and may allow us to predict the consequences of future species invasions of introductions on plant-flower visitor interactions. Both existing native flowering communities, the diversity of pollinator groups and the abundance and characteristics, all interact in a complex way. It is therefore important to understand the species-specific interactions between the flowers and their visiting pollinators.

Acknowledgements:

We would like to thank Eliška Konečná, Jan Buriánek, Jiří Hadrava, and Dagmar Hucková for help with sampling in the field or processing the samples. This study was supported by the Czech Science Foundation (projects GP14-10035P and GJ17-24795Y awarded to Jan Klecka).

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5. Pollination strategies of a plant in an unstable habitat: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae)

Photo: Asma Akter

Content of this chapter was submitted as:

Biella, P., Akter, A., Pajares, A.J.M., Federici, G., Galimberti, A., Jersáková, J., Labra, M., Mangili, F., Tommasi N. and Mangili, L. 2020. Pollination strategies of a plant in an unstable habitats: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae).

5.1. Pollination strategies of a plant in an unstable habitats: the case of the narrow-endemic toadflax *Linaria tonzigii* (Plantaginaceae)

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Abstract

Plant breeding system might be evolutionarily inherited or might represent an adaptation to a local environment, where self-pollination is expected in unstable habitats. To disentangle the dichotomy between habitat and phylogenetic effects, we focused on the reproductive ecology of a rare toadflax occurring in unstable high-altitude screes, *Linaria tonzigii* (Plantaginaceae). We focused on self-compatibility and spontaneous autogamy, daily nectar production, pollinator behaviour and pollen transfer. We also characterized the position of this species in a multi-marker phylogenetic analysis of the genus and mapped the occurrence of self-compatibility in the tree. The results showed that this species is self-compatible and it is pollinated mostly with spontaneous autogamy, that geitonogamy is frequent and self-pollinated seeds are as viable as cross-pollinated ones. Thus, self-reproduction is not avoided in this species and it could link to the recorded rarity of long-tongue visitors and the sedentary behaviour of small beetles, even if the plant provides a high nectar volume to diurnal pollinators. However, the phylogenetic analysis revealed that *Linaria tonzigii* is closely related to other self-compatible species. Therefore, this toadflax hints that selfing could be linked to the mixed effects of both pollinator rarity in unstable habitats and the evolutionary history of closely-related species.

Keywords: Pollination, Pollination ecology, Genetic diversity, Pollinator limitation, Rare species, Endemism, Outcrossing, ITS, Molecular marker, Phylogeny.

5.2. Introduction

The way plants reproduce can be linked to the habitat where they occur. Both self- and cross-pollination modes have been considered as adaptations to variable environments, although by means of different mechanisms (Charlesworth & Charlesworth, 1987; Igic, Lande, & Kohn, 2008; Yang & Kim, 2016). On one hand, self-pollination assures the establishment of successful phenotypes, on the other hand cross-reproduction increases genetic diversity and phenotypic plasticity (Busch & Delph, 2011). In other words, selfing limits gene flow among individuals and preserves gene combinations that confer high fitness in a local environment and permits seed sets when pollinating agents are unlikely to suffice to plant fitness (Schmitt & Gamble, 1990; Massol & Cheptou, 2011). For instance, remarkable cases of modulating the reproduction according to the environment are the plants that can iteratively switch from outcrossing to selfing (i.e., plants with both *chasmogamic* and *cleistogamic* flowers). In some plants with this type of modulation, it has been observed that either one or the other mode is activated in relation to the level of habitat stressors (Yang & Kim, 2016). Specifically, when the resources are limited and the plant grows in stressful conditions, self-pollination would assure reproduction without the need for the plant of allocating extra resources in pollinator attraction (Schemske, 1978).

In those plants with one obligate mode of reproduction, it is still quite unclear if selfing or crossing suits unstable environments. Specifically, selfing should occur in conditions where transferring two copies of the maternal genes to the seeds overweight the risks of inbreeding, such as when pollinators are scarce and the effective population size is low (Charlesworth & Charlesworth, 1987; Busch & Delph, 2011). In addition, transitions from self-incompatibility to self-compatibility occurred independently multiple times across the evolutionary history of plants (Igic *et al.*, 2008), even in recent geological times (Voillemot *et al.*, 2018), and can be linked to DNA purging such as after bottlenecks (Ness, Wright, & Barrett, 2010). However, in speciose plant genera, there is low consistency between reproductive strategy and habitat type. For example, in the genus *Senecio* both selfing species and crossing ones occur in Australian unstable habitats (Lawrence, 1985). Likewise, in the genus *Linaria*, selfing may occur in some species of stable habitats as grasslands and rock cliffs, but selfing also occurs in species of unstable habitats like sand dunes and crops (Carrió, Güemes, & Herreros, 2013). Thus, a direct link between growing environment and selfing mode is puzzling and a role may be played by the evolutionary history of populations, such as in groups of related *taxa* with monophyletic selfing traits (Miller & Tanksley, 1990). Thus, understanding how the plant reproductive strategies relate to both the growing environment and the species evolutionary dynamics is a key question of the reproductive biology of plants.

A wide variety of environments is colonized by species of *Linaria* (Plantaginaceae). This genus consists of about 150 species with zygomorphic, personate, occluded and spurred flowers (Sutton, 1988), from a relatively recent speciation radiation that lead to cryptic and recently described species with no clear monophily of seed or flower morphology traits (Fernández-Mazuecos *et al.*, 2013a; Fernández-Mazuecos, Blanco-Pastor, & Vargas, 2013b). While some species are distributed over large regions, a number of taxa occur in small areas and many *Linaria* species grow in peculiar environmental conditions, e.g. rocks, cliffs or sand dunes (Segarra-Moragues & Mateu-Andrés, 2007). A remarkable case is *L. tonzigii*, a calcicole toadflax of steep mountain screes, steno-endemic of the Central Alps, that is endangered at the global scale according to the IUCN, and that occurs in an endangered habitat listed in the EU Habitats Directive (European Commission, 2009; Mangili & Rinaldi, 2011). In addition, *L. tonzigii* has a high cultural value, being the logo of the local botanical association (F.A.B. – Flora Alpina Bergamasca, www.floralpinabergasca.net). In spite of the threatened status and value as species, its reproductive biology is unknown and this can jeopardize even the simplest conservation measures (Falk, 1992).

As *Linaria tonzigii* displays conspicuous zygomorphic flowers and a long nectar spur, a major role of long-tongued pollinators might be expected (Muchhala & Thomson, 2009; Vlašánková *et al.*, 2017). In addition, the timing of nectar production can reveal the plant preference for a specific pollinator group and possibly differentiate between diurnal and nocturnal visitors (Zimmerman, 1988; Amorim, Galetto, & Sazima, 2013). Moreover, the type of habitat where *L. tonzigii* occurs (steep alpine screes with sparse vegetation) might attract or even repel flower visitors (Dauber *et al.*, 2010; Barreto *et al.*, 2018). In this study, we have investigated the pollination biology of *Linaria tonzigii* and discussed it in relation to its habitat type and evolutionary history by studying (a) the self-compatibility, the spontaneous autogamy and the cross-pollination of this plant, (b) the pattern of nectar allocation, the pollinator fauna and their foraging behaviour, (c) and the phylogenetic placement of this species in the context of the genus *Linaria*.

5.3. Material and Methods

5.3.1. The plant and study area

Linaria tonzigii Lona is a plant endemic of the central Alps (Italy, Bergamasque Orobie mountains) with an extremely restricted distribution (convex hull of surface about 10km^2) and that lives in unstable habitats characterized by coarse gravel accumulations in a particularly steep mountain screes (Giupponi & Giorgi, 2019). It usually occurs in the alpine and subalpine altitudinal planes and exclusively on calcareous substrates.

Most of the field work took place in two populations located on the Monte Arera (N 45.933, E 9.804 at 2100 m a.s.l.; N 45.929, E 9.807 at 2000 m a.s.l.). An additional population located in Monte Cavallo was included in the pollinators survey (N 46.034, E 9.695 at 2100 m a.s.l.).

5.3.2. Pollination biology

In the field, the plants in pre-flowering stage were covered with bags of fine mash to exclude pollinators during the time of the investigation. For each of 20 plant individuals, the flowers were treated as follows: (1) Cross-pollination by hand, (2) Self-pollination by hand, (3) Autogamy (autonomous self-pollination), (4) Emasculation (Agamospermy). The seed set for each of these treatments were counted and compared to the seed set of plants exposed to open pollination. The seeds in each capsule of these categories were weighted and the total weight was divided by the number of seeds relative to each fruit (Kern ABT 120-5DNM, 0.00001 g). The seed viability in self- and cross-pollination treatments was tested with tetrazolium assay at 1% concentration for 24 hours after cutting the seeds in half for exposing the embryo to the solution.

These data were analysed as follows. The number and mean weight of seeds per capsule were a response variable in linear mixed effect models, pollination treatment was a predictor variable and a plant individual identity was a random intercept. The assumptions of normality were checked visually. The percentage of viable seeds per capsule was tested as a proportion of the total number of seeds (viable + non-viable seeds) in a generalized mixed effect model with binomial error distribution, the pollination treatment as a predictor variable and the plant individual identity as a random intercept.

5.3.3. Nectar production and sugar concentration

Sugar concentration in the nectar was tested by measuring the 24h production of nectar with a field refractometer (Eclipse Handheld Refractometer - Sugar, 0-50% - Bellingham + Stanley, London). For recording the pattern of nectar production during the day, the nectar volume was measured with microcapillaries (0.5 μL microcapillary tube Drummond Microcaps, Sigma-Aldrich) by comparing the volume of 24h with that produced after night and every 6 hours during the day (collections at 10:00 A.M, 15:00 A.M., 21:00 A.M.).

These data were analyses as follows. The volume of nectar production was a response variable in generalized mixed effect models with Gamma error distribution and log-link function, phase of the day was a predictor variable and a plant individual identity was a random intercept.

5.3.4. Pollinator monitoring, pollinator behaviour and pollen analyses

Flower visitors inside the corollas and touching the reproductive structures of the flowers were sampled with mouth aspirator and hand netting animals found inside the corollas. Surveys consisted in walking repeatedly across the screes during day and evening, approximately between 8 AM and 23:00 PM for 20 days distributed in two populations of Monte Arera in 2018 and 2019 and a population of Monte Cavallo in 2019. Additionally, video cameras with IR were placed both during day and night with continuous recording (SuperEye RJ0090-UK, OneThingCam^{TM}).

Pollen from the insects body was removed with fuchsin jelly (i.e. from the head of large bees and from the body of small beetles), melted on glass slide and inspected with an optic microscope (Leitz Laborulux K), and compared to a reference slide of pollen of *L. tonzigii* created by touching freshly opened *L. tonzigii* flowers with fuchsin gel (this reference slide is stored in MIB:ZPL herbarium Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy).

Flowers of 10 randomly-chosen stalks were dusted with UV-reflective dust (RadGlo RC 33, Radiant Color, Europark 1046 B 3530 Houthalen) on their inner surface of the corolla near to and on the anthers. At night, an UV emitting torch (MorPilot, 51 Led) was used for checking the inner corolla of all single flowers on the same stalk as the source UV flowers and on the stalks lying within one meter radius from the source.

5.3.5. DNA extraction and phylogenetic analysis

One to five plants of *L. tonzigii*, *L. vulgaris* and *L. alpina* (that are the three *Linaria* species occurring in the same administrative province where the study was conducted) were sampled at each population for subsequent laboratory analysis (Table S1), samples were vouchered following the protocol specified by the Global Registry of Biodiversity Repositories (http://grbio.org/) and the data standards for BARCODE Records (http://www.boldsystems.org/docs/dwg_data_standards-final.pdf). Young leaves were used for genetic screening (1-3 per plant) and they were stored immediately at -20°C until laboratory analysis. The specimens were stored in MIB:ZPL herbarium at the Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy.

One hundred milligrams of each sample were used for DNA extraction using DNeasy Isolation and Purification kit (Qiagen, Hilden, Germany). Purified DNA concentration and quality of each sample were estimated fluorometrically with a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, USA), by measuring the absorbance (Abs) at 260, 280, and 230 nm. DNA-based characterization of *L. tonzigii* was performed by amplification and sequencing of four DNA barcode markers (i.e., the plastidial rbcL, matK, the intergenic spacer trnH-psbA, and the nuclear ITS) and other three variable genomic regions used in studies of other plant species of a size of distribution similar to *L. tonzigii* (i.e., agt1, rpl32-trnL and trnL-trnF; (Blanco-Pastor & Vargas, 2013; Zecca et al., 2017). For each locus PCR amplification was performed by using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Freiburg, Germany) in a 25 μL reaction according to the manufacturer's instructions. PCR cycles consisted of an initial denaturation step for 7 min at 94 °C, 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at 50 °C for rbcL and trnL-trnF, 53 °C for matK and trnH-psbA, 55 °C for ITS, agt1 and rpl32-trnL), and extension (1 min at 72 °C), and a final extension at 72 °C for 7 min. The primer pairs used were 1F/724R for rbcL (Fay *et al.*, 1998), 390F/1326R for matK (Cuénoud *et al.*, 2002), psbA/trnH for trnH-psbA (Newmaster *et al.*, 2008), p5/u4 for ITS (Cheng *et al.*, 2016), AGT1_F1/AGT1_R1 for agt1 (Kovacova *et al.*, 2014), rpL32-F/trnL for rpl32-trnL (Shaw *et al.*, 2007) and trnT-F_cF/trnT-F_fR for trnL-trnF (Taberlet *et al.*, 1991).

PCR products were bidirectionally sequenced using an ABI 155 3730XL automated sequencer at Macrogen Inc., Korea. Sequence editing and alignment were performed using Bioedit (Hall, 1999) and the sequence data were submitted to the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI, see Table S1 in Supporting Information Appendix S1). The variability between *L. tonzigii* samples was tested for each sequenced locus by calculating the number of haplotypes, the haplotype diversity and the nucleotide diversity computed in DnaSP v5.10.1 software (Librado & Rozas, 2009).

To analyse the phylogeny, we downloaded sequences for nuclear (ITS and agt1) and plastidial (rbcL, matK, trnH-psbA, rpl32-trnL, and trnL-trnF) markers in all the *Linaria* species and subspecies available in Genbank using the *rentrez* package version 1.2.1 (Winter, 2017) in R (R Core Team, 2017). We also obtained sequences for these markers in *Maurandya scandens*, *Chaenorhinum macropodum*, *Antirrhinum majus*, and *A. graniticum* to be used as outgroups. Since the genetic identity of the analysed *L. tonzigii* specimens were very similar to each other (see results), only the sequence of the Mt. Arera haplotype was used in the phylogenetic analysis. Downloaded and newly sequenced markers were aligned using MAFFT version 7.307 (Katoh & Standley, 2013) and the best evolutionary model and partition scheme was estimated using PartitionFinder version 2.1 (Lanfear *et al.*, 2017). Phylogenetic trees were performed using both, Maximum Likelihood (as implemented in RaxML version 8.2.9, Stamatakis, 2014) and Bayesian Inference (as implemented in MrBayes version 3.2.6, Ronquist & Huelsenbeck, 2003) and visualized using the ape 5.1 library (Paradis & Schliep, 2018) in R.

5.4. Results

5.4.1. Pollination biology

The pollination treatment was a significant categorical predictor of the seed set (χ^2= 38.893, df=1, P = < 0.001; Figure 5.1A). Flowers exposed to open pollination produced less seeds than the hand cross- or self- pollinated ones, and open pollination produced a similar number of seeds as spontaneous autogamy (Table 5.1). The seed weight was significantly related to the pollination treatment (χ^2= 47.669, df=1, P = < 0.001; Figure 5.1B), however, there was no significant difference between open pollination and either hand cross- or self- pollination or spontaneous autogamy (Table 5.1). The rate of seed vitality was not significantly predicted by the pollination treatment (χ^2= 0.296, df=1, P = 0.862; Table 5.1, Figure 5.1C).

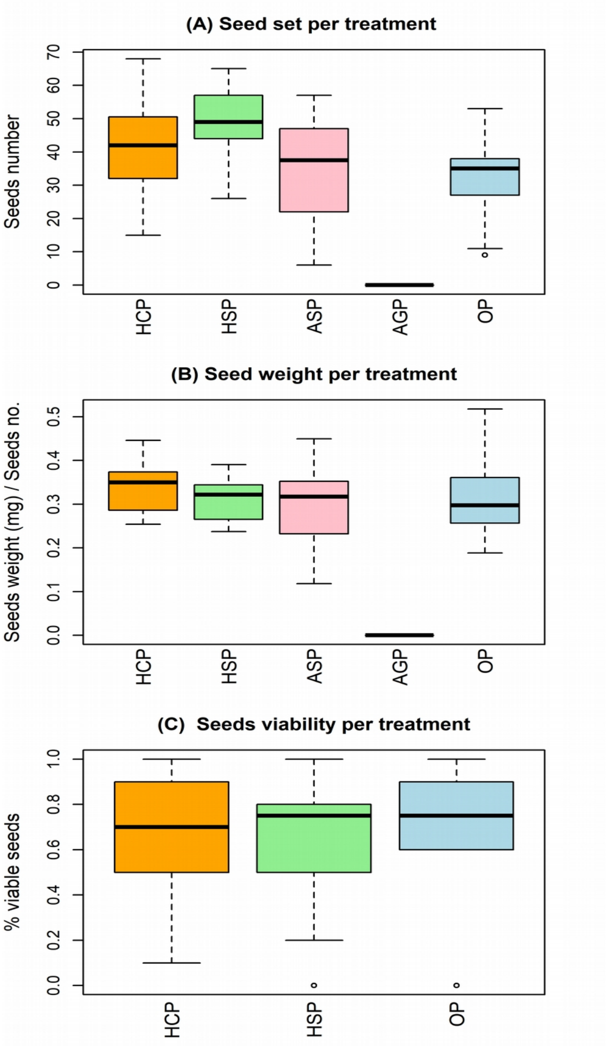

Figure 5.1 - Boxplots of seed set (A), seed weight (B) and seed viability (C) of *L. tonzigii* from the pollination treatments of hand cross-pollination (HCP), hand self-pollination (HSP), autonomous self-pollination (ASP), agamospermy (AGP) and control open pollination(OP). Significances are reported in Table 5.1.

Table 5.1 – Statistical comparison of seed set per capsule, seed weight (mg) and vitality (% of seeds) in open pollinated flowers (control, upper part of the table) and in different pollination treatments relative to open pollinated flowers (second part of the table); the statistical details are in Material and Methods. Treatments were the hand cross-pollination, hand self-pollination, spontaneous self-pollination (autogamy) and emasculation (agamospermy).

Response variable	**Pollination treatment**	**β**	**P value**
Seed set	Open pollination	33.086	**< 0.001**
Weight per seed	Open pollination	0.306	**< 0.001**
Seed vitality	Open pollination	0.78	0.274
Response variable	**Pollination treatment**	**$\beta_i - \beta_{\text{open pollination}}$**	**P value**
Seed set	Autogamy	0.973	0.8
Seed set	Cross-pollination	8.771	**< 0.05**
Seed set	Self-pollination	15.125	**< 0.001**
Seed set	Agamospermy	-33.45	**< 0.001**
Weight per seed	Autogamy	-0.009	0.663
Weight per seed	Cross-pollination	0.021	0.335
Weight per seed	Self-pollination	0.022	0.695
Weight per seed	Agamospermy	-0.303	**< 0.001**
Seed vitality	Cross-pollination	0.028	0.891
Seed vitality	Self-pollination	-0.015	0.944

5.4.2. Daily nectar production and sugar concentration

Sugar concentration in the nectar averaged around 35.4 %. The nectar volume produced changed during the day: the volume produced in 24h was similar to that produced in the morning, but it was lower higher than that produced around noon and evening (Table 5.2).

Table 5.2 – Statistical comparison of nectar volume (µl) in 24h (upper part of the table) and in different time phases of the day relative to the production during 24h (second part of the table); the statistical details are in Material and Methods.

Time phase	**Real mean (SD)**	**Estimated β**	**P value**
24h	1.04 (0.39)	0.708	**< 0.001**
Time phase	**Real mean (SD)**	**Estimated $\beta_i - \beta_{24h}$**	**P value**
Before 10:00	1.18 (1.03)	-0.078	0.279
10:00 - 15:00	0.75 (0.48)	-0.175	**< 0.01**
15:00 - 21:00	0.23 (0.21)	-0.889	**< 0.001**

5.4.3. Pollinator monitoring and behaviour

In total, 141 hours were video recorded and about 240 hours were of active sampling. In spite of the high amount of time spent in sampling (directly or with videocameras), few insects were found.

The direct sampling of pollinators yielded insects of different functional groups and taxonomical orders that were either inside the flowers or foraging on flower resources, and scattered in different flowers along the surveyed screes: 32 small thrips (Thysanoptera), 41 small beetles of Coleoptera: Staphylinidae (Staphylinidae: *Eusphalerum* sp.), 1 large moth of Lepidoptera: Noctuidae, 3 large bees of Hymenoptera: Apidae that were 2 queens of the bumblebee *Bombus monticola* and a female bee of *Anthophora quadrimaculata*; all these are stored in PB collection. Video recordings yielded additional flower visitors and their behaviour, namely: a large moth of Lepidoptera: Noctuidae and a butterfly of *Erebia sp.* (Lepidoptera: Nymphalidae) collecting nectar, a queen of the bumblebee *Bombus monticola* collecting nectar and a worker of a *Bombus* sp. collecting pollen. In addition an *Andrena rogenhoferi* bee was recorded and landed on the corolla of a *L. tonzigii* flowers carrying a lot of light-yellow pollen, which is a colour very similar to that of *L. tonzigii*, but it was not seen entering the flowers. Videos showed very clearly that while foraging, the bumblebees visited in fast succession a relatively high number of *L. tonzigii* flowers (19, 7, 6 flowers), while the moth and the butterfly visited only a few flowers (1 and 2 flowers respectively).

The captured specimens were examined in the laboratory and pollen of very similar morphology to *L. tonzigii* was found on the body of both small beetles and of the large bees, and large-bee carried a higher quantity of pollen on their body (i.e. their head) than the small beetles. The 99% of the pollen taken with the fuchsin jelly was identified as belonging to *L. tonzigii*. Pollen was not found on the one moth collected. UV-reflective dust indicated that most of the investigated flowers did not receive dust (47.92 %), while flowers of the dust-treated stalks received more dust than those of surrounding stalks (29.86 % and 22.22%, respectively).

5.4.4. Phylogenetic analysis and genetic identity

The genetic diversity between the analysed specimens was very low, with a minimum of one haplotype at matK and rbcL and a maximum of three poorly differentiated haplotypes at agt1, trnH-psbA and rpl32-trnL (Supporting Information, see Table S5.2 in Appendix S5.1).

A total of 140 species and subspecies of the genus *Linaria* and *Nuttallanthus* (*Linaria* s.l.) were included in the phylogenetic analysis. *Linaria tonzigii* clustered within the Clade E (sensu Fernández-Mazuecos *et al.*, 2013b), together with species of the Section Speciosae: *L. purpurea*, *L. repens*, and *L. capraria* (Figure 5.2). This position in the tree is consistently observed when nuclear and plastidial markers are used separately (Appendix S5.2 in Supplementary Information).

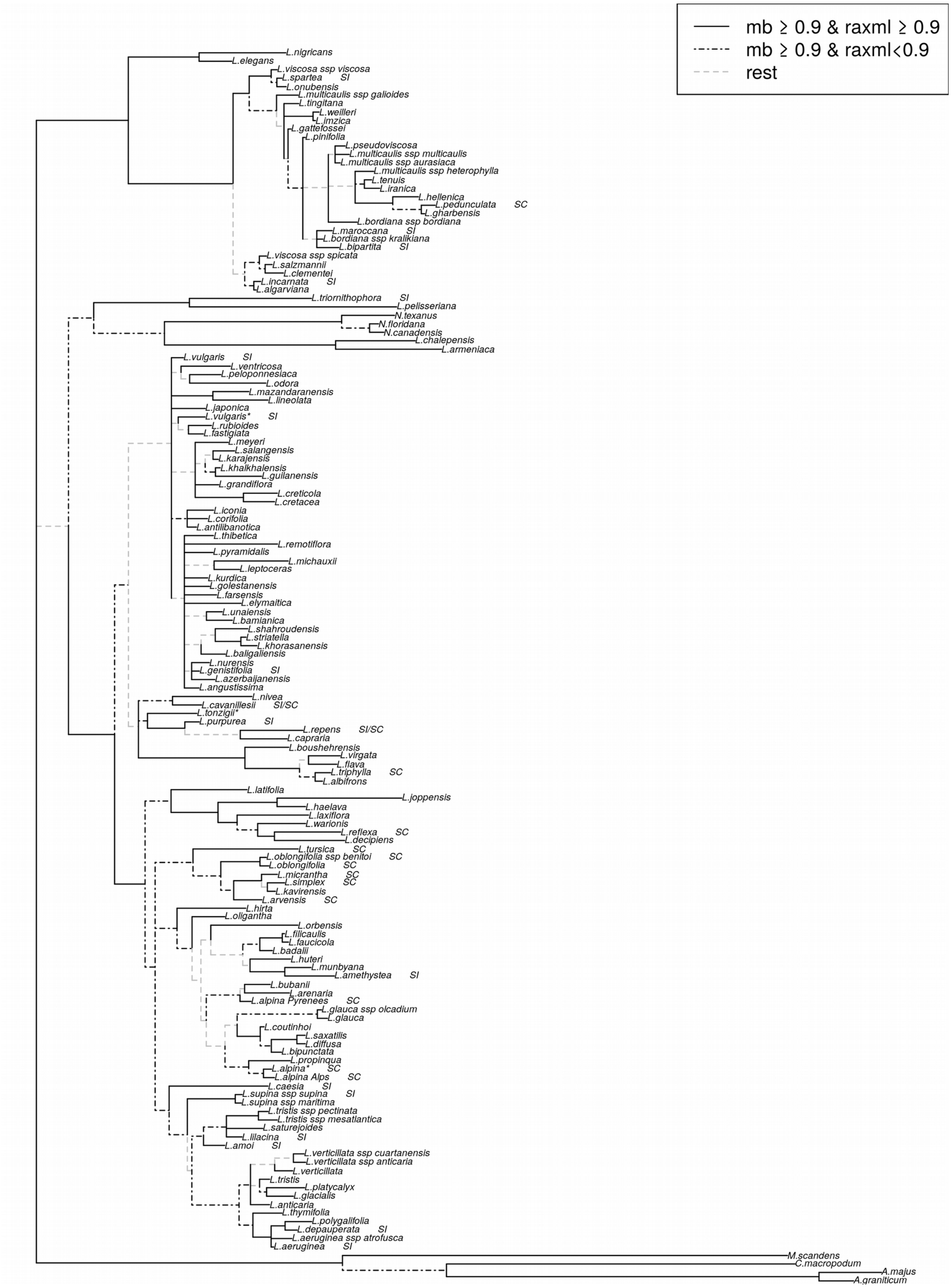

Figure 5.2 – Phylogenetic analysis of concatenated genetic markers (ITS, agt1, rbcL, matK, trnH-psbA, rpl32-trnL, trnL-trnF) of the genus *Linaria* s.l.. The asterisks indicate species exclusive of this study. Species reproductive mode as in Carrió *et al.*, 2013 and are indicated as SI (self-incompatible), SC (self-compatible) and SI/SC (both self-incompatible and self-compatible depending on the original study).

5.5. Discussion

In this study, we investigated several aspects of the pollination ecology and genetic identity of a species of the Mediterranean screes, that is a threatened habitat according to the IUCN (European Commission, 2009). The target species chosen for this study is *Linaria tonzigii*, which is a rare and steno-endemic plant that is threatened of extinction (EN in the IUCN Red List, Mangili & Rinaldi, 2011). Based on the literature records of other *Linaria* species and on the flower morphology (i.e. zygomorphic flowers with a nectar spur), we previously hypothesized that the pollinating fauna would be composed uniquely of long-tongued insects. In detail, other *Linaria* species are visited by a fauna of large bees, dipteran Bombyliidae and both diurnal and nocturnal Lepidopterans (Sutton, 1988; Stout, Allen, & Goulson, 2000; Fernández-Mazuecos *et al.*, 2013a), which are insects with long mouthparts. In addition, since the flowers of *L. tonzigii* have a very long spur, we hypothesised that if nectar is produced in the evening, a preference for nocturnal pollination could be expected (Amorim *et al.*, 2013).

This field study of *L. tonzigii* only partly confirmed these expectations. Our data showed a prevalence of diurnal pollinators, which also matched the highest production of nectar volume during morning hours. This result hints that the studied plant allocates resources to diurnal pollinators (Zimmerman, 1988). This is in congruence with a general pattern that pollinators usually visit flowers when the nectar resources are the highest (Real & Rathcke, 1991; Biella *et al.*, 2019). In addition, our survey indicated that not all flower visitors were long-tongued insects, and not all the pollinators were equals in terms of abundance and visitation behaviour. Large bees with long mouthparts were rarely found in the flowers, although they visited several flowers per foraging bout, while small beetles with short mouthparts were more abundant visitors but apparently very sedentary. Moreover, the seed number for each pollination treatment, and the pollen analysis, video recordings and pollen tracking indicated that the majority of the flow is within the same flower (spontaneous autogamy) or between flowers of the same stalk (geitonogamous pollination). Thus, these evidences suggest a prevalence of self-pollination in *Linaria tonzigii.* The reason why large and mobile pollinators rarely visited *L. tonzigii* might rely on the sparse distribution of the few plant species occurring in this habitat (Giupponi & Giorgi, 2019), and that highly-mobile winged pollinators would prefer foraging in patches with higher plant abundance (Redhead et al., 2016). This agrees with the evidence that the plant is also pollen limited, a clear indication of pollinator failure (Kalisz & Vogler, 2003), as indicated from the higher number of seeds produced by hand pollination then by other breeding types in the studied species.

Self- and cross- breeding experiments indicated that the species is self-compatible and that spontaneous autogamy produced as many seeds as open pollinated ones. Moreover, the self-pollinated seeds were as viable as cross-pollinated ones. All these results strongly indicate no avoidance of self-pollination and that that fruiting is more important than the source of the fecundating pollen (whether the pollen is from the same flower/individual or from different ones). Since the level of genetic diversity between populations can determine if selfing reproduction is or is not a sustainable strategy (Charlesworth & Charlesworth, 1987; Busch & Delph, 2011), we have checked for genetic differences between populations of *L. tonzigii* in a preliminary survey. From the genetic markers and the sample size used in this study, we have found a very low genetic diversity among individuals. Although preliminary, this result is coherent with a scenario of severe diversity reduction during the history of the species, likewise what reported in previous studies (Szczecińska *et al.*, 2016; Blambert *et al.*, 2016; Zecca *et al.*, 2017) and agrees with the theory that self-pollination is a viable strategy if genetic diversity is low (Yang & Kim, 2016).

Previous studies indicated that the majority of *Linaria* species are self-incompatible and they also doubted the evolutionary relationship between the self-compatible *Linaria* species (Bruun, 1937; Valdés, 1971). However, the multi-marker phylogenetic analysis of the *Linaria* genus located *L. tonzigii* in a subclade with other self-compatible species (i.e., *Linaria cavanillesii*, *Linaria repens*, *Linaria triphylla*), although another one is self-incompatible (i.e., *Linaria purpurea*) (data from Valdés, 1971; Docherty, 1982; Carrió *et al.*, 2013). In addition, it should be noted that also the subclade of *Linaria simplex* contains only self-compatible species (i.e. *L. tursica*, *L. oblongifolia*, *L. micrantha*, *L. simplex*, and *L. arvensis*). Therefore, mapping the position of the known self-compatible species (data from Carrió *et al.*, 2013) on the phylogenetic tree showed that (a) self-compatible species are scattered in the phylogenetic tree, (b) there are at least two subclades of recently diverged taxa that are self-compatible, but also that (c) for the majority of *Linaria* species no information on self-compatibility is available, and a conclusion on the evolutionary patterns of self-compatibility at the level of the entire genus might lack of full support. Questions as if self-compatibility is an inherited trait in some closely related *taxa* of *Linaria* or if it is an adaptation to the local habitat conditions, and what genetic structure self-compatibility could cause, are still open (Segarra-Moragues & Mateu-Andrés, 2007). Yet, in this case study of *Linaria tonzigii*, both the low pollinator activity and the possible plesiomorphy of self-compatibility are met. The advantage of both spontaneous and pollinator-induced self-pollination is that they can assure reproduction when the pollen flow is low, such as in unstable environments with unreliability of pollinator activity like the mountain screes inhabited by *L. tonzigii*.

Acknowledgements

The author thank the botanist association F.A.B. (Flora Alpina Bergamasca) for technical help and the administration of the protected area Parco delle Orobie Bergamasche for sampling permits (1428 and 1283MV/RC). The authors thank Adriana Nurtaza, Davide Magnani and Andrea Tapparo for their contribution in the laboratory tasks. AJMP was supported by a post-doctoral fellowship funded by the Portuguese Foundation for Science and Technology (SFRH/BPD/111015/2015).

Supplementary Material

Appendix S5.1 – Additional details on *Linaria* samples that were used for the genetic analyses and additional results on the genetic identity and variation in *Linaria tonzii*.

Appendix S5.2 – Phylogenetic analyses of concatenated sequences for nuclear (ITS and agt1) and plastidial (rbcL, matK, trnH-psbA, rpl32-trnL, and trnL-trnF) genetic markers of the genus *Linaria* s.l., inferred separately with RaxML and MrBayes.

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6. Changing pollinator communities along a disturbance gradient in the Sundarbans mangrove forest: a case study on *Acanthus ilicifolius* and *Avicennia officinalis*

Photo: Asma Akter

Content of this chapter was published as:

Akter, A., Biella, P., Batáry, P., Klecka, J. 2020. Changing pollinator communities along a disturbance gradient in the Sundarbans mangrove forest: a case study on *Acanthus ilicifolius* and *Avicennia officinalis*. *Global Biology and Conservation* 24:e01282.

6.1. Changing pollinator communities along a disturbance gradient in the Sundarbans mangrove forest: a case study on *Acanthus ilicifolius* and *Avicennia officinalis*

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Abstract

The Sundarbans, the largest mangrove forest in the world and a UNESCO world heritage site has been facing an increasing pressure of habitat destruction. Yet, no study has been conducted to test how human disturbances are affecting plant-pollinator interactions in this unique ecosystem. Hence, we aimed to provide the first insight of the impact of habitat loss and human disturbances on the pollinator communities in the Sundarbans. We selected 12 sites in the North-Western region of the Sundarbans, along a gradient of decreasing habitat loss and human activities from forest fragments near human settlements to continuous pristine forest, where we studied insect pollinators of two mangrove plant species, *Acanthus ilicifolius* and *Avicennia officinalis*. Our results show that different pollinator groups responded to the disturbance gradient differently. For example, the abundance of *Apis dorsata, one of the three local species of honey bees,* increased gradually from the village area towards the deep pristine forest. On the other hand, *A. cerana* and *A. florea* were found in the village sites and completely absent in the deep forest. Although pollinator community composition changed along the disturbance gradient, their efficacy in pollination did not seem to be significantly affected. However, lower plant diversity and low understory plant cover in the forest patches nearby the village indicated that human disturbances not only affected pollinator community composition but also played a major negative role in the regeneration of the forest. Our study provides first insights into plant-pollinator interactions in the Sundarbans and demonstrates that more research is needed to inform conservation of this unique habitat.

Key words: Conservation, mangroves, the Sundarbans, pollination, *Acanthus ilicifolius*, *Avicennia officinalis*

6.2. Introduction

Human destruction of natural habitats and alteration of landscapes are considered as major drivers of the world-wide forest loss and fragmentation (Aizen and Feinsinger, 1994; Fischer and David, 2007). This increasing disturbance and habitat loss not only change the distribution and abundance of different organisms but also affect species interactions, which may be amplified into long-term effects on the forest ecosystem (Fortuna and Bascompte, 2006). Plant-pollinator interactions play a crucial role in ecosystem function as around 90% of angiosperm species rely on pollinators at least to some extent for their sexual reproduction (Ollerton *et al.* 2011, Potts *et al*. 2016). This makes pollinators an essential component to maintain biodiversity and ecosystem integrity (Kearns *et al*. 1998; Potts *et al*. 2003).

In most forest ecosystems, the fringe of forest is generally under pressure of high human activities, e.g. illegal collection of wood for fuel, house building materials, and agricultural tools along with regular grazing of domestic animals. These frequent disturbances affect the forest structure and interrupt the ability of the understory species to regenerate (Smiet, 1992). Alterations of natural habitats can affect plant-pollinator interactions in different ways. On the one hand, pollinators can be affected by the lack of suitable habitat and resources, which may determine their performance (Ward and Johnson, 2005). From the pollinators’ perspective, destruction of habitats or reduction in the availability of food (nectar and pollen) and nesting sites are expected to reduce species richness, abundance and homogenize species composition (Sameiima *et al*., 2004, Steffan-Dewenter & Westphal, 2008, Biella *et al*. 2019, in press). Furthermore, increased flight distance among habitat fragments can cause less effective pollen transfer (Aizen and Harder 2007). Pollinator abundance can also decrease due to lower attractiveness of isolated fragments, small population size, or reduced density of flowering plants (Cheptou and Avendano, 2006). Consequently, plants may suffer reduced seed set (Ward and Johnson, 2005). Overall, the stability of plant-pollinator interactions tends to be altered when native habitat is changed or removed. Even small disturbances may cause disruption of plant–pollinator interactions within the remaining habitat patches in fragmented landscapes (Keitt, 2009). Plant’s evolutionary dependence on pollinator communities for the pollination and reproduction increases the susceptibility to habitat loss and human disturbances and in return, pollinator diversity, abundance and foraging behaviour might also get affected as a consequence (Quesada *et al.*, 2011). However, different pollinator communities may react to the forest loss and human disturbances at different scales and depend on the flower composition and environmental conditions both at the local and landscape scales (Hamer *et al*., 2000; Breitbach *et al.*, 2012).

Unlike most terrestrial ecosystems, mangroves are naturally fragmented, architecturally simple and often have limited species diversity, but with a number of uniquely adapted species (Vannucci, 2001, Alongi, 2002). Heavily populated coastal zones have accelerated the widespread clearing of mangroves for coastal development, aquaculture, or other resource uses (Polidoro *et al*., 2010) and have led to further forest destruction, fragmentation and habitat loss. Globally, around 20%- 35% of mangroves have been lost since the 1980s and approximately 1% of the mangrove areas are disappearing per year (Valiela *et al*. 2001; FAO, 2003; FAO, 2007). An extreme example of forest loss and habitat destruction is the Sundarbans mangrove forest, situated in the south-western Bangladesh, which is the world's largest continuous mangrove forest (Sarkar *et al*., 2016). Nearly 50% of the forest has been lost since the 1950s because of inadequate habitat protection, and large-scale habitat alteration (Feller *et al.*, 2010). Historical human pressures have severely degraded the Sundarbans ecosystem by depleting forest tree stock (Ellison *et al*., 2000) and causing habitat loss. While natural disturbances determine both regional and global forest dynamics and diversity (Masaki *et al.*, 1999; Sheil, 1999), anthropogenic activities may locally regulate the regeneration dynamics of forests and influencing the structure and floristic composition of the lowland forest (Horn and Hickey, 1991). A recent study by Sarkar *et al.,* (2019) also stated an increasing trend of compositional homogeneity in the plant diversity and radical shifts in species composition in the Sundarbans. Introduction of non-mangrove plants in the forest, either intentionally or accidentally, increasing population of invasive plant species, decreasing population of certain mangrove plant species (Sarkar *et al.,* 2019) and keeping honeybees (mainly *Apis cerana*) for apiculture along the forest edge for honey production are also sources of concern and their impact on this forest must be assessed to maintain local biodiversity. Despite the numerous ecosystem services provided by this mangrove forests (Walters *et al.*, 2008), very little is known about pollinator communities of this forest (Pandit and Choudhury, 2001; Hermansen, *et al*., 2014), and there have been no studies evaluating the impact of human disturbances and habitat loss on the pollinator communities, their interactions with local plants and plant reproduction.

While studies on the pollination ecology and biology of mangrove plants around the world are frequent (Aluri, 2019), studies on the pollinator communities and pollination efficacy in the Sundarbans are scarce. Only a few studies focused on the pollinator communities of the Indian part of the Sundarbans (Mitra *et al*., 2015; Chakraborti *et al.*, 2019). Generally, *Apis dorsata* is considered to be the most common pollinating insects in the Sundarbans (Gani, 2001; Mitra *et al*., 2015; Chakraborti *et al.*, 2019), especially in the major flowering season (from March to June), while other *Apis* species and solitary bees are also common in this forest and in other mangrove forests in the Indian subcontinent. Here, we targeted two plants species, *Acanthus ilicifolius* and *Avicennia officinalis*, to compare the pollinator communities along the disturbance gradient in the Sundarbans and test the impact of the disturbances on the plant-pollinator communities and pollination. Reproduction bilogy of these two species is well known (Aluri *et al*., 1994; Aluri *et al*., 2012; Aluri *et al*., 2017), although possible effects of anthropogenic activities and disturbances on their reproduction have not yet been studied. However, such studies are essential to predict the sustainability of a forest ecosystem and primary requirement to take any conservation decision. Therefore, we addressed four questions: i) Does the plant diversity and abundance of floral resources decrease with the increasing human disturbances? ii) Does the abundance of flower visitors decrease and the composition of their community change along the gradient of human impact? iii) Do differences in pollinator visitation along the gradient affect the level of pollination and seed production of selected plants, with seed set reduced in disturbed sites? iv) And, what kind of conservation measures should be taken to protect both plant and pollinator communities?

6.3. Methods

6.3.1. Study area

This study took place in the North Eastern part of Sundarban Mangrove Forest in Bangladesh, located nearby Munshigang, Shyamnagar, Satkhira (N 22°16’78, E 89°11’58). The Sundarbans is protected as a UNESCO world heritage site. There are three protected sites in this forest in the Bangladesh sites of the Sundarbans: East Wildlife Sanctuary (ES, 312 km2), South Wildlife Sanctuary (SS, 370 km2), and West Wildlife Sanctuary (WS, 715 km2) (Gopal and Chauhan, 2006).

However, the part of the forest we studied is outside of these protected areas and highly disturbed by human activities and facing a high rate of biodiversity loss. The forest is distinctly isolated by the river ‘Pankhali’ from the adjacent human settlements, though fragmented forest patches are still found inside the village areas.

Based on the distance of isolated forest patches from the forest, canopy and ground cover and intensity of human disturbances, we selected twelve sites (Fig. 6.1C, site characteristics: supplementary table 6.1). Therefore, our study sites expanded from the most fragmented and isolated forest patches in the village to the pristine forest sections and from the most to the least affected by anthropogenic activities. The maximum distance from the most disturbed site to the least disturbed site was ca. 10 km. Forest patches inside the village were adjacent to the high-density human settlement and completely exposed to their daily life activities. The grounds of these sites had no or very little understory vegetation and distance from the continuous forest was 1-2.5 km. Forest patches which were close to the river, were also exposed to high human activities and had little understory vegetation as well. On the other hand, sites on the opposite side of the river in the continuous forest with moderate human impact had around 50 % ground covered by understory plants. Finally, sites which were deep in the forest and the farthest from the village, were least or not disturbed at all, high in plant density and almost fully covered by the herbaceous and shrub plants (Data: https://doi.org/10.6084/m9.figshare.11877615). This part of the forest is only occasionally visited by the forest department for regular security checking and by the honey-collectors from wild *Apis dorsata* colonies, thus has the lowest human disturbance.

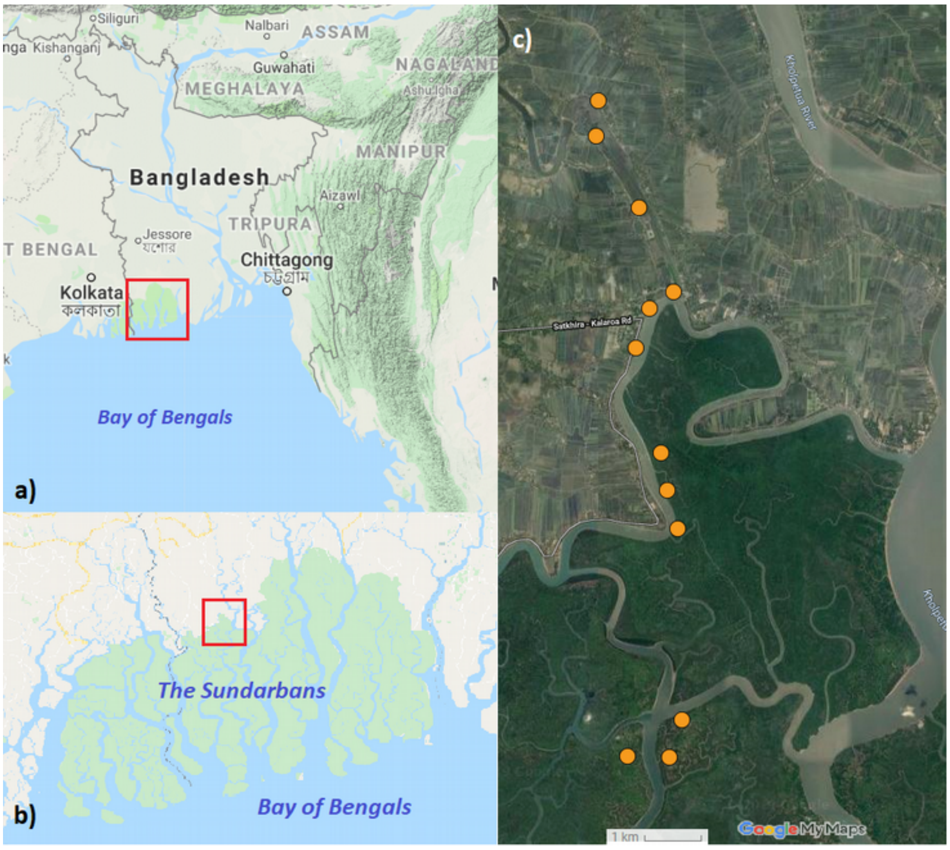

Figure 6.1: Location of the study area and the position of individual sites. Location of the Sundarbans mangrove forest in Bangladesh (A.) and location of our study area at the inland edge of the mangrove forest (B.). Location of individual sampling sites in the village and forest area (C.). Map data: Google, Imagery: TerraMetrics.

6.3.2. Plant diversity assessment

We surveyed the vegetation of all sites during our fieldwork. We identified all the species in each site and estimated total canopy cover, cover of the understory layer, and percentage cover by individual plant species. Only two plant species were flowering at all sites during the time of our study. Hence, these two species were chosen for a detailed study (Fig. 6.2).

***6.3.2.1. Acanthus ilicifollius* L.** (Lamiales: Acanthaceae) is an evergreen, non-viviparous, semi-woody spiny shrub, which grows up to two meters. It has a wide range of distribution; it occurs from Western India through the North-Eastern China to Southern Australia (Tomlinson, 1986). It is commonly found along the edges of estuaries, canals and river-banks. In our study site, they were found also found in the interior of the forest as only those forest patches were chosen which were flooded by the tidal flow and go underwater (Photos in the supplementary documents, S6.1). This species is very important for the accumulation of soil sediments and stabilization of the ground in brackish water areas. Inflorescence is spike, terminal, flower is large, showy, light blue to purple coloured, contains one large petal, four stamens, is bisexual and semi-tubular in shape (Aluri *et al*, 2017). The species produces nectar and pollen, has a mixed breeding system where out-crossing plays the most important role and it was reported to be pollinated by large bees (Aluri, 1990). Flowering time in the Sundarbans spans from April to June but can be different for other parts of its distribution zone (Ramasubramanian *et al*., 2003, Upadhyay and Mishra, 2010). Fruit is a capsule containing up to four seeds that disperse by effective anemochory especially during the dry season (Aluri *et al*., 2017).

Figure 6.2: The target plant species of our study. *Acanthus ilicifolius* plant and flower, being visited by *Apis dorsata* (left); *Avicennia officinalis* plant and flower (right).

6.3.2.2. Avicennia officinalis **L.** (Lamiales: Acanthaceae) is a common viviparous mangrove tree, which has a wide range of distribution from Southern India through Indo-Malaya, to New Guinea and the Eastern Australia (Tomlinson, 1986, Duke 1991). *A. officinalis* can tolerate a wide range of salinity and occurs dominantly in soils with high salinity, and frequent and long duration of tidal inundation, although their abundance is higher towards the landward sites in the Sundarbans (Joshi and Ghose, 2003). It is a medium-sized tree, typically twenty meters tall, but can be up to thirty meters tall and contains pneumatophores. Inflorescence is spike, flower is small, yet the largest among the *Avicennia* species, orange-yellow coloured with four petals, four stamens, bisexual, open (Aluri, *et al*., 2012). It produces both nectar and pollen, is self-compatible although it is protandric, has a long flowering period suggesting its adaptation for cross-pollination, and is mostly pollinated by bees and flies (Aluri, *et al.*, 2012). Flowering time is from April to August, depending on the location. Flowering is triggered by the rain and may vary even over a short distance (Opler *et al*, 1976; Reddy *et al.*, 1995). Like other *Avicennia spp*., *A. officinalis* contains 4 ovules but in general only one ovule develops into mature seed, which is non-dormant and germinates while the fruit is still attached to the tree, thus are crypto-viviparous in character (Tomlinson, 1986, Aluri, *et al.*, 2012).

6.3.3. Insect sampling

We observed and sampled flower-visiting insects form the two locally most abundant plant species, *Acanthus ilicifolius* and *Avicennia officinalis*. We surveyed them in May-June 2018, during the peak flowering time. We conducted our observations and sampled floral visitors for ten days. In each site, we sampled for 20-30 minutes in each session, replicated six times, which resulted into 120-160 minutes of observation for each species per site. During the high tide, a vast area of the forest is flooded, which restricted our fieldwork to 4-6 hours per day (Data: https://doi.org/10.6084/m9.figshare.11877615). For our observations we set up three collection windows with a size of 1 m^2 for each plant species and we always sampled in the same windows. For both species, we counted the number of inflorescences and number of flowers per inflorescence within the window. For the *Avicennia officinalis*, we also measured the total flower cover in each window. We had three windows in every site for both species. Insects were observed and collected by netting, from 7 a.m. to 5 p.m., in sunny and warm condition, no observation was made under rain, storm or high winds. We determined the flower visitors when they touched the reproductive parts of the flower or entered the flower with a tubular shape. The three honey bee species and some other conspicuous flower visitors were released after counting, as they were easily recognizable.

The rest of the captured insect were stored in the freezer after the collection and later mounted and stored dry in boxes for identification. Insects were identified by the authors and experts by using their expertise and various identification keys and taxonomical revisions of individual genera (Brunetti, 1923, Curran, 1947; Kumar & Sharma, 2015; Goulet & Huber, 1993; Pesenko & Pauly, 2005, Schmid-Egger, 2011). Bees, wasps and hoverflies were identified at the species or genus level, while other insects were identified up to family and only in few cases up to superfamily. We used the concept of ‘morphospecies’ denoted as sp.1, sp.2 etc. when species-level identification was not possible.

6.3.4. Pistil collection and pollen tubes analysis

In order to measure the pollination efficacy by the pollinators in different sites, we counted the number of pollen tubes in pistils as a proxy to pollen deposition. Although counting the number of pollen tubes does not differentiate between self- and cross-pollination, the number of pollen tubes growing in pistils is linked to the deposition of viable conspecific pollen and to seed production, hence provides information about pollination efficacy (Alonso *et al.*, 2012, Biellla *et al*., 2019). We collected pistils at the end of our fieldwork to determine the impact of pollinator efficacy for each plant. Pistils were collected from 30 flowers per site excluding the plants where pollinators were observed and only form those flowers where female phase was over and stigmas were no longer receptive for pollen. Collected stigmas were stored in Formalin-Acetic-Acid solution (FAA) at room temperature. To assess the pollen tube growth, pistils were softened and stained by following the technique of Martin (1959). Pistils of both species were softened in 1M NAOH for 24 hours. After softening, they were stained with 0.1% Aniline blue in 0.1M K_2HPO_4 for 15 hours in the dark. After completing staining, pistils were washed and mounted in 50% Glycerine drop on glass slides, flattened evenly and covered with coverslips for observing under the fluorescence microscope and counted (Fig. 6.3). All the processes were done at room temperature and after the observations, samples were stored at 4°C for future reference.

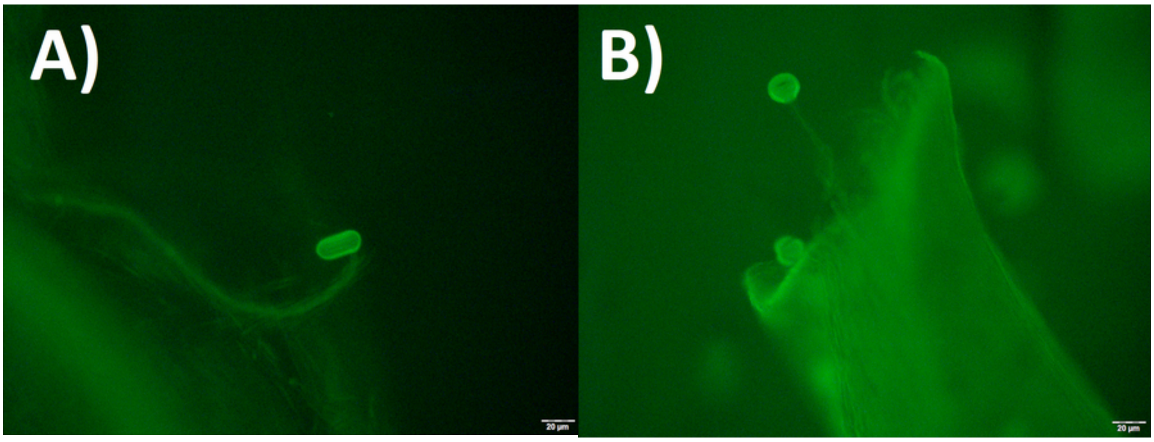

Figure 6.3: Pollen grains and tubes in the pistils: Images from a fluorescence microscope of *A. ilicifolius* (A.) and *A. officinalis* (B.).

6.3.5. Fruit and seed collection

Fruits were only collected from *Acanthus ilicifolius* from each site. *A. ilicifolius* starts flowering in March and its fruits were available at the time of our fieldwork. Fruit production of *A. ilicifolius* was assessed as the number of fruits collected per infructescence (3-12 infructescences per site) and seed production was estimated by counting the number of seeds per fruit in each infructescence. The number of seeds per fruit ranges from zero up to four in a fully seeded pod. Fruiting of *Avicennia officinalis* in that area occurs during July-August, which is at the peak of the rainy season when the forest is inaccessible and fruit collection was thus not feasible for every site. Local collectors were unable to reach sites inside deep forest due to the high-water level and unavailability of transport.

6.3.6. Statistical analysis

Shannon's Diversity Index (Shannon, 1948) was used to compare plant and pollinator species diversity between the sites. We analysed the impact of the position of the sites along the gradient from highly disturbed to the least disturbed parts of the forest (expressed as the distance of the sites from the village in km) on plant abundance and diversity using generalized linear models (GLM). We used Gaussian error distribution for plant species richness and Shannon's diversity index, and binomial error distribution with overdispersion ("quasibinomial") for proportion of plant cover. Multiple values of flower abundance, insect visitation, and pollen grains deposited on stigmas were measured repeatedly at each site, so we used generalized linear mixed-effects model (GLMM) with site identity as a random factor and Poisson error distribution for these response variables. We also used the duration of the observation period and the number of flowers in the observation window (both log-transformed) as an offset in the GLMM of insect visitation to properly analyse variation in visitation per flower per hour (Reitan & Nielsen, 2016). We also performed a redundancy analysis (RDA) to test for changes in the composition of the flower visitor assemblages with the increasing distance from the village area, separately for the two plant species. Finally, we analysed fruit and seed production in *A. ilicifolius* using similarly constructed GLMMs, with the number of flowers in an inflorescence used as an offset (log-transformed) when analysing the number of fruits per flower, and the number of fruits used as an offset (log-transformed) when analysing the number of seeds per fruit. We used R 3.4.4. (R Core Team 2018) for all analyses and plots; GLMMs were fitted using the lme4 package (Bates *et al.* 2015).

6. 4. Results

6.4.1. Plant diversity and abundance

Overall, we found 13 plant species of 9 families in the sampled sites (data: https://doi.org/10.6084/m9.figshare.11877615). However, more plant species can be found in the wider area.

We observed the lowest plant species richness and diversity in the forest patches nearby the village (Fig. 6.4A and 6.4B), which were dominated by mostly *Sonneratia apetala* Buch-Ham., *Excoecaria agallocha* L., and *Avicennia officinalis* L., all of them are tree species. Plant species richness and diversity increased along the gradient from the village towards the undisturbed forest interior (GLM, F=6.4, P=0.030 for species richness, and F=4.5, P=0.060 for Shannon's diversity index). *A. ilicifolius* was the only shrub plant in the forest patches in the village area. The understory plant cover increased significantly towards the forest interior (GLM, F=29.6, P=0.0003; Fig. 6.4D), unlike the canopy cover (GLM, F=3.4, P=0.096; Fig. 6.4C)

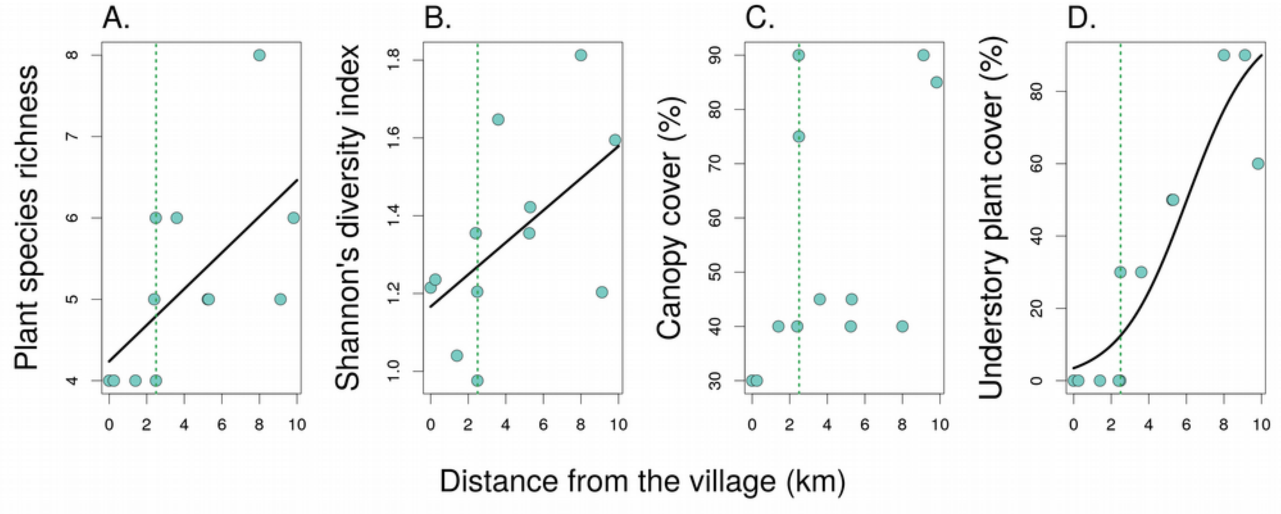

Figure 6.4: Plant diversity and abundance along the disturbance gradient and distance from the village towards the pristine forest: plant species richness (A.), Shannon's diversity index (B.), canopy cover (C.) and understory plant cover (D.) for each site.

We also estimated the plant cover individually for our two target plant species and counted the number of flowers/m^2 for both plant species to assess their floral abundance. Plant cover of the shrub *A. ilicifolius* gradually increased from the village towards the forest interior (GLM, F=56.3, P<0.0001; Fig. 6.5A) but did not change significantly in case of *A. officinalis* (GLM, F=0.32, P=0.58; Fig. 6.5B). Flower density of *A. ilicifolius* did not vary significantly along the gradient (GLMM, X^2=0.95, P=0.33; Fig. 6.5C), while *A. officinalis* showed decreasing flower density from the village towards the deep forest (GLMM, X^2=8.9, P=0.0029; Fig. 6.5D).

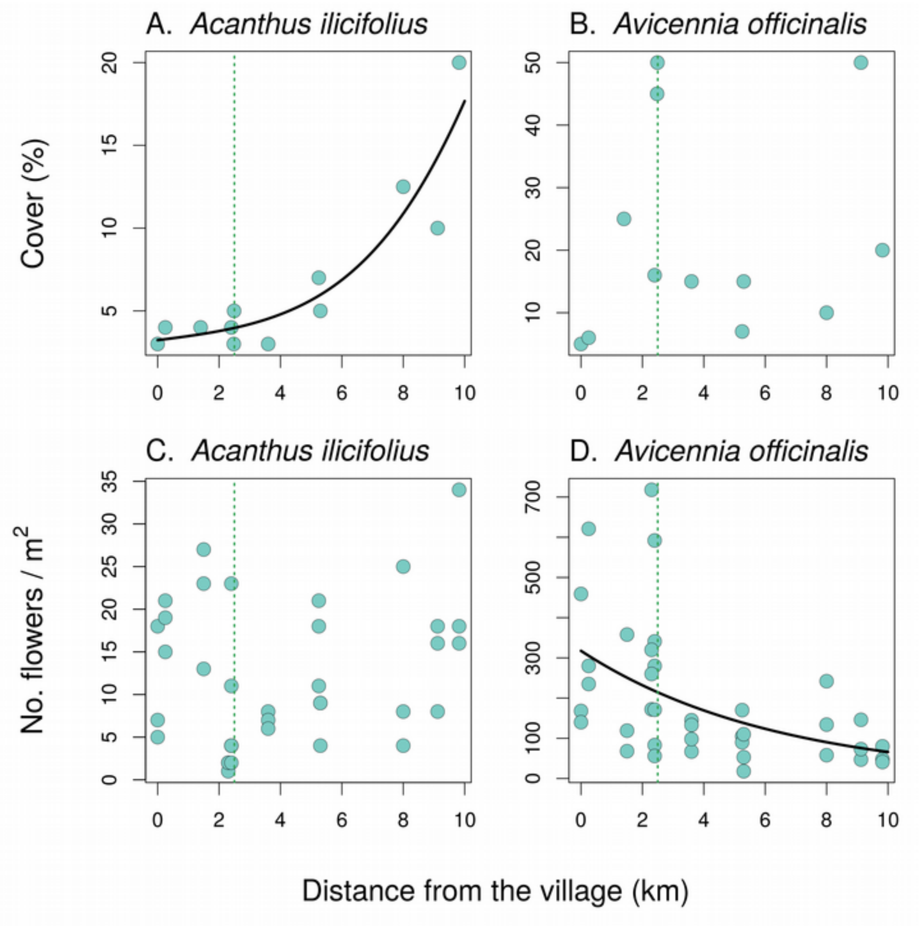

Figure 6.5: Individual plant cover and number of flowers for two target species: A. Plant cover for *A. ilicifolius* (shrub). **B.** Plant cover for *A. officinalis* (tree). **C.** Flower abundance for *A. ilicifolius* and **D.** Flower abundance for *A. officinalis*. X-axis showing the distance of the sites along the gradient from the village towards the undisturbed forest interior (**A-D**).

6.4.2. Insect diversity and flower visitation rate

Flower visitor community in our sampling period of this part of the forest consisted of the major insect groups, such as Hymenoptera, Diptera, Coleoptera and Lepidoptera. We observed total 4431 pollinating insects and randomly collected total 536 pollinating insects excluding three *Apis* species some easily recognised insects. We identified 105 insect species or morphospecies from at least 27 families (list of species: https://doi.org/10.6084/m9.figshare.11877615). Hymenoptera made up to 80% of the total number of pollinator individuals. Among them, bees were the biggest group and around 44% of them belonged to the genus *Apis*. *A. dorsata* Fabricius, 1793, also known as the giant honey bee, was the most abundant overall both in the village and inside the forest. Additionally, *Apis cerana* Fabricius, 1793, the eastern honey bee or the Asiatic honey bee, and *Apis florea* Fabricius, 1787, the dwarf honey bee, were found in the forest patches near the village but were completely absent in the deep forest. Next to the three honey bee species, solitary bees were the major insect groups among pollinators, consisting 38 species from 7 families. Wasps from the family Vespidae were the most diverse insect family with 22 morphospecies. Among the non-bee pollinators, flies, beetles and butterflies made up to 15% of the total pollinators. No bird was observed as pollinator for *A. ilicifolius*.

We found no significant changes in the total visitation of the two plant species by pollinators along the gradient from the village to the undisturbed forest interior (GLMM, X^2=1.5, P=0.22 for *A. ilicifolius*, and X^2=2.8, P=0.096 for *A. officinalis*). In addition, there were no significant changes in species richness of pollinators along the gradient for both plant species (GLM, F=0.49, P=0.050 in *A. ilicifolius* and F=1.4, P=0.26 in *A. officinalis*). The Shannon's diversity index of the pollinator community also did not change in *A. ilicifolius* (GLM, F=2.1, P=0.17) and *A. officinalis* (GLM, F=1.5, P=0.25). However, species composition of the pollinators varied along the gradient as revealed by the redundancy analysis (RDA). The distance of the sites from the village explained 21.36% of total variance in species composition in the flower visitors of *A. ilicifolius* (RDA, F=2.72, P=0.0078) and 23.83% in A. *officinalis* (RDA, F=3.13, P=0.0021) with some species or groups more abundant in the sites close to the village area (e.g. *Apis cerana*, *A. florea*, and Diptera) and others (*A. dorsata* and Coleoptera) in the forest interior (Fig. 6.6, Fig. 6.7, Fig. 6.8, Table 6.1).

Table 6.1: Visitation rate by different groups of pollinators on the two plant species. Results of statistical tests (GLMM) of the changes of the visitation rate by insect orders and individual species of honey bees along the disturbance gradient from the village towards the forest interior. Likelihood ratio test was used to test the statistical significance of each fitted relationship.

	Acanthus ilicifolius			***Avicennia officinalis***		
Insect order	**slope**	**X^2**	**P**	**slope**	**X^2**	**P**
Hymenoptera	0.06	1.37	0.2419	0.11	3.15	0.0758
Diptera	-0.10	4.40	**0.0359**	-0.08	0.49	0.4828
Coleoptera	0.26	4.43	**0.0353**	0.31	7.56	**0.0060**
Lepidoptera	-0.26	0.98	0.3227	-0.19	0.11	0.7420
Honey bee species						
Apis dorsata	0.34	13.56	**0.0002**	0.24	11.54	**0.0007**
Apis cerana	-0.52	5.31	**0.0212**	-0.41	13.42	**0.0002**
Apis florea	-0.50	6.85	**0.0089**	-1.21	6.90	**0.0086**

Both plants were visited mostly by Hymenoptera at a rate which did not vary along the disturbance gradient, while the visitation rate by Diptera on *A. ilicifolius* decreased and visitation rate by Coleoptera increased along the gradient on both plants (Table 6.61). Lepidoptera was observed rarely and mostly at sites along the edge of the continuous portion of the forest (Fig. 6.7). The three honey bee species of the genus *Apis* were the most frequent visitors on flowers of both plants, but they responded differently to the disturbance gradient (Fig. 6.8). *A. cerana* and *A. florea* abundances decreased with the distances of forest patches from the village towards the forest, while the number of *A. dorsata* increased gradually with the increasing distances of forest patches from the village (Fig. 6.8).

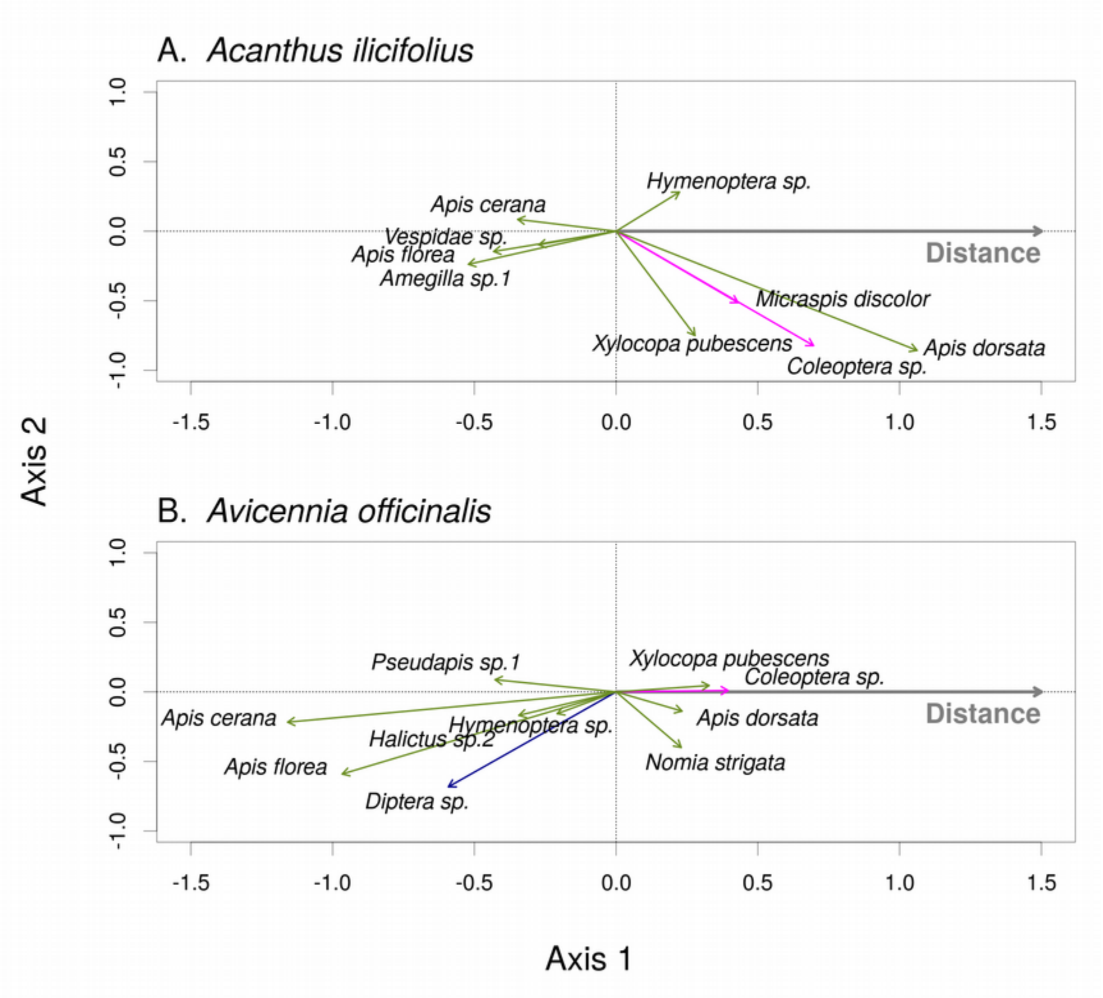

Figure 6.6: The composition of flowers visitors of *Acanthus ilicifolius* (A.) and *Avicennia officinalis* (B.) changed along the forest disturbance gradient. Results of RDA which show how abundance of individual flower visitor species on the two plant species changed with increasing distance from the village. Hymenoptera are displayed by green arrows, Coleoptera by magenta, and Diptera by blue arrows. Species whose abundance was little affected by the distance from the village (species with scores on Axis 1 <|0.2|) are not shown for clarity. Species with arrows pointing to the left were associated mostly with the fragmented forest close to the village, while species with arrows pointing to the right were found mostly in the deep forest far from the village.

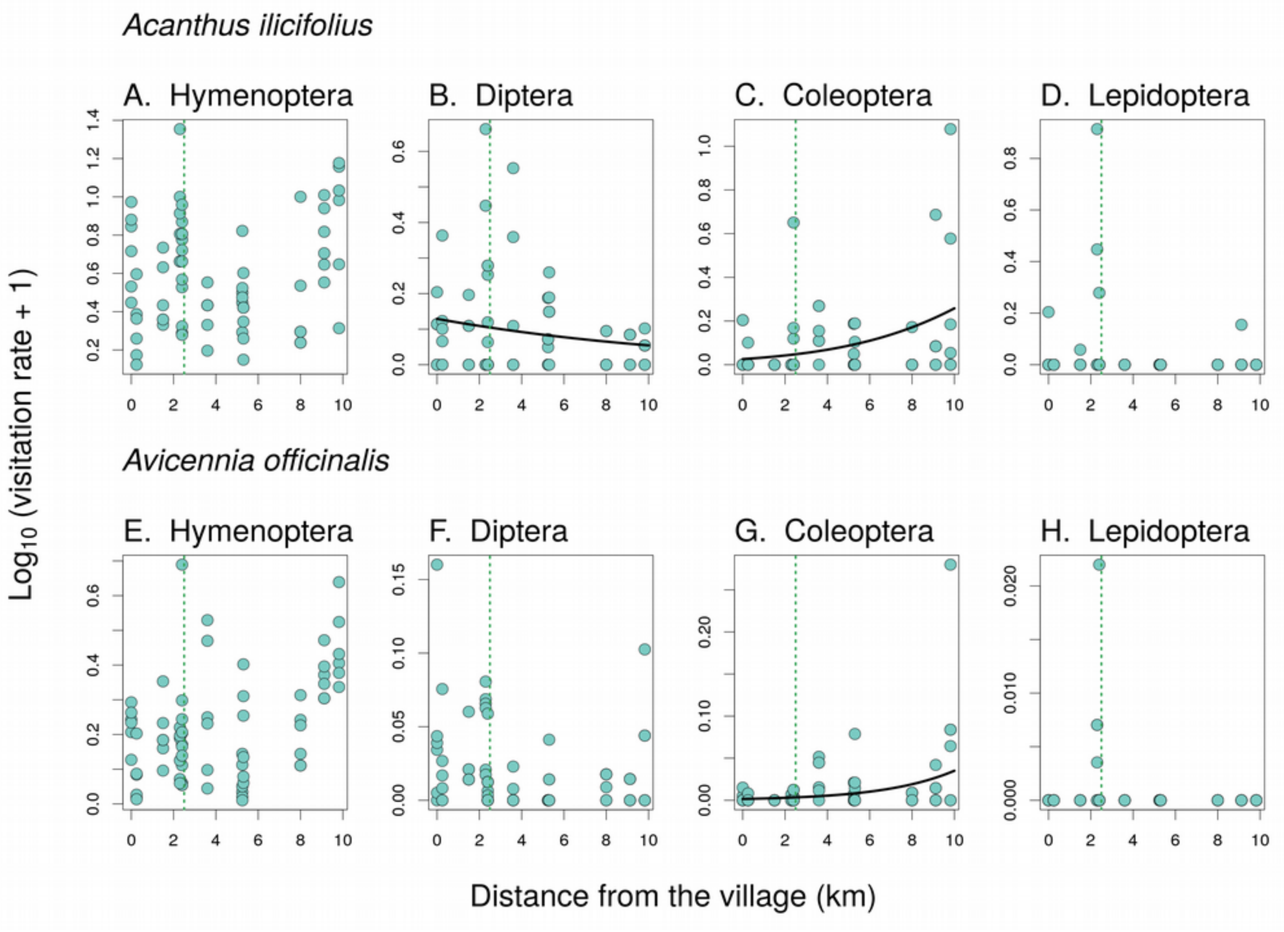

Figure 6.7: Visitation rate of insect orders on the two plants. The number of visits per flower and hour on *Acanthus ilicifolius* (A. - D.) and *Avicennia officinalis* (E. - H.). The estimated relationship is plotted as a line only in cases where it was statistically significant according to a likelihood ratio test (see Table 1). The vertical dotted green line shows the point along the disturbance gradient where the continuous forest begins and continues further away from the village.

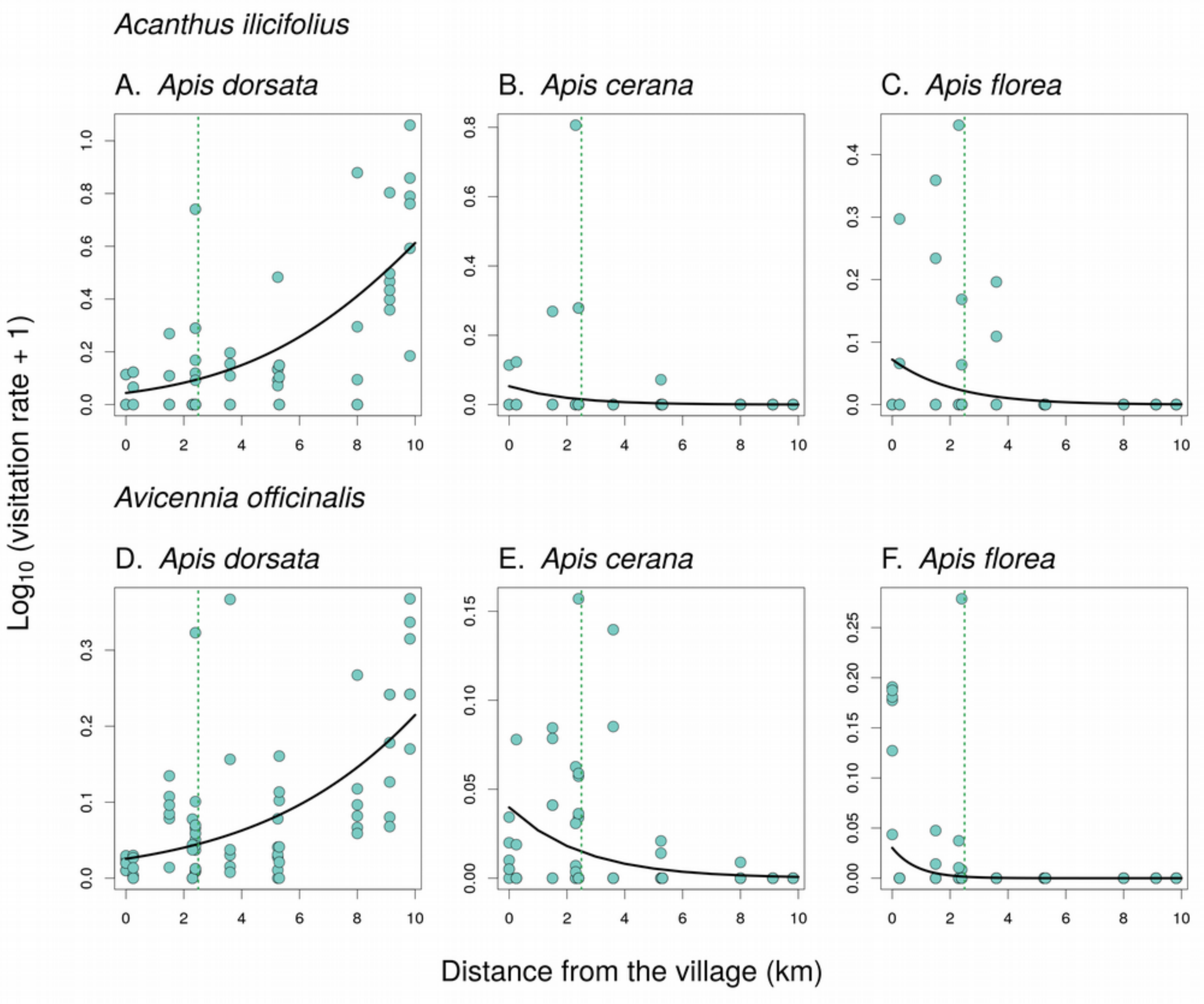

Figure 6.8: Visitation rate of the three species of honey bees on the two plants. The number of visits per flower and hour on *Acanthus ilicifolius* (A. - C.) and *Avicennia officinalis* (D. - F.). The relationship between the visitation rate and distance from the village was statistically significant in all cases according to a likelihood ratio test (see Table 1). The vertical dotted green line shows the point along the disturbance gradient where the continuous forest begins and continues further away from the village.

6.4.3 Pollen deposition and seed production

Although we observed significant variation in the composition of pollinator communities of both plants along the disturbance gradient from the village to the forest interior, pollination was not highly affected by these variations. The number of pollen tubes in the *A. ilicifolius* did show a significant increase in the total number of pollen grains deposited on its stigmas with the distance from the village towards the forest interior (GLMM, X^2=4.2, P=0.041; Fig. 6.9A.), but this did not translate into differences in fruit and seed production. That is, the number of fruits per infructescence was not affected by the distance along the gradient from the village towards the forest interior (GLMM, X^2=0.29, P=0.59), the same holds for the number of seeds per fruit (GLMM, X^2=0.069, P=0.79). The second species, *A. officinalis*, showed no differences in the total number of pollen grains deposited per flower along the disturbance gradient (GLMM, X^2=1.3, P=0.25; Fig. 6.9B.), while seed set data were not available.

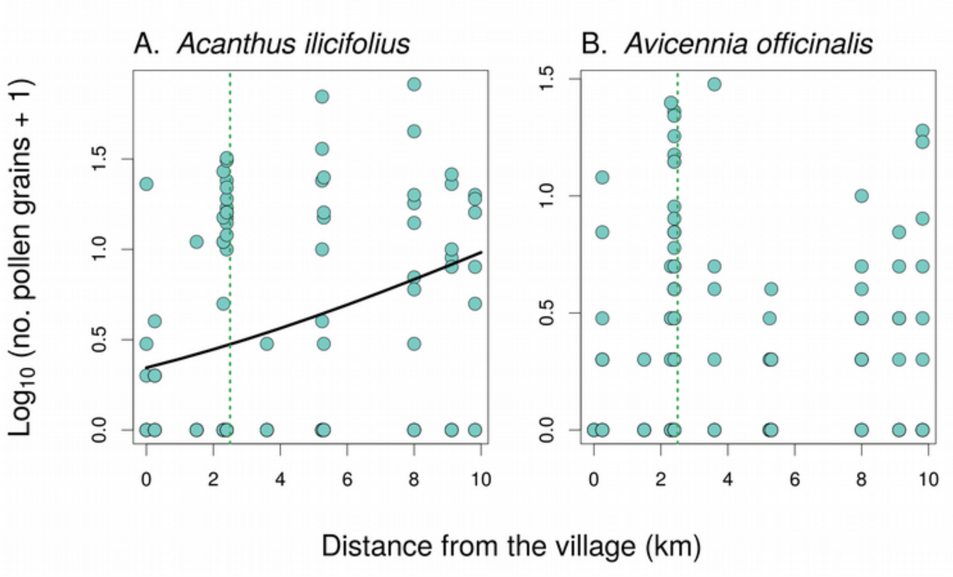

Figure 6.9: Pollen deposition on stigmas of the two plant species. The number of pollen grains deposited on stigmas of *A. ilicifolius* (A.) and *A. officinalis* (B.) in relation to the distance from the village towards the forest interior. The relationship was statistically significant only in *A. ilicifolius*. The vertical dotted green line shows the point along the disturbance gradient where the continuous forest begins and continues further away from the village.

6.5. Discussion

6.5.1. Impact of human disturbances on plant diversity, pollinator communities and pollination

Our research shows changes in the plant community structure, composition of the pollinator communities, and flower visitation patterns along the gradient of decreasing human disturbance from village area towards a relatively pristine forest interior. Forest patches nearby the village had the lowest number of plant species and the lowest values of the Shannon’s diversity index. These fragmented forest patches are used by the local people as grazing land for their domestic animals and plant leaves and stems were regularly collected for fuel and fodder and intentionally kept clear to deter tigers as they prefer to hide in the bushes for hunting (Badhwar, 1988). Furthermore, pollutants from boats and households may hamper the regrowth of plants in such forest patches (Santos *et al*., 2012) and resulted to have almost no understory and very low plant species richness and diversity. Likewise, *A. ilicifolius* was lower in the patches nearby the villages and increased significantly towards the deep forest. Although plant cover increased with the distance from the village, flower production per unit area did not show any differences. Contrastingly, the percentage cover of *A. officinalis* did not show any change along the disturbance gradient, but its flower abundances decreased towards the deep forest. This can be due to the rain deficiency in this part of the forest as *Avicennia* species flowering time shows a high sensitivity to rainfall (Opler *et al*., 1976; Reddy *et al*., 1995) or due to the increasing salinity as several studies showed delayed flowering in plants due to the increasing salinity (Maas and Poss, 1989, Khatun and Flowers, 1995). This forest is lying in the Bay of Bengal delta and with the advantage towards the deep forest from the village leads to higher salinity as the sea gets closer (Haque and Reza, 2017). We also noticed that *A. officinalis* had an unusually high cover in some of the patches at the transition between the village and continuous forest due to the partial plantation by the forest department to support the restoration of the forest (Saenger and Siddique, 1993; Rahman and Rahman, 2015).

The giant honeybee (*A. dorsata)* was the major pollinator for both species in every site with a sharp increase from the village towards the forest interior. Although *A. dorsata* was reported to be a vital pollinator for both cultivated crops and wild plants (Robinson, 2012), they usually forage in more abundant flower resources (Punchihewa *et al*., 1985) and may be a poorer competitor than the other two *Apis* species (Koeniger and Vorwohl, 1979). Studies showed that our three honey bee species compete for food, with *A. florea* and *A. cerana* being the stronger competitors than *A. dorsata*, and this competition can be avoided by differentiation of foraging (Koeniger & Vorwohl, 1979). This may explain why the abundance of *A. dorsata* is lower in forest patches nearby the village and higher in the deep forest as *A. cerana* beehives were located in the village patches and *A. florea* was only present in the village sites. It has been reported that the presence of domesticated *A. cerana may* affect the abundance of *A. dorsata* in human disturbed areas (Samejima *et al*., 2004). Moreover, forest patches nearby the village are more exposed to both professional and non-professional honey collectors and naturally occurring *A. dorsata* hives are frequently disturbed, extracted, and even destroyed by the honey-collectors which may lead to low number of hives in the village areas. On the other hand, *A. cerana* is completely domesticated in that area and they were able to forage both in the forest and village patches within their foraging distance (Partap, 2011) while they were absent in the deeper forest. Among the three species, *A. florea* has a distinct habitat preference and was only found in village areas. This smaller bee prefers to build their nest in lower branches, in sunny location (Whitcombe, 1984) and forest patches near the village offer more suitable nesting sites in terms of their habitat preference, compared to the deeper mangrove forest. *A. florea* tend to swarm and transfer nest swiftly and prefer to stay close to the abundant food and habitat resources (Whitcombe, 1985). Unlike other *Apis* species, this species does not migrate when the flower resources are scarce and shortage of their flight range make them more aggressive towards other bees but generally niche compartmentalization between the flower resources would minimize the competition (Koeniger & Vorwohl, 1979) and different studies on the *Apis* species showed that these three species can co-occur without any significant competition (Punchihewa, *et al*., 1985, Oldroyd, *et al*., 1992, Koetz, 2013). However, interactions between the domesticated *A. cerana* and other pollinators are not well-known. A number of recent studies on European honeybees *Apis mellifera* showed their strong negative effects on wild pollinators (Magrach *et al*. 2017, Henry & Rodet 2018, Hung *et al*. 2019). Exploring the interactions between the Asian honeybees *A. cerana* and wild pollinators in similar detail will thus be an important topic for future research.

Although three species of *Apis* made up half of the pollinating insects for both targeted plants, solitary bees played the second most important role in visiting the flowers. The overall abundance of Hymenoptera increased towards the deeper forest for *A. ilicifolius* but decreased for *A. officinalis*, but this can be the result of decreased flower abundance of this species. On the other hand, the abundance of *Xylocopa pubescens* increased towards the deep forest for both plant species, although their abundance was not as significant as other pollinators, despite their well established role as a pollinator for *A. ilicifolius*. We did not observe any birds visiting *A. ilicifolius*, although Primack and Tomlinson (1980) reported sunbirds as pollinator for this species in Australia. Unlike *Apis* spp., our study found that the disturbance gradient had little effect on the total flower visitor abundances and diversity of solitary bees. Although some studies suggested that the species richness and population density of solitary bees may decrease proportionally with the increasing human disturbances (Inoue *et al*., 1990; Liow *et al*., 2001), another study showed the opposite where wild be communities were reported to be persistent against the human disturbances or even to benefit under particular circumstances (Stein *et al.*, 2018). However, we lack detailed information about the biology and foraging behaviour of individual species, apart from *Apis* spp. discussed above, which prevents more detailed assessment.

6.5.2. Perspectives for forest conservation

Many plants in the mangrove forest are dependent on the insect pollination and similarly, mangrove provides an excellent forage for bees (Lacerda, 2002) and other insects. Human disturbance impacts on both plant and animal diversity are likely to be severe, therefore, we need to focus on developing sound conservation policies for the mangrove forests, such as the Sundarbans. Based on our results, it seems that changes in the composition of the pollinator community along the gradient of human disturbances did not affect the pollination success of the studied species much, but the plant diversity and cover of the understory plants were significantly lower in patches close to the village. This suggests that despite the successful pollination and seed production, human exploitation interrupts forest regeneration and likewise affects the pollinator community. Moreover, overexploitation of the wild-living giant honey bees, *A. dorsata*, was likely responsible for the lower abundance of this species in forest patches near the villages compared to the pristine part of the forest. However, almost no information is available on how honey hunting affects the colony survival, growth and migration of *A. dorsata.* Continuous destruction of its nests and habitat may lead to further decline of the giant honey bee. Local extinctions of *A. dorsata* have been reported across their vast distribution range (Oldroyd and Nanork, 2009) and deserve attention regarding their conservation. On the other hand, based on the population growth rate and the rate of harvesting of *A. florea*, the other wild-living honey bee species, it is unlikely that this species will be affected by human disturbances at the same rate as other honey bees (Oldroyd & Wongsiri, 2006). The third local honey bee species, *A. cerana*, is domesticated and kept by the local beekeepers and it is unlikely to go extinct. Studies in other low-land forest areas in Asia showed that *A. dorsata* immigrates into the forests in the mass flowering season and when the amount of floral resources drop, they leave the forest (Itioka *et al.*, 2001). In contrast, the resident *Apis* species and other solitary bees, stay year around and pollinate the flowering plants for entire period (Sakai, 2002). Hence, keeping domestic honey bees in the mangrove areas is widely accepted as non-harmful from the conservation point of view. However, more intensive research will be needed to decide whether keeping domestic beehives in this area is beneficial or harmful for the local pollinator communities and plant diversity.

Although our results show that changes of the composition of the flower visitor community along the gradient of human disturbance in our case likely do not affect the reproduction of the studied plants, human activities negatively affect the mangrove forest in other ways, mostly by disrupting forest regeneration by clearing the understory. Also, we conclude that bees are the most important pollinators in this forest, but *Apis dorsata* is threatened by human activities, in particular by harvesting of its honey. The forest provides vital resources for the local people, so to prevent further deterioration of the state of the forest, it is necessary to initiate more intensive conservation approaches, e.g. mangrove tree plantation with a focus on rare species, and increase awareness about the necessity of mangrove conservation among the locals and involve them directly through the community based approaches (López-Portillo *et al.*, 2017). While honey harvest is an important source of income for the local people, honey hunters should be encouraged and trained to harvest honey in a non-destructive sustainable way with proper equipment to minimise the impact on the bee hives (Purwanto *et al*., 2000; Waring and Jump, 2004).

Acknowledgements

We would like to thank students from the University of Chittagong and Khulna University, Bangladesh for their help in the field work and the technical support from both Universities. Our thanks to Jiří Hadrava and Martin Šlachta for help with species identification of Diptera and Hymenoptera. We also thank the Forest Department of Bangladesh for granting the permission and security to work in this forest. We are also grateful to Jana Jersáková for the help with laboratory analysis. This project was funded by National Geographic Society (Grant-EC-376R-18) and also partly supported by the Czech Science Foundation (GJ17-24795Y) and by the University of South Bohemia in České Budějovice, Czech Republic. The funders had no role in the study design, data collection, analysis and preparing the manuscript.

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Summary

Pollination is a key ecosystem service provided by the interacting organisms in the ecosystem, benefits both human and wildlife. Animal-mediated pollination is critical for natural and agricultural production system but increasingly affected by the changing climate, habitat loss, invasive species and land-use change. These changes have an interacting impact on the pollination and not well understood in many cases. In this study we considered few several important aspects of environmental change which directly affects the floral resource variability, arrangement and their impact on pollination and seed production.

In chapter two, with a manipulative climatic growth conditions, we showed that plant morphological growth, flowering time and quality of floral resources greatly depend on the growth condition related to climatic changing drivers. Climate changing factors have individual and interactive impact on plant growth, flower production and seed production. Here, we demonstrated how temperature variation, amount of water and N_2 supplementation affect the plant morphology and flower and nectar production and how consequently, this variation affects the pollinator perception and the quality of seed production.

Chapter three focuses on the response of pollinator's foraging behaviour based on the arrangement of floral resources. *Dracocephlum moldavica* was grown in the greenhouse and then brought to the field when started to flower. We arranged the flowering plants in clusters containing plant from 1 to 37 with a variable number of flowers and observed the pollinator visitation rate and foraging behaviour. This study added the information on the distribution of resources but did not conform the predictions based on optimal foraging theory and the ideal free distribution model.

Chapter four deals with the idea of introducing new floral resources into plant community and the responses from the pollinator community. Here, we introduced two different colour and shaped floral resources into the community and observed the impact of newly introduced non-native flowering plant on the total pollinator visitation both on both native and non-native flowers. We also relate the flower visitors mediated by the floral traits, such as colour and shape of flowers.

Chapter five explains the pollination strategies of plant in an unstable habitat. Here, we studied the pollination ecology of *Linara tonzigii*, a rare endemic plant in the high-altitude Italian Alps. We focused on the self-pollination and autogamy of this species as these are the common pollination

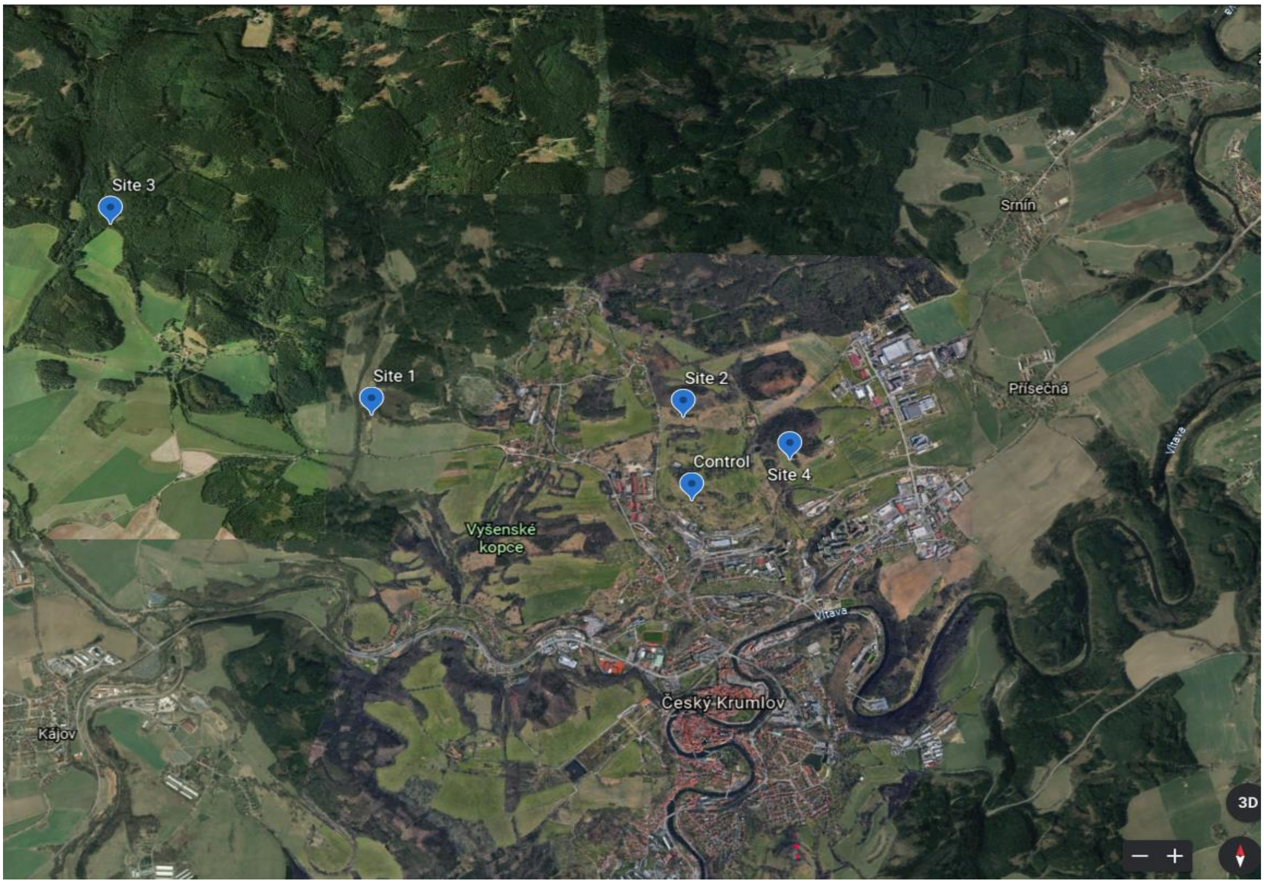
strategies for environmentally pollination limited plants. We also observed the daily nectar production rate, pollinator's foraging behaviour and pollen transfer of this plant. The results revealed the self-compatibility characteristics of this species and its close phylogenetic relation to other self-pollinated species.

Finally, chapter six indicates the possible consequences of habitat loss on the plant communities and its direct impact on the pollinator communities, which is closely tied with human interference. This study was carried out in the Sundarbans Mangrove forest, an increasingly disturbed natural forest with its own pollinating system. This study showed how habitat destruction and human disturbances directly affect the pollinator communities and its consequences on pollination.

Taken together, all chapters demonstrate that any changes in the environment, whether it's the growing condition for plants, or the resource variation for flower foragers, or even the impact of habitat loss and other interferences, all has it's unique impact on the plant-pollinator interactions and the dynamics of this changes are both individual and interactive. Our study also revealed that the plant-pollinator community is resilient in nature and capable of adjusting with the ever-changing environment despite its own limitation.

Supplementary documents:

S.4.1. Chapter 4:



Supplementary Figure 4.1: Map of the location of four experimental sites and control site of our studied communities.

Supplementary Figure 4.2: Photos of our experimental sites respectively; a) site 1, b) site, c) site 3 and d) site 4.

Supplementary Table 4.1: List of sites and their description

Site	Co-ordinates	
Control	48.8249681N, 14.3158347E	This was the closest site to the city Cesky krumlov with an area of 0.48 acres. It was part of an open meadow on the slope of a hill with a forest nearby. This site represented a rich vegetation with tall grasses, herbs and scattered shrubs. Density of flower plants was higher throughout the season with an adequate flowering.
Site 1	48.8352853N, 14.2601628E	This site was close to the village Lazec, near Cesky Krumlov and surrounded by trees in three sides with one open side. This site was about 0.58 acres and connected to a big open meadow through the open side. The place presented mostly grass, herb and few shrubs with an adequate flower density.
Site 2	48.8287556N, 14.3139625E	This site was close to the Cesky Krumlov city and was 0.2 acres. Surrounded by conifers in three sides and open in one side to a small meadow, this place was occupied by tall grasses and herbs with very few shrubs. The density of flowering plant was quite higher of this site than any other site.
Site 3	48.8308256N, 14.2875694E	This site situated near the village Vysny in Cesky Krumlov. A forest occupied mostly by tall grasses, herbs and trees with scattered shrubs. With an area of 0.43 acres, this place was a mixture of forest and open places near by a big open meadow. The vegetation of this site was a little different and density of flowering plants were lower compare to other sites.
Site 4	48.8284525N, 14.3233717E	This site was also close to Cesky Krumlov, within a forest with alongside a small meadow. With an area of 0.2 acres, this site was also a mixture of forest trees and tall grasses with herbs and shrubs. The flowering plant density was as adequate in the beginning but decreased at the end of the season.

Supplementary Table 4.2: List of plant species with family and flower morphology traits

Plants	Family	Flower shape	Flower colour
Achillea millefolium	Asteraceae	Ray-disc	White
Aegopodium ramosum	Apiaceae	Zygomorphic	White
Agrimonia eupatoria	Rosaceae	Short-corolla	Yellow
Allium scorodoprasum	Amaryllidaceae	Funnel	White
Anthericum ramosum	Asparagaceae	Open	White
Astragalus glycyphyllos	Fabaceae	Zygomorphic	White
Campanula patula	Campanulaceae	Funnel	Blue
Campanula rapunculoides	Campanulaceae	Funnel	Blue
Centaurea jacea	Asteraceae	Ray-disc	Pink
Centaurea scabiosa	Asteraceae	Ray-disc	Pink
Centaurium erythraea	Gentianaceae	Open	Pink
Cirsium arvense	Asteraceae	Long-corolla	Pink
Clinopodium vulgare	Lamiaceae	Zygomorphic	Pink
Convolvulus arvensis	Convolvulaceae	Funnel	White
Coronilla varia	Fabaceae	Zygomorphic	Pink
Daucus carota	Apiaceae	Open	white
Dianthus deltoides	Caryophyllaceae	Long-corolla	Pink
Echinops sphaerocephalus	Asteraceae	Long-corolla	Blue
Echium vulgare	Boraginaceae	Funnel	Blue
Erigeron annuus	Asteraceae	Ray-disc	Yellow
Galium mollugo	Rubiaceae	Open	white
Galium verum	Rubiaceae	Open	Yellow
Gentiana cruciata	Gentianaceae	Short-corolla	Blue
Helianthemum grandiflorum	Cistaceae	Open	Yellow
Hypericum perforatum	Hypericaceae	Open	Yellow
Inula salicina	Asteraceae	Long-corolla	Yellow
Knautia arvensis	Caprifoliaceae	Long-corolla	Pink
Lathyrus pratensis	Fabaceae	Zygomorphic	Yellow
Leontodon autumnalis	Asteraceae	Ray-disc	Yellow
Leucanthemum vulgare	Asteraceae	Ray-disc	White
Lotus corniculatus	Fabaceae	Zygomorphic	Yellow
Malva moschata	Malvaceae	Open	Pink
Medicago lupulina	Fabaceae	Zygomorphic	Yellow
Melilotus officinalis	Fabaceae	Zygomorphic	Yellow
Melilotus albus	Fabaceae	Zygomorphic	White
Origanum vulgare	Lamiaceae	Zygomorphic	Pink
Pastinaca sativa	Apiaceae	Open	Yellow
Pimpinella saxifraga	Apiaceae	Open	White
Plantago lanceolata	Plantaginaceae	Open	White
Potentilla erecta	Rosaceae	Open	Yellow

Prunella vulgaris	Lamiaceae	Zygomorphic	Blue
Rhinanthus major	Orobanchaceae	Zygomorphic	Yellow
Rubus sp.	Rosaceae	Open	White
Salvia verticilliata	Lamiaceae	Zygomorphic	Blue
Senecio jacobaea	Asteraceae	Ray-disc	Yellow
Stellaria graminea	Caryophyllaceae	Open	White
Teucrium chamaedrys	Lamiaceae	Zygomorphic	Pink
Thymus sp.	Lamiaceae	Zygomorphic	Blue
Trifolium aureum	Fabaceae	Zygomorphic	Yellow
Trifolium medium	Fabaceae	Zygomorphic	Pink
Trifolium pratense	Fabaceae	Zygomorphic	Pink
Trifolium repens	Fabaceae	Zygomorphic	White
Verbascum chaixii	Scrophulariaceae	Open	Yellow
Vicia sativa	Fabaceae	Zygomorphic	Blue

Supplementary Figure 4.3: *D. moldavica* and *C. officinalis* in the greenhouse

Supplementary Table 4.3: Observation schedule with the type of plants

Date	Site	Induced plant	No of Induced plant	Total transect walk
2.7.15	Site 1	Before	0	17
3.7.15	Site 1	None	0	21
6.7.15	Site 1	*Calendula officinalis*	36	23
7.7.15	Site 1	*Calendula officinalis*	36	22
10.7.15	Site 1	None	0	19
15.7.15	Site 1	*Dracocephalum moldavica*	36	18
	Site 2	Before	0	21
17.7.15	Site 1	None	0	17
	Site 2	*Dracocephalum moldavica*	36	15
	Site 3	Before	0	10
19.7.15	Site 1	Mixed	36	18
	Site 2	None	0	12
	Site 3	Mixed	36	10
	Site 4	Before	0	16
21.7.15	Site 1	None	0	17
	Site 2	*Calendula officinalis*	36	16
	Site 3	None	0	18
	Site 4	*Dracocephalum moldavica*	36	19
23.7.15	Site 2	None	0	6
	Site 3	*Calendula officinalis*	36	4
	Site 4	None	0	2
24.7.15	Site 2	None	0	15
	Site 3	*Calendula officinalis*	36	13
	Site 4	None	36	16
26.7.15	Site 2	Mixed	36	18
	Site 3	None	0	14
	Site 4	Mixed	36	13
28.7.15	Site 2	None	0	16
	Site 3	*Dracocephalum moldavica*	36	21
	Site 4	None	0	14
30.7.15	Site 3	None	0	9
	Site 4	*Calendula officinalis*	36	22

01.8.15	Site 4	None	36	4

2. Chapter 5:

Supplementary Appendix 5.1 – Genetic identity and variation in *Linaria tonzigii*

Young leaves (1-3 per plant) from each plant specimen were used for genetic screening (Table S1).

The sequences obtained in this study are the very first molecular data regarding *L. tonzigii* ever deposited in public international archives (Table S1).

The results of the genetic survey show that aligned sequences had no insertion/deletion (indels) sites, with the only exception of the two intergenic regions trnH-psbA (11 indels in LTM1) and trnL-trnF (10 indels in LTS1 and LTS2). When comparing sequences between populations, the genetic diversity was very low, with a minimum of only one haplotype at matK and rbcL and a maximum of three haplotypes at agt1, trnH-psbA and rpl32-trnL (Table S2). The sample from Mt. Cavallo showed exclusive haplotypes at three out of seven sequenced loci, whereas the population from Mt. Secco showed exclusive haplotypes at two loci. Overall, for each locus, most of the sampled populations shared the same haplotypes (Table S2)

Supplementary Table 5.1- Sampling details and haplotypes of DNA sequences produced in this study. GenBank accession numbers are reported within brackets.

Voucher	Field ID	Species	Population	Coordindates	AGT1	ITS	matK	rbcL	trnH-psbA	rpl32-trnL	trnL-trnF
MIB:ZPL:07787	LTA1	*L. tonzigii*	Mt. Arera	45.930611, 9.804611	AG1 (LR746142)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP1 (LR746156)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07788	LTA2	*L. tonzigii*	Mt. Arera	45.930611, 9.804611	AG1 (LR746142)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP1 (LR746156)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07789	LTA3	*L. tonzigii*	Mt. Arera	45.934194, 9.804472	AG1 (LR746142)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07790	LTC1	*L. tonzigii*	Mt. Cavallo	46.034483, 9.693883	AG2 (LR746143)	IT2 (LR746139)	MK1 (LR746148)	RB1 (LR746145)	TP2 (LR746157)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07791	LTF1	*L. tonzigii*	Mt. Ferrante	45.974583, 10.030139	AG3 (LR746144)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP1 (LR746156)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07792	LTM1	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP3 (LR746158)	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07793	LTM2	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07794	LTM3	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07795	LTM4	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07796	LTM5	*L. tonzigii*	Mt. Menna	45.930222, 9.747694	AG3 (LR746144)	–	–	–	–	RT1 (LR746151)	LF1 (LR746161)
MIB:ZPL:07797	LTS1	*L. tonzigii*	Mt. Secco	45.924139, 9.882333	AG3 (LR746144)	IT1 (LR746138)	MK1 (LR746148)	RB1 (LR746145)	TP1 (LR746156)	RT2 (LR746152)	LF2 (LR746162)
MIB:ZPL:07798	LTS2	*L. tonzigii*	Mt. Secco	45.924139, 9.882333	AG3 (LR746144)	–	–	–	–	RT3 (LR746153)	LF2 (LR746162)
MIB:ZPL:07799	LA	*L. alpina*	Mt. Triomen	46.021513, 9.592167	_	LR746140	LR746149	LR746146	LR746159	LR746154	LR746163

MIB:ZPL:07800	LV	*L. vulgaris*	Mapello	45.713570, 9.560710	_	LR746141	LR746150	LR746147	LR746160	LR746155	LR746164

Supplementary Table 5.2 – Genetic differences among samples of *L. tonzigii*. For each investigated locus, the length of aligned sequences (or the length range in case of indels), the number of segregating sites (S), the number of haplotypes (h), the haplotype diversity (Hd) and the nucleotide diversity and its standard deviation are reported:

	agt1	**ITS**	**matK**	**rbcL**	**trnH-psbA**	**rpl32-trnL**	**trnL-trnF**
length (bp)	846	707	836	703	320-331	804	848-858
S:	2	2	0	0	4	2	0
h:	3	2	1	1	3	3	2
Hd	0.530	0.333	0	0	0.333	0.318	0.303
π (SD)	0.00106 (0.00027)	0.00094 (0.00061)	0 (0)	0 (0)	0.00417 (0.00269)	0.00041 (0.00023)	0 (0)

Supplementary Appendix 5.2 – Phylogenetic analyses of nuclear and plastidial genetic markers

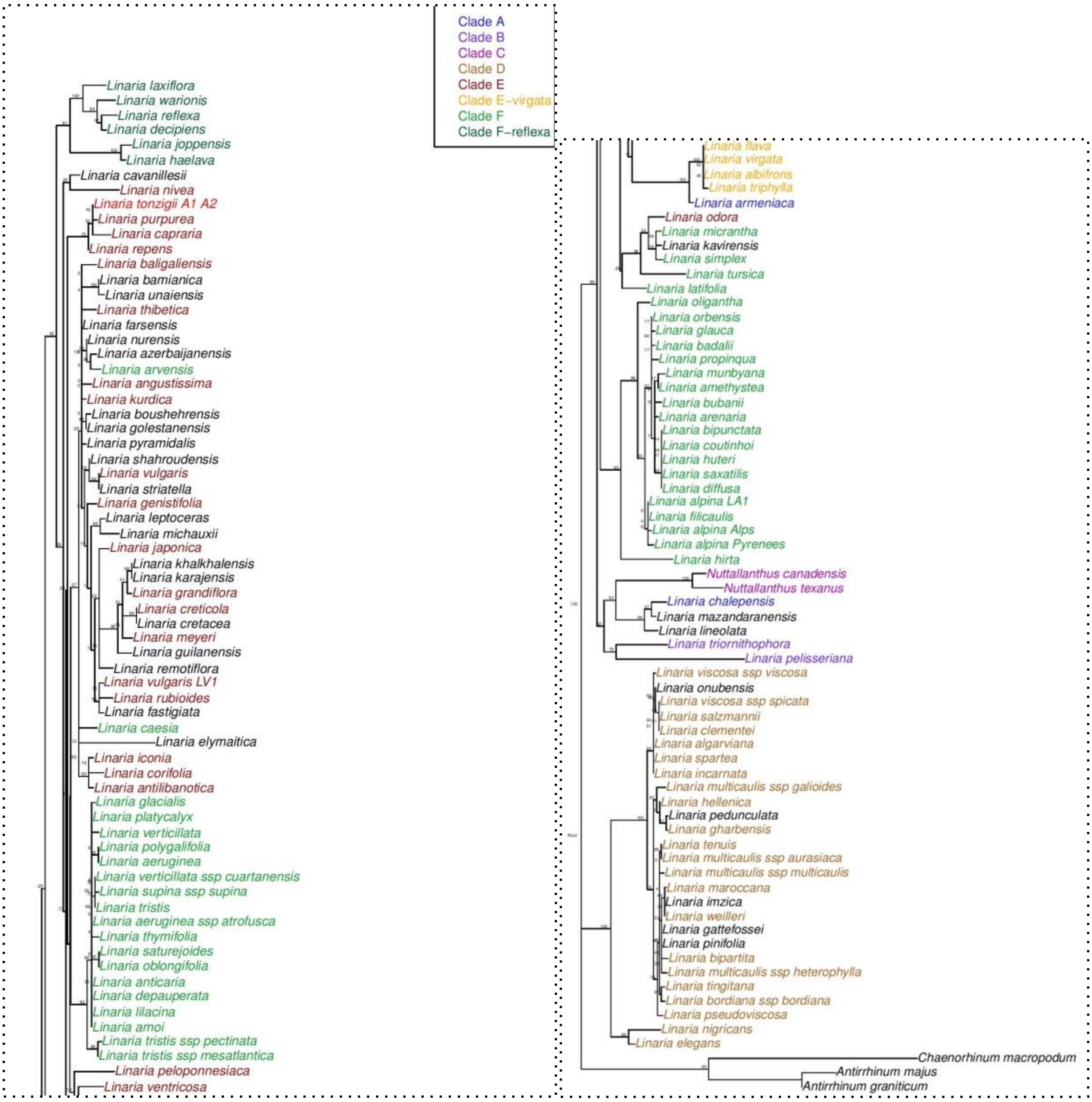
The following pages contain phylogenetic analyses of concatenated sequences for nuclear (ITS and agt1) and plastidial (matK, rpl32-trnL and trnL-trnF) genetic markers of the genus *Linaria*, inferred separately with RaxML and MrBayes. The colors indicate the *taxa* in the clades described by Fernández-Mazuecos *et al.*, 2013b, but *taxa* that were not included in that study are in black.

Supplementary Figure 5.1: Phylogenetic analysis of nuclear (ITS and agt1) genetic markers with MrBayes

Supplementary Figure 5.2 - Phylogenetic analysis of nuclear (ITS and agt1) genetic markers with RaxML

Supplementary Figure 5.3 - Phylogenetic analysis of plastidial (matK, rpl32-trnL, and trnL-trnF) genetic markers with MrBayes

Supplementary Figure 5.4 - Phylogenetic analysis of plastidial (matK, rpl32-trnL, and trnL-trnF) genetic markers with RaxML



S.3. Chapter 6:

Supplementary Table 6.1: Characteristics of the sites

Forest types	Distance of forest patches from the river edge km	Canopy cover, %	Ground cover, %	Characteristics
1.	1 -2.5	30-40	0	Farthest forest patches from the continuous forest, situated inside village and highly affected by the human activities.
2.	0.1-0.2	40-60	0-10	Forest patches nearby the river Pankhali, where human impact is highest, and the continuous forest is in the opposite side of the river.
3.	1.1- 2.8	40-45	30-60	Forest patches in the continuous forest and moderately affected by the human activities.
4.	5.5-7.3	60-90	60-90	Location inside the continuous forest, farthest from the human settlement and least affected by the anthropogenic activities.

Supplementary Table 6.2: List of plant species

plant	local_name	family	no_sites
Avicennia officinalis L.	bain	Acanthaceae	12
Acanthus ilicifolius L.	hargoza	Acanthaceae	12
Sonneratia apetala Buch.-Ham.	keora	Lythraceae	12
Excoecaria agallocha L.	geoa	Euphorbiaceae	10
Oryza coarctata Roxb.	nona dhan	Poaceae	7
Ceriops decandra Ding Hou	goran	Rhizophoraceae	2
Nypa fruticans Wurmb	golpata	Arecaceae	1
Rhizophora mucronata Lam.	lal garzan	Rhizophoraceae	1
Imperata cylindrica (L.) P.Beauv.	shon	Poaceae	1
Heritiera fomes Buch.-Ham.	sundari	Malvaceae	1
Derris trifoliata Lour.	kalilata	Fabaceae	1
Bruguiera gymnorhiza (L.) Lam.	lal kakra	Rhizophoraceae	1
Xylocarpus granatum K.D.Koenig	dhundal	Meliaceae	1

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Ph.D. Thesis, 2020

Printed in the Czech Republic by D Print

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