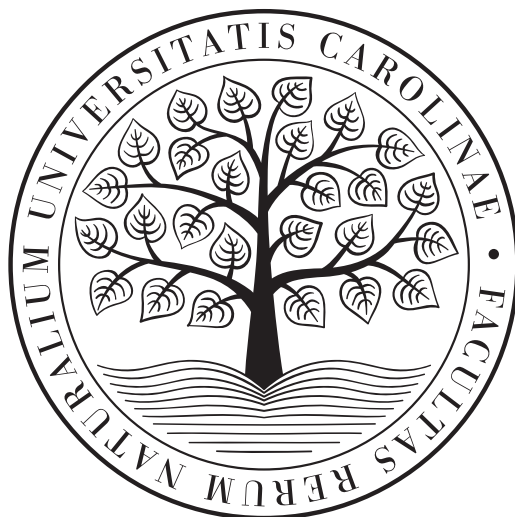


Charles University

Faculty of Science

Study programme: Botany

Study branch: Botany



Mgr. Jakub Štenc

Processes Driving Pollen Transfer in Generalised
Pollination Systems

Procesy ovlivňující přenos pylu v generalizovaných
opylovacích systémech

Doctoral thesis

Supervisor: RNDr. Zdeněk Janovský, Ph.D.

Praha, 2024

Declaration

Hereby, I declare that I made this thesis independently and I acknowledged contribution of coauthors to individual parts of the thesis. I did not submit this thesis nor its part for any other degree or diploma.

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny spoluautory jednotlivých částí této práce. Tuto práci ani její podstatnou část jsem nepředložil k získání jiného nebo stejného akademického titulu.

Prague, 30th of April 2024 V Praze dne 30. dubna 2024

Jakub Štenc

Acknowledgement

I am thankful to many people who somehow guided me through one of the most challenging journey of my life. Here, I would like to express my gratitude to some of them:

Muchas gracias BB, tu eres mi luciernaga quien ilumina mi noche. Y tu iluminaste mi camino a aquí. T'estime.

Díky Maminko, Mariáne, Babičko, Pepo, Kelzo a Kubo!

Zdeňku, thanks for everything you did and also for what you did not. I could hardly ever express the whole gradient of my gratitude and feelings. I hope that despite everything, you will remember me well. Takk skal du ha.

Then, I would like to thank the people I met while working on my PhD: Marcos, Rorro, Javi, Moha, Jesus, miarma Ana, Carlos, Mireia, Andrés, Jesus as well as people who somehow stuck with me from previous times: Ančé, Adam, Eliška, Venca, Bára, Marie, Evča, Lucka, Culka, Karel, Lenka and many others.

Furthermore, I would like to thank the PEE and FEG groups and namely their members: Tomáš Herben, Tomáš Koubek, Louskáček, Jitka Klimešová, Adam Klimeš, Bára Lepková, Jana Duchoslavová and Terka Mašková. Then to my collaborators: Jirka Hadrava, Míša Mikát, Evča Matoušková, Lukáš Jánošík, Alice Haveldová, Tadeáš Ryšan, Martin Freudenfeld, Hela Pijálková, Josef Nováček and many others. A vast proportion of the data presented in this thesis was collected by the “Opylovači” collective: over 70 people who selflessly carried out all the experiments over 13 years. And it is my honour that the majority of them I can call friends.

For both technical and moral support, I owe many thanks to Ondřej “Šebík” Šebesta and Martin Schätz. Without you guys, I would not have counted my pollen.

Dear members of the Department, my colleagues. Today is the end of my studies at this very remarkable institution. But alas, it is nine (sic!) years, which is far too short a time to live, think, study, and work among such great and admirable researchers and people. I do not know half of you half as well as I should like, and I like less than half of you half as well as you deserve. I have things to do, and I regret to announce that this is the end of my PhD. I bid you all a very fond farewell. Kuba got a sock.

I also acknowledge financial support for particular parts of my thesis. Two manuscripts from Chapter 2 were financially supported by the START project (START/SCI/141), and two manuscripts from Chapter 3 were financially supported by the Grant Agency of Charles University (GAUK no. 842120). Moreover, two internships, a total length of 3 months, were supported by the START project (START/SCI/141).

Abstract

Sexual reproduction of most plant species in the world depends on pollen transfer mediated by animal pollinators. However, pollen transfer is challenging and inefficient due to conflicting interests between plants and pollinators. Consequently, only a small fraction of produced pollen grains successfully reach stigmas, highlighting the pressure on plants to adopt strategies that increase the efficiency and specificity of pollen transfer. This thesis examines three important factors shaping pollen transfer: the generalization of plant-pollinator spectra, pollen presentation mechanisms, and pollinator behaviour.

The first chapter explores the generalization of plant-pollinator spectra, revealing that the majority of plant species in temperate Europe exhibit highly generalized pollinator spectra, with plant niche width and plant dominance being significant factors shaping these spectra. The second chapter delves into pollen presentation mechanisms, showing how pollen release matches the pollinator visitation activity. Diurnal dynamic in pollen availability on flowers and subsequent pollen transfer may then increase the specificity of pollen transfer in communities with a higher degree of generalization, highlighting the importance of timing in plant-pollinator interactions. Finally, the third chapter focuses on pollinator foraging behaviour, examining the impact of floral traits and pollinator experience on flower preference and constancy. Experimental studies with *Eristalis tenax*, L. Syrphidae hoverflies reveal intricate interactions between floral traits and experience, influencing foraging decisions and flower constancy.

By integrating diverse methodological approaches and experiments, this thesis provides insights into the intricate dynamics of pollen transfer. Understanding these drivers is crucial for comprehending the structure and resilience of plant-pollinator networks, with implications for conservation and ecosystem stability.

Keywords

pollination; pollen transfer; generalization-specialization; pollen presentation; pollinator behaviour; flower constancy

Abstrakt

Pohlavní rozmnožování většiny rostlin na světě závisí na přenosu pylu zprostředkovaného opylovači. Nicméně, přenos pylu je velmi neefektivní proces při kterém dochází k velkým ztrátám pylu v důsledku rozdílných zájmů rostlin a opylovačů. V důsledku je tak jen velmi malá část z vytvořených pylových zrn úspěšně přenesena na blizny rostlin, což zvyšuje tlak na rostliny vyvinout strategie zvyšující efektivitu a specifitu přenosu pylu. V této práci se zabývám třemi významnými faktory ovlivňujícími přenos pylu: generalizaci spektra opylovačů rostliny, mechanismy prezentace pylu a chováním opylovačů.

První kapitola prozkoumává generalizovanost spektra návštěvníků rostlin a ukazuje, že většina druhů rostlin v temperátní Evropě je navštěvována širokým spektrem opylovačů, přičemž šířka spektra rostliny a její schopnost dominance v porostu měla významný vliv na složení spektra opylovačů. Druhá kapitola se zaměřuje na mechanismy prezentace pylu a jak mohou ovlivnit návštěvnost rostlin opylovači. Následně pak ukazuje, jak prezentace pylu souvisí s přenosem pylu a jak může zvýšit specifitu přenosu pylu. Třetí kapitola se pak zaměřuje na chování opylovačů s důrazem na vliv květních vlastností a zkušeností opylovače na preferenci ke květům a věrnost opylovače. Pomocí manipulativních experimentů s pestřenkou trubcovou (*Eristalis tenax*, L. Syrphidae) ukazuje silný vliv barvy a velikosti, nicméně jen slabý vliv a symetrie květů na preference a následně rozdíl v chování mezi naivními a zkušenými zvířaty ve věrnosti k jednotlivým formám květů.

Kombinací různých metodologických přístupů a experimentů tato práce poskytuje pohled na složité téma opylování a přenos pylu. Pochopení mechanismů, které ovlivňují přenos pylu je pak zásadní předpoklad pro pochopení struktury a odolnosti sítí vztah mezi opylovači a rostlinami s důsledky pro jejich poznání a ochranu

Klíčová slova

opylování; přenos pylu; generalizace-specializace; prezentace pylu; chování opylovače; věrnost opylovače

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1 Introduction

Plants are (usually) strictly limited in their ability to actively move and tend to stay in the same place for the majority of their lives. Yet, plants also usually benefit from exchanging their gametes among individuals, which is considerably limited by their inability to move. Thus, vascular plants adopted several mechanisms to exchange gametes among distant individuals, involving wind, water, or animals as pollen vectors. About 80-90 % of vascular plant species depend on their pollen transfer to animal pollinators (Ollerton et al., 2011). In most cases, insect pollinators serve as pollen vectors, transferring pollen from anthers to stigmas of conspecific plant individuals and consequently affecting seed production of a wide range of plant species.

However, animal pollinators are imperfect vehicles for pollen transfer, as they have their own needs and preferences, often in conflict with plant needs. From the plant perspective, pollinators should ideally carry pollen without losing it, eating it or placing it on flowers of another species. However, pollinators are not ideal pollen vectors and tend to optimize their foraging to collect suitable resources, find mates for reproduction and avoid predators and pathogens. As a result of the conflicting interests between plants and pollinators, only a tiny proportion of pollen grains produced by a plant successfully reach the stigma and fertilize ovules of another individual of the same species, with estimates that only about 1-10% of produced pollen grains are reaching stigma successfully (Thøstesen and Olesen, 1996; Johnson et al., 2004; Watts et al., 2013; Gong and Huang, 2014; Parker et al., 2016) and even less fertilize the ovules (Wist and Davis, 2013).

To secure pollen transfer, plants need to attract pollinators to carry pollen. However, pollinators significantly differ in their ability to transfer pollen and contribute to plant sexual reproduction (Willmer and Finlayson, 2014; Page et al., 2021). Moreover, some flower-visiting animals may even be considered pollen or nectar thieves (Castro et al., 2013; Parker et al., 2016) rather than pollinators and may even have negative impact on plant reproduction (Burkle et al., 2007; Bergamo et al., 2018; Rojas-Nossa et al., 2021). Thus, plants are under strong pressure to specialize in the most suitable set of pollinators in order to secure successful pollen transfer. Similarly, pollinators visit flowers which are attractive and promise suitable rewards. Consequently, plants and pollinators may co-evolve in their traits to increase the mutual benefits from their interactions and fitness, resulting in the development of distinct “pollination syndromes”, i.e., suites of traits shared among plant species corresponding to the traits of their pollinators with the highest impact on plant fitness (Faegri and Pijl, 1979; Fenster et al., 2004).

The concept of pollination syndromes promised an elegant and straightforward approach how to predict the “main” pollinator responsible for the majority of the pollen transfer (Fenster et al., 2004) and to which the plant should be specialised (Stebbins, 1970). This can be nicely demonstrated by the example of *Angraecum sesquipedale* Thouars, known as Darwin’s orchid, for which the identity of pollinator was successfully predicted based on the presence of a long spur allowing access to the reward only to pollinators with similarly long proboscis (the story

is reviewed in Arditti et al. (2012)). Based on this example, researchers in past decades have investigated multiple examples of pollination syndromes, e.g., for birds (Wester and Claßen-Bockhoff, 2011; Chmel et al., 2021), flies (Chen et al., 2015) or tangle-veined flies (Pauw et al., 2022). Furthermore, it has been proposed that pollination syndromes play a significant role in the structure of plant-pollinator networks (Lázaro et al., 2008; Danieli-Silva et al., 2012) and in the evolution of flower traits (Assis, 2023), which is highlighted by switches between the syndromes. Hence, the concept of pollination syndromes has played a crucial role in pollination ecology in past decades.

However, pollination syndromes have only limited ability to predict the whole composition of pollinators visiting plant species (Ollerton et al., 2009; Rosas-Guerrero et al., 2014; Abrahamczyk et al., 2017). This is most likely caused by the fact that a significant proportion of plant-pollinator interactions are subjected to variation on multiple spatial (Price et al., 2005; Gómez and Perfecttiy, 2010; Zych et al., 2019) and temporal (Schwarz et al., 2020; Tobajas et al., 2023) scales and thus it is complicated for both researchers and plants to predict the species identity of the pollinator visiting flowers and transferring pollen in particular plant population without empiric experience. With the increasing degree of stochasticity in the pollinator spectra and plant populations, close specialisation with a limited set of partners is potentially risky for both plants and pollinators, increasing the threat of co-extinction if one of the partners is removed from the system. Similarly, plant reproduction may be hampered by a decrease in abundance of the pollinator species (Delmas et al., 2015). Hence, generalisation of the plant pollinator spectra may help to secure pollination in the case of local extinction or decrease in abundance of pollinator species as plants can utilize another pollinator species to deliver pollen successfully. However, the degree of generalisation in plant-pollinator systems in the temperate zone is still largely unknown, with only limited evidence suggesting a high degree of generalisation (Waser et al., 1996; Olesen and Jordano, 2002; Ollerton et al., 2007; Herrera, 2020). Similarly, despite the general expectation that plant-pollinator interactions are more specialised in the tropics, current knowledge is still insufficient (Johnson and Steiner, 2000).

Hence, significant proportion of plants has to deal with uncertainty originating in the wide spectra of possible flower visitors and potential pollinators. Plants thus adopted several mechanisms allowing them to increase probability and specificity of pollen transfer, including (but not limited to) attracting wide spectra of pollinators by possessing attractive floral traits (Bukovac et al., 2017), timing interactions with pollinators to avoid competition (Stone et al., 1998; Štenc et al., 2023) separating in space (Kipling and Warren, 2014) and promoting pollinator flower constancy (Waser, 1986a; Huang et al., 2015; Brosi, 2016; Us Van Der Niet et al., 2020). Moreover, all of the mentioned mechanisms (and even more other ones) are interacting together and, in conclusion, lead to more specialised interactions on the level of individuals than could be expected from the observations on the population level (Brosi, 2016). This is highly important as we still do not fully understand all the mechanisms leading to temporal specialization and the effect on pollen transfer separately, and knowledge of their interactions is basically anecdotal. Here are more detailed examples on the timing of pollen release and flower constancy and how

these mechanisms contribute to the temporal specialisation in pollen transfer.

Depending on the composition of plant-pollinator spectra, plants encounter a dilemma regarding optimising the amount of pollen they should present to pollinators during a visit. Variations in pollinator abilities to carry pollen and in visitation activity contribute to this challenge: Plants may suffer from low visitation rates by their primary pollinators, potentially limiting pollen transfer (Delmas *et al.*, 2015), or they may be visited by numerous pollen-wasting pollinators, increasing the loss of available pollen (Parker *et al.*, 2016). Therefore, the Pollen Presentation Theory (PPT) predicts that plants should optimize the amount of pollen presented to a single pollinator to limit potential losses caused by pollen-wasting pollinators (Harder and Thomson, 1989; Thomson, 2006a). Theoretical models predict that partitioning and gradual pollen dispensing may be advantageous for plants with abundant but less efficient pollinators (Harder and Thomson, 1989; LeBuhn and Holsinger, 1998). In contrast, plants with less abundant but highly efficient pollinators are supposed to benefit from placing as much pollen as possible on the pollinator during a single visit (Castellanos *et al.*, 2006). Different pollen presentation strategies are allowed by various pollen packing and dispensing mechanisms (Lloyd and Yates, 1982). For example, some plants possess explosive pollen release mechanisms (reviewed in Aluri and Reddi (1995)), allowing rapid release of a vast proportion of the pollen from the flower during the first pollinator visit. Other plants dispense pollen by gradual maturation of anthers within the flower, presenting to pollinators pollen from only a small fraction of anthers (Ren and Bu, 2014). However, the interplay between pollen presentation, pollen pick-up from flowers by pollinators and consequent timing of pollen transfer and deposition still needs to be clarified.

A higher degree of generalisation within the plant-pollinator community causes multiple pollinators to be shared among plant species. Sharing pollinators helps to attract pollinators (Feldman *et al.*, 2004; Mesgaran *et al.*, 2017), which may be crucial in preventing the collapse of plant-pollinator interactions in the event of local extinctions (Memmott *et al.*, 2004; Kaiser-Bunbury *et al.*, 2010), but sharing pollinators often leads to heterospecific pollen transfer, i.e., transfer of pollen among different plant species (Ashman *et al.*, 2020), decreasing both male and female reproduction success (Ashman *et al.*, 2004; Ashman *et al.*, 2020). To limit heterospecific pollen transfer, plants may motivate pollinators to remain flower constant i. e., temporally specializing in visiting only one plant species during the foraging sequence (Waser, 1986b; Montgomery, 2009). Flower constancy is then predicted to be a crucial factor in species co-existence in a co-flowering community with a higher degree of generalisation (Brosi, 2016; Janovský *et al.*, 2017). Up to now, flower constancy was confirmed in bees (Waser, 1986b; Gegear and Laverty, 2005; Raine and Chittka, 2007), butterflies (Goulson and Cory, 1993), and hoverflies (Goulson and Wright, 1998). Studies showed that to stimulate pollinator constancy, plants have to possess traits that allow to pollinators to distinguish them (Ne'eman and Kevan, 2001). However, only a few studies experimentally compared the effect of variation in more than two traits on the stimulation of flower constancy (e.g., Gegear and Laverty, 2005). Therefore, further investigation is necessary to properly answer how flower traits interact and how the interactions among

them affect pollinator behaviour.

Pollination and pollen transfer are complex phenomena affected by numerous drivers (see [Minnaar et al., 2019](#) for review). Therefore, it is challenging to investigate all the mechanisms and drivers simultaneously, especially in a limited time. Hence, the focus of this thesis is limited to 3 main topics corresponding to three important drivers affecting pollen transfer (Fig 1.) without the ambition to cover all the topics comprehensively. The topics of the thesis are highlighted in the introduction and correspond to individual chapters of the thesis: composition of plant pollinator spectra, pollen presentation and pollinator behaviour (Figs. 2 and 3).

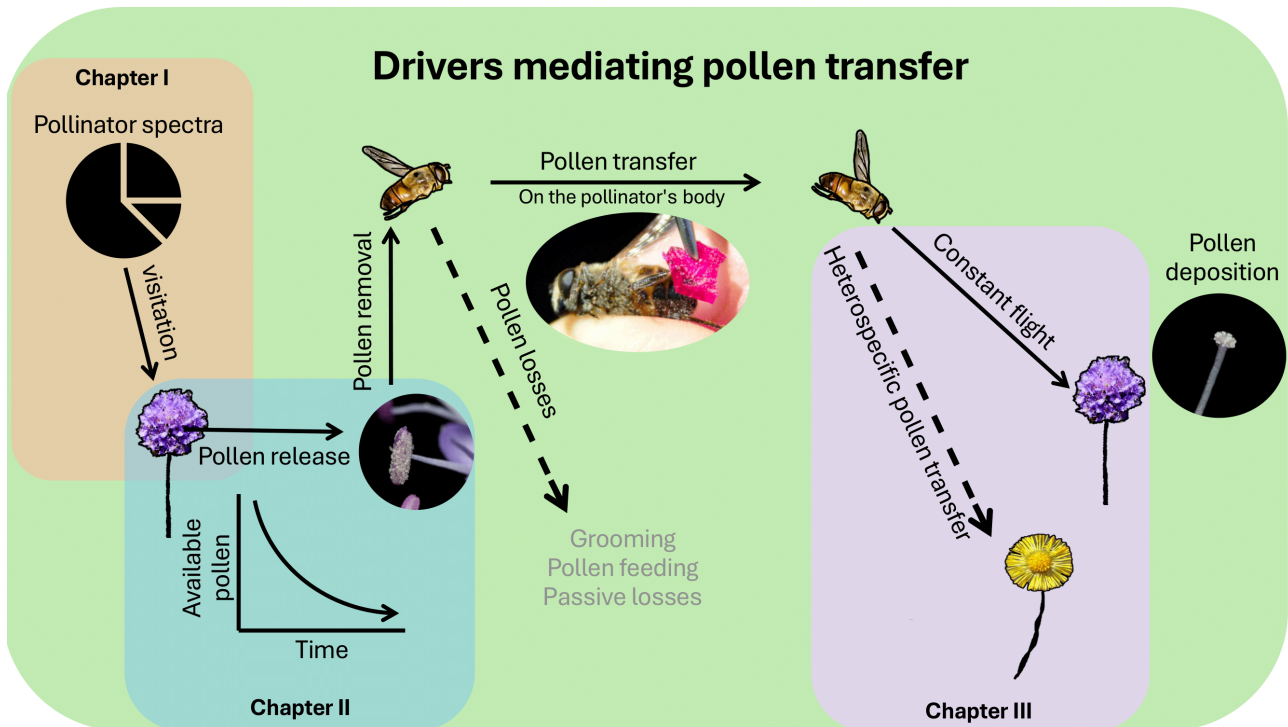


Fig 1. Illustration of pollen transfer. Topics of individual chapters are in coloured rectangles.

2 Aims

In general, this thesis aims to investigate selected drivers affecting pollen transfer in generalized plant-pollination system. In particular, this thesis study how composition of plant pollinator spectra, pollen presentation and pollinator behaviour can shape the pollen transfer.

I ask three specific questions:

- 1) What determines the composition of plant-pollinator spectra? (Chapter I article 1)
- 2) How does pollen presentation affect the pattern of pollinator visitation and pollen transfer? (Chapter II, articles 2 and 3)
- 3) How do flower traits and pollinator experience influence pollinator behaviour and pollen transfer? (Chapter III, articles 4 and 5)

To answer these questions, I combined diverse methodological approaches and several experiments (see Fig 2). I contributed to investigating plant pollinator spectra based on published data (Chapter I, article 1). I conducted fieldwork experiments in the long-term studied plant-pollinator community, focusing on the mechanisms of pollen release in three co-flowering plant species and the link to pollinator visitation activity (Chapter II, article 2). Based on the pollen release mechanisms, I studied the diurnal dynamic in pollen load composition on pollinator bodies (Chapter II, article 3). In collaboration with my colleagues, I performed manipulative experiments to test the effect of floral traits (Chapter III, article 4) and experience (Chapter III, article 5) on the foraging behaviour of *Eristalis tenax*, a model species of hoverfly pollinator.

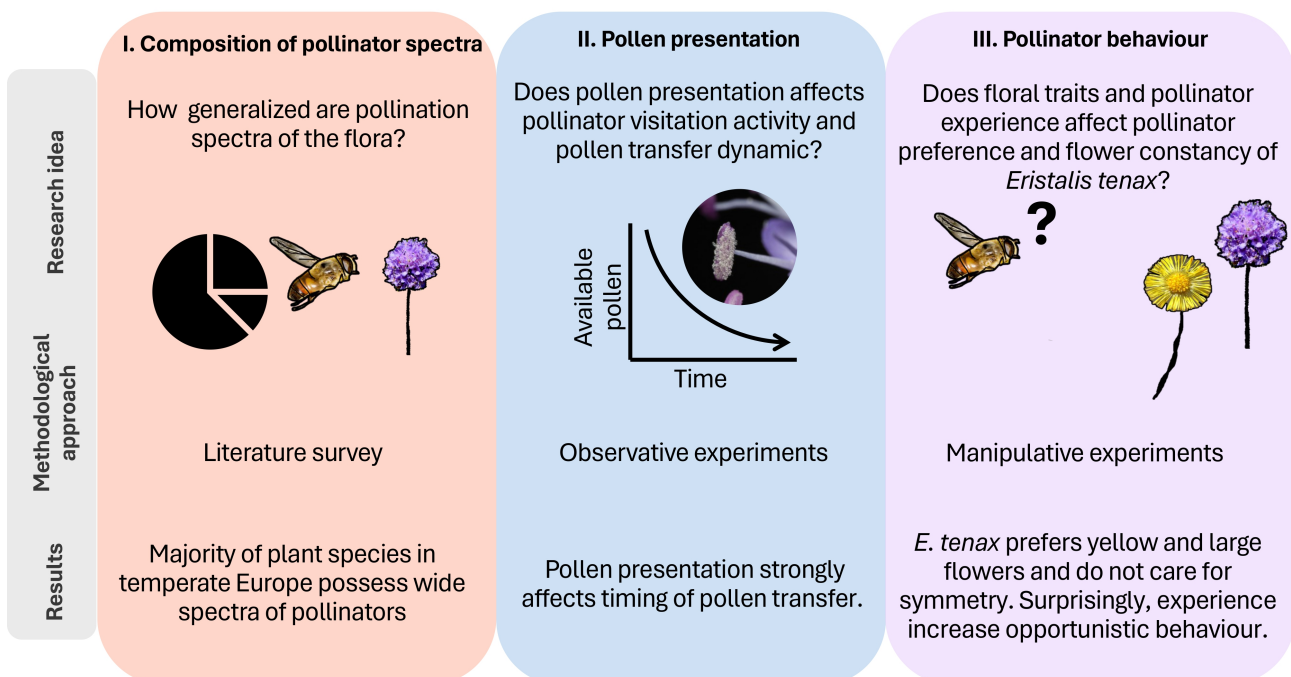


Fig. 2: Illustration of particular chapters with outlined research ideas, methodological approaches and results.

3 Chapter I: Composition of pollinator spectra

The knowledge of plant-pollinator spectra is essential for a basic understanding of the effectiveness of plant pollination Gong and Huang (2014) as well as the susceptibility of the plant species to declines in pollinator diversity (e.g. Biesmeijer et al. (2006), Powney et al. (2019)). The composition of a plant's pollinator spectrum has been hypothesised to be mainly connected with plant traits and phylogeny (Ellis and Ellis-Adam, 1993; Fenster et al., 2004), formulating the so-called pollination syndromes, which predict that plants should possess a particular suit of traits based on their primary pollinator (Faegri and Pijl, 1979; Fenster et al., 2004). However, the power of floral traits to predict a plant's pollinator spectrum is limited (Ollerton et al., 2009; Wang et al., 2020), especially for species with floral traits allowing generalised pollinator spectra, which make up the majority of plant communities (Waser et al., 1996; Herrera, 2020).

Why does the generalisation of plant pollinator spectra matter? According to Stebbins (1970), plants should evolve in close relationship with the pollinator responsible for most plant sexual reproduction via pollen transfer. This was widely demonstrated in systems where switches in the pollinator syndrome are apparent, especially from bee to bird-pollinated plants (Castellanos et al., 2004). However, a substantial proportion of plants is visited by broad spectra of pollinators, which may contribute similarly to pollen transfer (Waser et al., 1996; Herrera, 2020). Moreover, the composition of plant-pollinator spectra may be variable across space (Thompson, 1997; Thompson, 2001; Moeller, 2005; Brunet and Sweet, 2006; Gómez and Perfecttiy, 2010; Niemirski and Zych, 2011) and time (Petanidou et al., 2008; Gruchowski-Woitowicz et al., 2023; Tobajas et al., 2023), resulting in different selection pressures in individual populations which may hinder the adaptation of floral traits toward one pollinator. To address this challenging issue, Ohashi et al. (2021) presented a model of trade-off mitigation, proposing a theoretical framework to understand how the selection of floral traits by multiple pollinators works simultaneously on the selection of floral phenotype, demonstrating that adaptive generalization is at least theoretically possible.

Besides selection pressure on flower traits, possessing highly generalised pollinator spectra may also be challenging in a co-flowering plant community (Kooi et al., 2016). On the one hand, the ability to utilise multiple pollinators may help avoid pollen limitation caused by the decline of the pollinator population. Depending on a single pollinator puts plants at a higher risk of pollen limitation (Albrecht et al., 2012). On the other hand, broader spectra of pollinators increase the probability of sharing pollinators with co-flowering plants which may led to a higher degree of heterospecific pollen transfer (Bruninga-Socolar et al., 2023).

Besides floral traits, other niche-related ecological characteristics, such as niche width or ability to dominate a local community, are likely to contribute to the structure of pollinator spectra of plant species with floral traits, allowing plant generalisation. The niche width of the flowering plant is directly connected with the variability of co-flowering community composition, i.e., species with wider niches are more likely to co-flower with a wider variety of co-flowering

communities and vice versa. The species composition of a community of co-flowering species reportedly affects pollinator spectra (Potts et al., 2003; Lázaro et al., 2009) with both negative (Moeller, 2005; Ghazoul, 2006) and positive (Bergamo et al., 2020) effects on the pollen transfer of the co-flowering plant species. Similarly, local plant abundance affects abundance (Hegland et al., 2009; Janovský et al., 2013) and composition (Hegland and Boeke, 2006; Stang et al., 2006; Vázquez et al., 2009; Janovský et al., 2013) of plant pollinator spectra. Based on the plant-pollinator network studies (Stang et al., 2006; Vázquez et al., 2009) and theoretical model (Sargent and Otto, 2006) an increase in local abundance (i.e., dominance) may be expected to increase the degree of plant pollinator spectra generalisation. Moreover, increase in local abundance may increase the attractiveness of the plant species to particular pollinators, e.g., social hymenoptera, especially honeybees (Ginsberg, 1983; Tschardt and Steffan-Dewenter, 2000).

3.1 Research summary of the chapter I

3.1.1 Pollinator community and generalisation of pollinator spectra changes with plant niche width and local dominance

Zdeněk Janovský, **Jakub Štenc**

In this study we focused on the composition and degree of generalisation of pollinator spectra of plant species in a database compiled from mainly Central European plant species. We hypothesize that: 1) locally dominant plants can be expected to have more generalized pollinator spectra with a higher proportion of pollinator functional groups either specializing in highly concentrated resources (honeybee) or of pollinator functional groups which are numerous and rather opportune (e.g., muscids having alternative food sources); and 2) plant species with wider niches should have more generalized pollinator spectra. We test these hypotheses by compiling a database of pollinator spectra of 259 Central European plant species from literature and relating it to information on species' niche widths and local dominance derived from the vegetation plots obtained from Czech National Phytosociological Database (Chytrý and Rafajlová, 2003). We focus our analyses on the overall composition of pollinator functional groups and their Simpson diversity, which we take as a measure of generalisation of the pollinator spectrum. We also analyse specific responses of proportions of the pollinator functional groups in pollinator spectra. This allows us to detect any specific responses of pollinator spectrum composition to plant local dominance and niche width.

Our results showed that the composition of plant-pollinator spectra is formed by two main gradients: From bumblebee-dominated plants to other pollinators which are dominated by flies or hoverflies and nitinulid beetles on the other side of the second gradient. However, majority of plants form a bulk of generalized species without any inclination to the extremes of the gradients. Moreover, our results showed effect of both plant niche width and plant ability to dominate in the vegetation on the composition of pollinator spectra.

The manuscript was published in *Functional Ecology* in article called “in co-authorship of Zdeněk Janovský (supervisor) and Jakub Štenc ([Janovský and Štenc, 2023](#)).

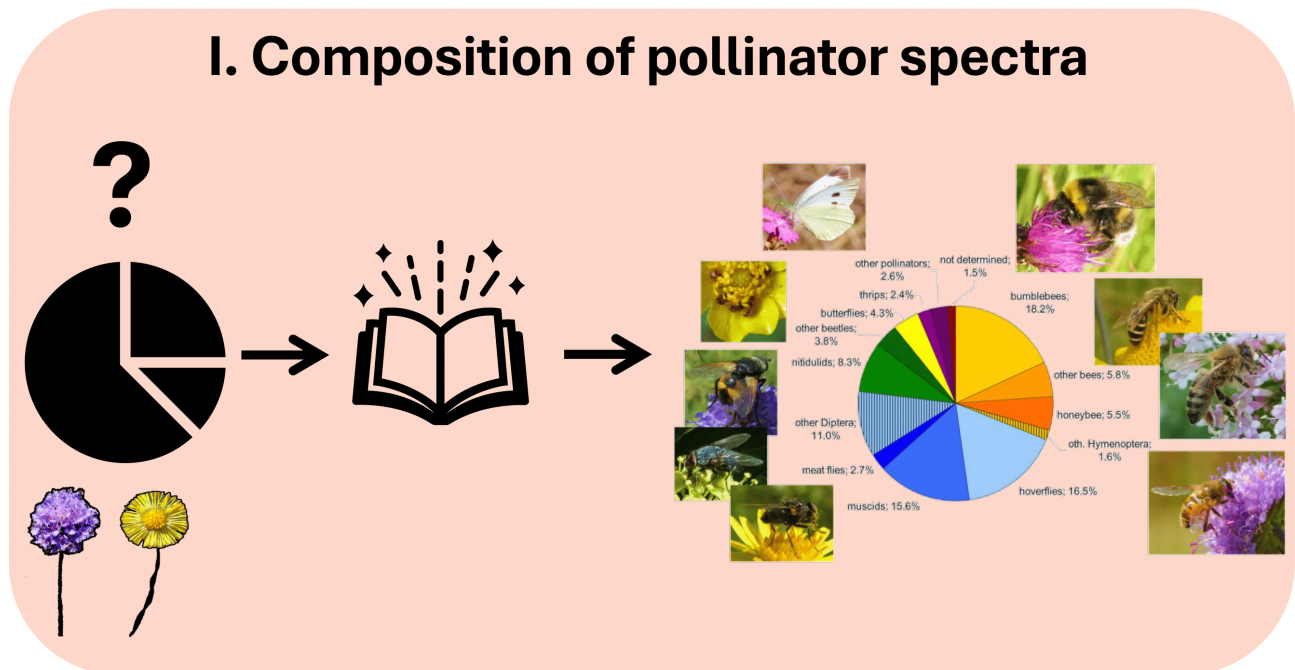


Fig. 4: Graphical summary of chapter I.

4 Chapter II: Pollen presentation and diurnal structure of plant-pollinator interactions

The low pollen transfer efficiency and losses of pollen caused by pollinators strongly affect the economics of plant investments in the male component of fitness (Harder and Thomson, 1989; Johnson and Harder, 2023). Thus, the selective pressure to increase pollen transfer efficiency may be driving the evolution of plant adaptation, such as flower morphology, which may help to restrict or allow access to pollen depending on the most suitable strategy. This may be demonstrated by the wide variety of pollen presentation strategies which have developed over plant species. However, it is the pollinators with interspecific variation in pollination efficiency and visitation rate that link pollen release strategy and pollen transfer efficiency (Harder and Thomson, 1989).

According to Pollen Presentation Theory (PPT), plants may optimize the amount of pollen presented to a single pollinator based on pollinator efficiency and frequency (Harder and Thomson, 1989). While plants visited by efficient and less frequent pollinators may benefit from presenting a higher amount of pollen to a single pollinator (e.g., orchids), plants with less efficient, but frequent pollinators may present pollen in small doses or packages gradually to several visiting pollinators to decrease the probability of losing all pollen by pollinator feeding on the pollen, inefficient transfer or simply by pollinators visiting other plant species less constantly in following visits.

Different pollen dispensing mechanisms have evolved among plants to adapt pollen-presenting strategies (e.g., Howell et al., 1993; Aluri and Reddi, 1995; Erbar and Leins, 1995). For example, within the Fabaceae family, mechanisms can be found for explosive pollen release for rapid pollen release (Galloni et al., 2007; Figueroa Fleming and Etcheverry, 2017), but also mechanisms allowing gradual pollen release (Figueroa Fleming and Etcheverry, 2017). Few families (Asteraceae, Campanulaceae, etc.) possess so-called pumping mechanisms where stigma growing through a closed flower pump up pollen to the upper part of the flower, where it can be collected by pollinators (Erbar and Leins, 1995). Another example is the so-called buzz-pollination mechanism (Vallejo-Marín, 2019; Kemp and Vallejo-Marín, 2021), when plants present pollen within poricidal anthers, i. e., anthers opening by one hole and effectively working as a salt-shaker. To collect pollen grains, the pollinator has to vibrate the anther in a particular range of frequencies (Kemp and Vallejo-Marín, 2021), allowing controlled pollen removal from anthers (Harder and Barclay, 1994). Pollen releasing mechanisms then represent a gradient of strategies that demonstrate plant ability to adopt different strategies (even within a plant family) and optimize the amount of pollen presented to their pollinators at the time (Harder and Thomson, 1989).

Moreover, inflorescent architecture may also play a significant role in pollen presentation and plant-pollinator interactions (Harder et al., 2004). Pollinators tend to visit more flowers within a single inflorescence (Jordan and Harder, 2006), but they usually adopt particular a visitation

pattern, e.g., bees tend to first visit lower flowers within the inflorescence and prospecting it in the upward direction (Corbet, 1978; Jordan and Harder, 2006; Ishii et al., 2008). Similarly, by adopting a particular pollen release pattern within the inflorescence plants may be able to actively affect the efficiency of pollen transfer (Barrett et al., 1994; Ishii et al., 2008; Iwata et al., 2012; Liao and Harder, 2014).

By combining the inflorescence development and pollen presentation, plants may develop a pollen presentation schedule, i.e., release pollen at a particular time during the day. The benefits of adopting a pollen presentation schedule are generally twofold: i) increased pollen transfer by fitting the presentation schedule to the timing of pollinator activity, e.g., releasing pollen during the night hours in the case of plants with nocturnal pollinators; ii) mitigating competition for pollinators due to their temporal specialization (Brosi, 2016), i.e., if plants share pollinators, they can avoid transfer of heterospecific pollen by releasing pollen at different periods of pollinator activity (e.g., Stone et al., 1998).

Despite vast evidence on different pollen-releasing strategies and flower/inflorescence morphology and architecture, our knowledge of the ecological significance of pollen-release strategy in structuring the plant-pollinator community is very limited. Studies such as that of Stone et al. (1998) are rare examples of plants mitigating competition through diurnal stratification of pollen release, which limits our knowledge about the general impact of diurnal stratification on plant-pollinator communities. Therefore, investigation of the diurnal pollen release schedule coupled with patterns of pollinator visitation activity and linked with proxies of pollen transfer (e.g., the composition of transferred or deposited pollen, seed set) are crucial for further understanding competition and facilitation for pollinators among plant species.

4.1 Research summary of the chapter II

In this chapter, I conducted a set of experiments on estimating the availability of pollen on the flowers of three co-flowering plant species during the day and the amount of pollen transferred on pollinators' bodies. I coupled the data with the diurnal pattern of pollinator visitation activity to estimate the difference in pollinator contribution to pollen transfer during the day. The experiments demonstrated the diurnal structure in plant-pollinator interactions driven by the pattern of pollen release (Štenc et al., 2023) and the consequent effect on the amount of pollen transferred on pollinator bodies (Chapter II, Article 3, manuscript in preparation).

4.1.1 Pollinator visitation closely tracks diurnal patterns in pollen release

Jakub Štenc, Lukáš Jánošík, Eva Matoušková, Jiří Hadrava, Michael Mikát, Zdeněk Janovský

In this study, we investigate pollen presentation schedule (i.e., the amount of pollen available at the time to the flower visitor) of three co-flowering species and the diurnal visitation pattern of their pollinators, aiming to explore how closely pollinator activity tracks pollen availability. For our study, we selected three plant species exhibiting differences in their pollen release

mechanism, flower and inflorescence organization and partial overlap in their pollinator spectra. Particularly, we address the following questions: I. Does pollen availability show a pattern during the day? II. Does pollinator visitation activity track pollen availability?

To achieve our aims, we conducted experiment with three plant species: *Trifolium hybridum*, *Centaurea jacea* and *Succisa pratensis*. The study species differ in their pollen dispensing mechanisms: *Trifolium* flowers are closed, with an explosive release of pollen towards the front part of the pollinator body as was described in other Fabaceae genera (Galloni et al., 2007); *Centaurea* presents pollen by so-called pump mechanism allowing gradual pollen release, where pollen is moved up from closed tube by the growing style (as described in Erbar and Leins (1995)); *Succisa* releases pollen openly on four anthers sticking out from the flower and the pollen is easily accessible to pollinators. All studied species possess flowers organized into inflorescences.

To estimate the availability of pollen during the day, we collected flowers from the locality in one hour intervals from 8 to 17 hours in 2 consequent days. During the third day we estimated the number of active flowers in randomly collected inflorescences from the locality. Then we estimated the amount of pollen available per inflorescence during the day. To link this pattern with the pollinator activity, we utilized data from 11 years of observation of pollinator activity with records on the time of the interaction.

Results showed differences in pollen availability among studied species (Fig. 7), which all possessed different patterns in pollen availability: *Trifolium* presented the majority of the pollen in late hours (14-15), *Centaurea* dispensed pollen gradually, peaking at noon and *Succisa* presented pollen in morning hours, quickly depleting all pollen from flowers. Pollinator activity roughly tracked pollen availability, with considerable delay on *Succisa*, possibly caused by lower pollinator activity in colder morning hours affected with morning dew (Fig. 8).

The project was supported by START project (START/SCI/141). The manuscript was published in *American Journal of Botany* as part of a part of joint special issues of the *American Journal of Botany* and *Applications in Plant Sciences*: “Pollen as the Link Between Phenotype and Fitness” (Štenc et al., 2023).

4.1.2 Diurnal stratification of pollen presentation structures the pattern of pollen transfer and mitigate competition for pollinators

Manuscript submitted to *Journal of Ecology*, 7. 12. 2023

Jakub Štenc, Lukáš Janošík, Martin Freudenfeld, Eva Matoušková, Jiří Hadrava, Michael Mikát, Klára Daňková, Tereza Hadravová, Tadeáš Ryšan, Jasna Simonová, Klára Koupilová, Alice Havelková, Eliška Konečná, Jan Martínek, Barbora Jelínková, Stanislav Vosolsobě, Karolína Dobešová, Marie Smyčková, Jan Smyčka, Jitka Smyčková, Lucie Studená, Josef Nováček, Štěpán Káně, Kateřina Knotková, Tomáš Figura, Jan Simon-Pražák, Kateřina Čermáková, Jiří Janoušek, Václav Bočan, Vojtěch Brož, Anna Suchá, Eva Horčíčková, Tomáš Dvořák, Tomáš

Jor, Doubravka Požárová, Karel Plavec, Zdeněk Janovský

To connect the pattern of pollen presentation and pollinator visitation activity with the pattern of pollen transfer, we investigated pollen carried by individual pollinators during the day. We counted both conspecific and heterospecific pollen carried by pollinator regarding the last plant species visited by the pollinator as an measurement of pollen quantity and quality, respectively. Then we estimated visitation density for the pollinators of respected plant species in 10 minutes long intervals during the activity period.

Our results showed different patter of pollen transfer during the day of different plant species, which we explain by the differences in pollen releasing mechanism and pollinator foraging behavior: *Trifolium hybridum* posses explosive pollen release mechanism leading to specific placement of pollen on the pollinator body and possibly more precise pollen transfer even with lower density of visits. *Centaurea jacea* stimulates flower constancy and specificity of pollen transfer due to high plant density, with increase of carried pollen by pollinators in time during the day. *Succisa pratensis* possess strict pollen release schedule, followed by a close pollinator visitation pattern, resulting in high specificity of pollen transfer for a short period of time in the morning hours.

We conclude that our results unravel the diurnal structuring of pollen transfer and the plant's ability to mitigate the negative effect of pollinator sharing. Further, we discuss how diurnal schedule of pollen transfer may affect inner temporal structure of plant-pollinator network, allowing long-term coexistence in generalized plant-pollinator community.

The project was supported by START project (START/SCI/141).

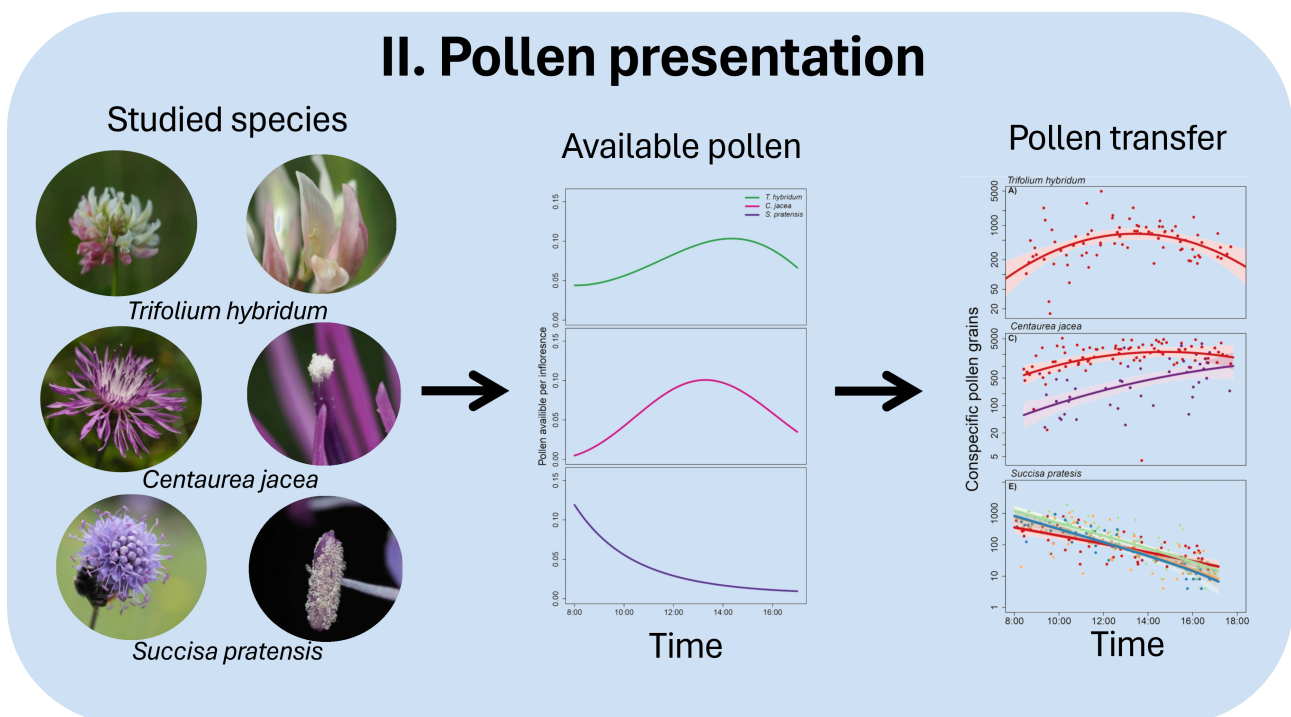


Fig. 4: Graphical summary of chapter II.

5 Chapter III: Pollinator foraging behaviour - effect of preference and flower constancy

Pollination and pollen transfer happen on a very fine scale, i.e., an individual pollinator visits an individual flower from which it transports pollen to another individual flower. The sum of these interactions later forms plant-pollinator interaction networks (Tur et al., 2014). Thus, while studying the whole networks of species interaction may be informative about the overall ecology of the plant-pollinator community, investigation of individual pollinator behaviour provides further insight into how and why the networks are constituted (Brosi, 2016). Therefore, our understanding of the factors affecting pollinator behaviour and visitation choices such as pollinator preferences and flower constancy, is crucial for a deeper understanding of how the structure of plant-pollinator networks is formed.

For example, individual pollinators possess particular preferences based on several floral and inflorescence traits, which are consequently responsible for pollinator visitation choices. Thus, variation in flower traits such as colour, size and/or another trait affects pollinator visitation behaviour and, consequently, pollen removal and deposition (Johnson et al., 1995). Pollinator preferences may, therefore, be one of the main evolutionary drivers of flower evolution in order to increase the pollinator visitation rate (Schiestl and Johnson, 2013; Trunschke et al., 2021). Preferences differ among pollinator taxa (Lunau and Maier, 1995); however, despite decades of research, the experimental evidence is strictly limited to only a few taxa with the main emphasis on bees. Moreover, it has been reported that preferences can be shaped by pollinator experience (Hill et al., 1997; Gumbert, 2000; Plowright et al., 2017) and depend on the context of both the composition of possible choices (Gegear and Laverty, 2005; Hersch and Roy, 2007) and environmental conditions (Finnell and Koski, 2021), our understanding of which needs to be deepened.

Flower constancy, i.e., consequent visits to the same flower form in the presence of other flower forms (Waser, 1986a), can increase pollen transfer efficiency and secure successful pollen transfer even in a plant community that shares pollinators (Goulson, 1994; Montgomery, 2009; Janovský et al., 2017). Thus, to understand the structure of the plant-pollinator network, we need to first understand how the individual pollinators choose the flowers to visit during their foraging bouts (Brosi, 2016). While some traits are considered to stimulate flower constancy (e.g. flower colour (Gegear and Thomson, 2004) and size (Gegear and Laverty, 2005)), their relative importance is still largely unknown. During their lives, pollinators may encounter multiple flowers, with various traits and reward levels, resulting in different experiences of foraging individuals and levels of flower constancy (Goulson and Cory, 1993; Hill et al., 1997; Raine and Chittka, 2007). Thus, manipulative experiments with full-factorial design are necessary to disentangle the complex nature of pollinator decision-making.

5.1 Research summary of the chapter III

In this chapter, I employed two sets of experiments with individuals of *Eristalis tenax* L. (Syrphidae), a common dipteran pollinator with known innate preferences toward yellow colour. To perform the experiments, I used artificial flowers with constant rewards prepared on the 3D printer to easily manipulate floral traits. In the first experiment naive individuals of *E. tenax* showed expected preferences toward yellow and large flower forms, but unexpectedly strong interactions between the traits boosting their preferences (Matoušková et al., 2023). In the second experiment, experienced hoverflies showed surprisingly lower preferences and flower constancy, suggesting that previous experience may cause an increase in the opportunistic behaviour of *E. tenax* (Chapter II, Article 3, manuscript in preparation).

5.1.1 Innate preferences of *Eristalis tenax* L. (Syrphidae) for flower colour, size and symmetry are more intricate than the simple additive model

Published in *Biological Journal of the Linnean Society*, 03 July 2023 , <https://doi.org/10.1093/biolinnean/blad035>

Eva Matoušková, **Jakub Štenc**, Zdeněk Janovský

In this study we focused on the preferences of naive individuals of *Eristalis tenax* - important and abundant pollinator, known for visiting a broad spectrum of flowering plants with different flower traits. To test the effect of different floral traits on pollinator preference we used arrays of artificial flowers printed on a 3D printer. Artificial flowers were designed to vary in 3 traits: size (big x small), colour (yellow x blue) and symmetry (actinomorphic x zygomorphic). In our experiment, we used flowers of all possible combinations of these traits in arrays consisting of 10 flowers of two “forms” (20 flowers per array in total). This resulted in 32 treatments combining all morph pairs in a full factorial design. The experiment was conducted in the laboratory conditions with reared naive individuals of *E. tenax*, allowing us to study the behavioral response of inexperienced individuals.

Naive individuals of *E. tenax* showed strong preference toward yellow colour, amplified by the flower size. However, flower symmetry does not seem to affect *E. tenax* preferences, which is in contrast with experiments with bees. We conclude, that the *E. tenax* preferences are affected by interaction among the floral traits, which is more intricate than could be assumed by simple additive model.

The project was supported by GAUK (842120). The manuscript named “Innate preferences of *Eristalis tenax* L. (Syrphidae) for flower colour, size and symmetry are more intricate than the simple additive model” was published in *Biological Journal of the Linnean Society* (Matoušková et al., 2023).

5.1.2 Learning to be unfaithful - Flower constancy of *Eristalis tenax* L. (Syrphidae) decreases with experience

Manuscript in preparation

Alice Haveldová, **Jakub Štenc**, Eva Matoušková, Zdeněk Janovský

Following previous experiment, we compared both preference and flower constancy of naive and experienced *Eristalis tenax*. We aimed to study the effect of learning on both aspects of *E. tenax* foraging behaviour with reared naive individuals and individuals with previous experience with foraging in the diverse flowering plant community, with known visitation pattern. We tested the preference and flower constancy using artificial flowers differing in colour (yellow x white) and size (big x small). Flowers were arranged in array consisting from two forms and pollinator behaviour was recorded with camera in laboratory and field conditions under the same experimental setup.

Experienced individuals of *E. tenax* showed lower preferences and flower constancy, suggesting that previous foraging experience may increase the tendency toward opportunistic behaviour. We conclude, that despite strong innate preference toward yellow flowers, individuals of *E. tenax* are changing their behaviour based on the flower community they forage in, allowing them to adapt to the different flower resources.

The project was supported by GAUK (842120).

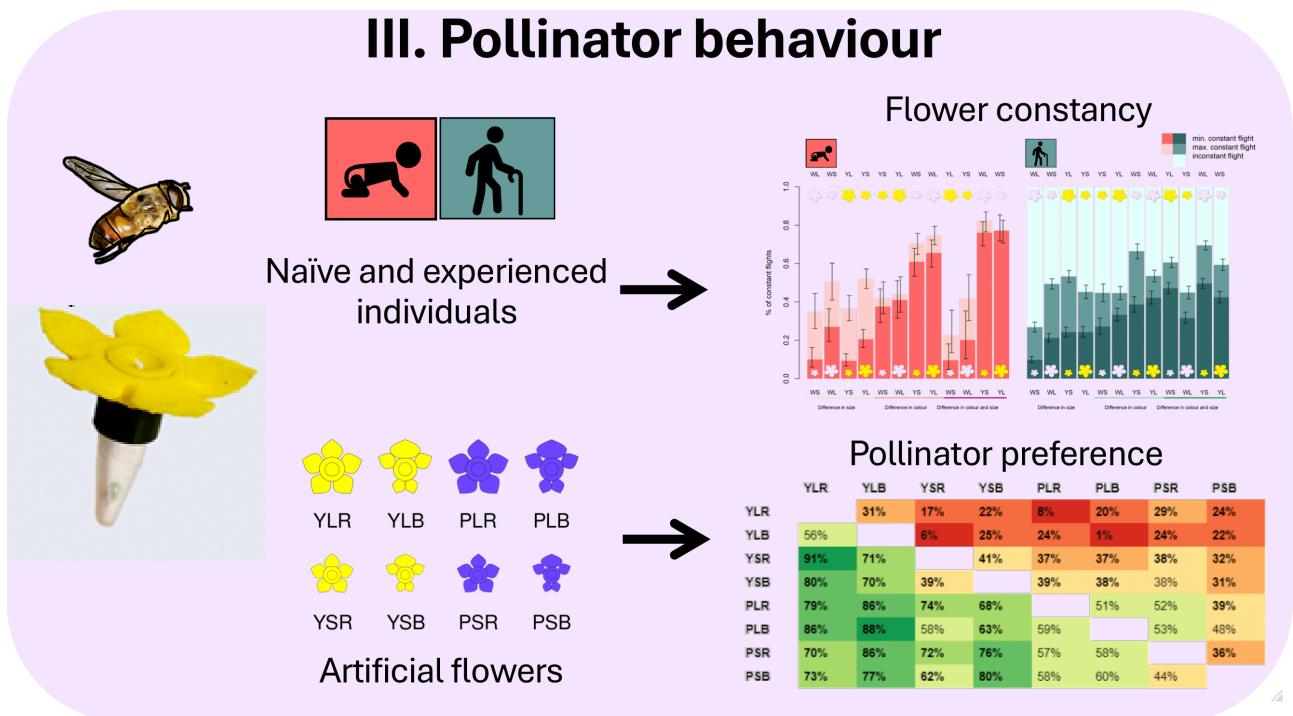


Fig. 5: Graphical summary of chapter III.

6 General conclusions

Plant-pollinator interactions and pollen transfer are intricate ecological phenomena with profound implications for ecosystems and our lives. This thesis presents the summary of my research, consisting of five original research articles focusing on pollination and pollen transfer. I conducted several experiments centered around three key drivers of pollen transfer: the composition of plant-pollinator spectra, pollen presentation and diurnal dynamics in pollen transfer, and pollinator behavior.

In Chapter I, I examined how plant local dominance and niche width influence the of pollinator spectra of the plant species. I found that plants with broader niches attract a more generalised set of visitors, whereas those with higher local dominance tend to be visited predominantly by specific groups, e.g., honeybees and flies. These results highlight the importance of plant life-history traits alongside flower characteristics in shaping pollinator spectra. Furthermore, my study presents data on many plant species from temperate Europe, revealing a high degree of generalization in their pollinator spectra.

Chapter II investigated how pollen presentation influences pollen transfer by structuring pollinator visitation patterns. The results suggest that plants can temporally increase the specificity of pollen transfer by presenting pollen at different times of the day. This finding has implications for our understanding of plant-pollinator networks, emphasizing the importance of considering interactions at finer temporal scales.

In Chapter III, I explored the behaviour of *Eristalis tenax*, a common pollinator species, through manipulative experiments. These experiments revealed strong preferences of *E. tenax* for yellow-coloured and larger flowers and the impact of experience on flower constancy. The results indicate that *E. tenax* can adapt opportunistic behaviour in species-rich environments, potentially leading to higher levels of heterospecific pollen transfer. Plant adaptation mechanisms, such as specific pollen placement, may mitigate this effect.

In summary, my research underscores that while plant-pollinator interactions in temperate Europe are predominantly generalised, plants possess mechanisms to increase the temporal specificity of their interactions with pollinators, facilitating the long-term coexistence of plant-pollinator communities. However, the complexity of these interactions exceeds the scope of a single thesis, and further research is needed. Understanding the structure of plant-pollinator networks at various levels is crucial for predicting the effects of global changes on community stability. By unraveling individual mechanisms and their interactions, we can better anticipate the impacts of shifting interactions on plant-pollinator communities.

7 Author contribution statement

The presented thesis consists of general introduction, three published papers, two manuscripts under review and one unpublished manuscript. Author contributions to individual paper are as follows:

7.1 Chapter I: generalisation of pollinator spectra

7.1.1 Pollinator community and generalisation of pollinator spectra changes with plant niche width and local dominance

Zdeněk Janovský, **Jakub Štenc**

Published in *Functional Ecology*, 9 September 2023, <https://doi.org/10.1111/1365-2435.14439>

Zdeněk Janovský initiated the idea, collected data from the literature, Zdeněk Janovský and **Jakub Štenc** collected part of the data in the field, Zdeněk Janovský performed the analysis. Zdeněk Janovský and **Jakub Štenc** wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Jakub Štenc led the correspondence and review process with help of Tomáš Herben.

7.2 Chapter II: Pollen presentation and diurnal structure of plant-pollinator interactions

7.2.1 Pollinator visitation closely tracks diurnal patterns in pollen release

Jakub Štenc, Lukáš Jánošík, Eva Matoušková, Jiří Hadrava, Michael Mikát, Zdeněk Janovský

Published in *American Journal of Botany*, 18 May 2023, <https://doi.org/10.1002/ajb2.16179>

Zdeněk Janovský and **Jakub Štenc** conceived the idea of research. **Jakub Štenc** and Lukáš Janošík designed the method for sampling pollen and collected the pollen and inflorescence data. Eva Matoušková, Jiří Hadrava, Michael Mikát and Zdeněk Janovský collected the data on plant and pollinator abundances. Zdeněk Janovský and **Jakub Štenc** analysed the data. **Jakub Štenc** and Zdeněk Janovský wrote the manuscript with edits and comments from the rest of the authors.

Jakub Štenc led the correspondence and review process with help from co-authors.

7.2.2 Diurnal stratification of pollen presentation structures the pattern of pollen transfer and mitigate competition for pollinators

Manuscript in preparation

Jakub Štenc, Lukáš Janošík, Martin Freudenfeld, Eva Matoušková, Jiří Hadrava, Michael Mikát, Klára Daňková, Tereza Hadravová, Tadeáš Ryšan, Jasna Simonová, Klára Koupilová, Al-

ice Haveldová, Eliška Konečná, Jan Martínek, Barbora Jelínková, Stanislav Vosolsobě, Karolína Dobešová, Marie Smyčková, Jan Smyčka, Jitka Smyčková, Lucie Studená, Josef Nováček, Štěpán Káně, Kateřina Knotková, Tomáš Figura, Jan Simon-Pražák, Kateřina Čermáková, Jiří Janoušek, Václav Bočan, Vojtěch Brož, Anna Suchá, Eva Horčíčková, Tomáš Dvořák, Tomáš Jor, Doubravka Požárová, Karel Plavec, Zdeněk Janovský

The research idea: **Jakub Štenc**, Zdeněk Janovský, Lukáš Janošík; Data collection: **Jakub Štenc**, Lukáš Janošík, Martin Freudenfeld, Eva Matoušková, Jiří Hadrava, Michael Mikát, Klára Daňková, Tereza Hadravová, Tadeáš Ryšan, Jasna Simonová, Klára Koupilová, Alice Haveldová, Eliška Konečná, Jan Martínek, Barbora Jelínková, Stanislav Vosolsobě, Karolína Dobešová, Marie Smyčková, Jan Smyčka, Jitka Smyčková, Lucie Studená, Josef Nováček, Štěpán Káně, Kateřina Knotková, Tomáš Figura, Jan Simon-Pražák, Kateřina Čermáková, Jiří Janoušek, Václav Bočan, Vojtěch Brož, Anna Suchá, Eva Horčíčková, Tomáš Dvořák, Tomáš Jor, Doubravka Požárová, Karel Plavec, Zdeněk Janovský; Samples processing: **Jakub Štenc**, Lukáš Janošík, Martin Freudenfeld; Analysis: Zdeněk Janovský, **Jakub Štenc**; Funding: **Jakub Štenc**, Lukáš Janošík, Zdeněk Janovský; **Jakub Štenc** led writing of the manuscript, other coauthors commented on the text. All authors agreed with the final version of the manuscript.

Jakub Štenc is leading the correspondence and review process with help from co-authors.

7.3 Chapter III: Pollinator foraging behaviour

7.3.1 Innate preferences of *Eristalis tenax* L. (Syrphidae) for flower colour, size and symmetry are more intricate than the simple additive model

Published in *Biological Journal of the Linnean Society*, 03 July 2023, <https://doi.org/10.1093/biolinnean/blad035>

Eva Matoušková, **Jakub Štenc**, Zdeněk Janovský

Eva Matoušková, Zdeněk Janovský and **Jakub Štenc** conceived the research idea. Eva Matoušková and Zdeněk Janovský designed the method for testing *E. tenax* preferences. Eva Matoušková and **Jakub Štenc** designed and produced artificial flowers. Eva Matoušková collected the data. Eva Matoušková and Zdeněk Janovský analysed the data. **Jakub Štenc**, Eva Matoušková and Zdeněk Janovský provided funding. Eva Matoušková, **Jakub Štenc** and Zdeněk Janovský wrote the manuscript.

Jakub Štenc led the correspondence and review process with help from co-authors.

7.3.2 Learning to be unfaithful - Flower constancy of *Eristalis tenax* L. (Syrphidae) decreases with experience

Manuscript in preparation

Alice Haveldová, **Jakub Štenc**, Eva Matoušková, Zdeněk Janovský

Alice Haveldová, Zdeněk Janovský and **Jakub Štenc** conceived the research idea. Alice Haveldová and Zdeněk Janovský designed the method for testing *E. tenax* preferences. Eva Matoušková and **Jakub Štenc** designed and produced artificial flowers. Alice Haveldová collected the data and Zdeněk Janovský analysed the data. **Jakub Štenc**, Eva Matoušková and Zdeněk Janovský provided funding. **Jakub Štenc** and Alice Haveldová led writing of the manuscript with contribution of all coauthors to the final manuscript.

Jakub Štenc will lead the correspondence and review process with help from co-authors.

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RNDr. Zdeněk Janovský, Ph.D.

Supervisor

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Mgr. Jakub Štenc

Student

8 Articles of the thesis

8.1 Chapter I: Composition of pollinator spectra

8.1.1 Pollinator community and generalisation of pollinator spectra changes with plant niche width and local dominance

Zdeněk Janovský¹, Jakub Štenc^{2*}

¹Svatý Jan t. Krsovice 1, 285 04 Uhlířské Janovice, Czech Republic

²Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 41 Prague, Czech Republic

*Corresponding author, e-mail address: jakubstenc@gmail.com.

8.1.1.0.1 Abstract

1. Floral traits are assumed to be the main determinants of plant's pollinator spectrum, yet a majority of temperate plant species possess traits allowing to a certain degree of generalisation of their pollinator spectrum. The actual level of pollinator spectrum generalisation is likely to be the result of both plant floral traits and the diversity and abundance of plant species a plant co-occurs with. We expect that plant species co-occurring in highly diverse plant communities should host more generalised pollinator spectra.
2. In the present study, we explore the degree to which the composition and generalisation of plant pollinator spectra depend on the plant's niche width (measured as co-occurrence with other plant species) and its local dominance (as measured by mean cover in the community). For this purpose, we compiled a database of 250 plant species pollinator spectra from the literature and coupled it with data on plant niche width and local dominance based on the Czech National Phytosociological Database.
3. Species with wider niches had on average more generalised pollinator spectra, ranging from bumblebee-dominated spectra to diptera- and/or nitidulid beetle-dominated spectra, which was strongly related to plant phylogeny. Plants with bumblebee-dominated spectra had neither wide niches nor niche specialists.
4. The majority of plants had either muscid-, hoverfly- or nitidulid-dominated or completely generalised pollinator spectra. Among such plants, higher local dominance increased the proportion of opportune muscids in pollinator spectrum, while hoverflies showed the opposite pattern. Honeybees although rather infrequent in pollinator spectra also showed a strong preference for locally dominant plant species.
5. Synthesis: The composition of a plant's pollinator spectrum is not independent of other aspects of the plant's life-history, namely, niche width and the ability to dominate the community. Wider plant species niches result in more generalised pollinator spectra, supporting our hypothesis that habitat generalists are less prone to specialisation on particular pollinator groups. Conversely, the ability to dominate local plant communities influenced pollinator spectra mainly through specific responses of individual pollinator groups.

8.1.1.0.2 Keywords Pollination, honeybee, bumblebee, hoverfly, nitidulid beetles, *Apis*, *Bombus*, *Syrphidae*, *Nitidulidae*, plant-pollinator interactions, pollinator spectrum generalisation, pollinator spectrum composition

8.1.1.0.3 Introduction Specialization-generalization of plant-pollinator interaction has been widely debated throughout decades majority of the attention focused on explaining the plant-pollinator relationships by the fit of the flower traits with particular pollinator functional group, known as pollinator syndromes (Faegri and Pijl, 1979; Fenster et al., 2004). However, the power of floral traits to predict a plant's pollinator spectrum is only limited (Ollerton et al., 2009; Wang et al., 2020) and growing evidence shows that the plant-pollinator interaction are more generalized than was expected (Waser et al., 1996; Olesen and Jordano, 2002; Ollerton et al., 2007; Herrera, 2020). Apart from floral traits, ecologically important factors affecting directly pollinator foraging, such as plant species density and composition of co-flowering community (Goulson, 1994; Brosi, 2016), are usually neglected despite possibly serving as filters driving the structure of plant-pollinator interactions (Johnson and Steiner, 2000; Niet et al., 2014). The composition of co-flowering community tend to affect plant pollinator spectra (Potts et al., 2003; Lázaro et al., 2009), with reported both negative (Bergamo et al., 2020) and positive (Moeller, 2005; Ghazoul, 2006) effects of the co-flowering plant species on pollen transfer and reproductive success). The diversity of the floral context, as well as the particular plant species involved affect both pollinator density (Hegland et al., 2009) and composition (Lázaro et al., 2009; Janovský et al., 2013; Biella et al., 2020). Several studies have also shown evidence of variable pollinator spectra across a plant species' range (e.g., Thompson, 1997; Thompson, 2001; Moeller, 2005; Brunet and Sweet, 2006; Niemirski and Zych, 2011), corresponding to the expectation that plant species possibly encountering variable pollinator assemblages should maintain generalised overall pollinator spectra (Waser et al., 1996), despite encountering locally limited number of pollinator species within particular floral context (Herrera, 2005; Lázaro et al., 2009). Being able to achieve local dominance in the community (such as highly competitive or some clonal species do) allows a plant species to attract more pollinators (e.g., Hegland et al., 2009; Janovský et al., 2013), but in environments with limited number of pollinators it may limit their ability to successfully pollinate individual flowers (Goulson, 2000; Akter et al., 2017). Moreover, the pollinator assemblage on locally dominant species is also more diverse (Stang et al., 2006; Hegland et al., 2009; Vázquez et al., 2009), likely including both effective and less effective pollinator groups. This disadvantage of pollinator generalisation is offset in locally dominant plant species by improved pollination effectiveness due to an increased proportion of conspecific pollen transfers (Levin and Anderson, 1970; Goulson, 1994), which especially helps to increase the effectiveness of less flower-constant pollinators. Based on the potential increase of the proportion of conspecific pollen transfer with increasing local density (Goulson, 1994), it has been also hypothesised that plant species local density may play a significant role in evolution toward generalized pollinator spectra (Sargent and Otto, 2006). Conversely, it raises the potential importance of more abundant pollinators in securing sufficient pollen transfer (Harder and Thomson, 1989). Plant niche width and the tendency to locally dominate communities can also affect the plant pollinator spectrum indirectly through the responses of the pollinator functional groups. For example, social hymenopterans (mainly honeybees and bumblebees) concentrate on locally dominant

plants (Ginsberg, 1983; Tschardtke and Steffan-Dewenter, 2000), and they are able to locate them in wide surroundings around their nests (e.g., Osborne et al., 1999; Steffan-Dewenter and Kühn, 2003; Westphal et al., 2003). However, many bee, butterfly and hoverfly species show distinct habitat preferences (e.g., Kratochwil, 1989; Sommaggio, 1999; Gutierrez et al., 2001; Potts et al., 2003) thus, they are likely to occur only in a fraction of habitats in which a plant species with wide niches occurs. This diversity of pollinator habitat preferences is thus likely to further reinforce the expectation that plant species with wide ecological niches have diverse generalised pollinator spectra. In this study, we test following hypotheses: 1) based on evidence from plant-pollinator network studies (Stang et al., 2006; Vázquez et al., 2009) and theoretical model (Sargent and Otto, 2006) we predict that plants achieving high local abundance (i.e., dominance) are possessing more generalised pollinator spectra with higher proportion of locally abundant pollinators (e.g., honeybees and muscid flies). 2) We expect that plant species with wider ecological niches should have more generalised pollinator spectra due to more probable overlapping with ecological niche of wide variety of pollinators and spatial turnover of pollinator species (Moeller, 2005; Gómez and Perfecttiy, 2010; Herrera, 2020). We tested these hypotheses by compiling a database of pollinator spectra of 259 Central European plant species from the literature and relating it to information on species niche widths and local dominance derived from vegetation plots obtained from the Czech National Phytosociological Database (Chytrý and Rafajová, 2003). We focused our analyses on the overall composition of pollinator functional groups and their Simpson diversity, which we took as a measure of generalisation of the pollinator spectrum. We also analysed specific responses of proportions of pollinator functional groups in pollinator spectra. This allowed us to detect any specific responses of pollinator spectrum composition to plant local dominance and niche width.

8.1.1.0.4 Methods Floral Visitor Database

We retrieved data on plant species pollinator spectra from the Floral Visitor Database v. 1.1 (hereafter FloViD) compiled as a part of the PLADIAS project aimed at integrating all available data on Central European plant species present in the flora of the Czech Republic (Chytrý et al. (2021); data available at www.pladias.cz). We restricted the selection only to data on pollinator spectra originating from Central, Northwest and Northern Europe (essentially Europe north of the Alps) and to data on plant species for which at least 25 pollinator individuals were recorded. These selection criteria were met by 259 Central European plant species corresponding to more than 200,000 recorded pollinator individuals. In FloViD, pollinators are classified into 14 functional groups : honeybee – *Apis mellifera*; bumblebees – *Bombus* spp. Incl. *Psithyrus*; other bees – anthophilous pollen-collecting taxa from Apoidea other than honeybees and bumblebees; other Hymenoptera – hymenopterans other than pollen-collecting Apoidea; hoverflies – *Syrphidae*; muscids – flies from the families *Muscidae*, *Anthomyiidae*, *Fanniidae*, *Scathophagidae* and *Sphaeroceridae*; meat flies – meat flies s.l., families *Sarcophagidae*, *Calliphoridae* and *Rhinophoridae*; other Diptera – Diptera other than hoverflies, muscids and

meat flies; nitidulids – small floricolous beetles with aggregated distribution across flowers from the families Nitidulidae, Kateridae, Byturidae and Phalacridae; other beetles – beetles other than nitidulids; butterflies – all Lepidoptera (including moths and sphingids); thrips – all Thysanoptera; other pollinators – non-accidental flower visitors outside the orders Diptera, Hymenoptera, Lepidoptera, Coleoptera and Thysanoptera; not determined – flower visitors which could not be unambiguously identified to any of the preceding functional groups. A more detailed description of pollinator classification can be found in Chytrý et al. (2021). We calculated the relative proportions of pollinator functional groups per plant species to control for different numbers of pollinator individuals sampled. Additionally, we calculated Simpson's diversity index (1-Simpson index; hereafter Simpson diversity) based on the relative proportions of pollinator functional groups in order to obtain a measure of generalisation of the plant's pollinator spectrum [sensu functional generalisation; Ollerton et al. (2007)]. The index takes into account both the number of functional groups pollinating the plant species and their evenness (see e.g. Gong and Huang, 2011 for a similar approach). The higher the Simpson diversity (the closer to 1), the more generalised a plant's pollinator spectrum is.

Plant local dominance and niche width

We defined plant local dominance and niche width based on the Czech National Phytosociological Database of vegetation plots (CNPd; Chytrý and Rafajová (2003)). The CNPD consists of records of plant species and their abundance in the plot (measured as cover on standardised semi-quantitative scale) collected across the vast majority of vegetation types occurring in Central Europe. For all statistical analyses we used the geographically stratified selection from the CNPD reflecting the frequency of occurrence of individual vegetation types in the Czech Republic to avoid over-representation of predominantly sampled areas (data obtained from Herben et al. (2016), further description of the stratification in Chytrý et al. (2005)). We calculated a plant species' local dominance in communities as the square root of the mean cover of the species in the vegetation plots where it was present. We used the Ecological Specialisation Index [ESI; Zelený and Chytrý (2019)] as a measure of niche width based on the beta diversity of the range of communities where the species occurs following Fridley et al. (2007). The advantage of the ESI is that it describes the width of the realised species niche along multiple gradients and on standardised range. Moreover, it is relatively easy to obtain from a reliable data source (CNPd). The index ranges from 0 to 10 (but typically only from 3-8). High ESI values indicate species with narrow niches, i.e., typically occurring in similar floral neighbourhoods. Since the ESI was not defined for some of the species included in the FloViD, the final number of plant species included in analyses was 251.

Data analysis

To assess the relationship of plant local dominance and its niche width with the plant pollinator spectrum, we conducted a two-step analysis. First, we identified the main gradients in the composition of plant pollinator spectra by means of phylogenetic principal component analysis (phylPCA). Second, we regressed plant species' positions in the phylPCA ordination space as

well as the Simpson diversity of pollinator spectra and relative proportions of the pollinator functional groups against plant local dominance and niche width using the Phylogenetic Generalised Least Squares (pGLS). For both steps of the analysis, we assumed a Brownian model of evolution of the continuous response variables and used the maximum likelihood estimate of Pagel's lambda to control for the effects of phylogeny (Freckleton et al. 2002). We then used the DAPHNE phylogenetic tree (Durka & Michalski 2012). We used the plant species' scores on principal components of the phyPCA to describe the species' position in the ordination space of pollinator spectra. The broken stick rule (Jackson 1993) indicated that only the first three principal components (hereafter PC1 to PC3) were informative and thus to be used in the subsequent pGLS analyses. We used the data on all 251 plant species available for pGLS analyses of responses concerning the whole pollinator spectrum (PC1 to PC3 and Simpson diversity). For pGLS analyses with proportions of particular pollinator functional groups, we used only those plant species where the pollinator group was recorded at least once. Functional groups 'others,' 'not determined' and 'thrips' were only included in the phyPCA and not in the pGLS analyses since the first two are not informative and 'thrips' were recorded only in ca 50 plant species. The relative proportions of pollinator functional groups were square-root transformed prior to analyses to meet the assumption of homogeneity of variance of the phylogeny-corrected residuals. To test also for unimodal relationships between the predictors and responses, we included quadratic terms for both predictors (in addition to the linear ones). All analyses were conducted in R statistical environment v. 3.6.1 (R Core Team, 2022a, available at www.r-project.org). We used the base installation, package phytools 0.6-99 (Revell, 2012) and package caper 1.0.1 Orme et al. (2018); all available at <http://CRAN.R-project.org>).

8.1.1.0.5 Results Structure of plant pollinator spectra Overall, the pollinator functional groups most represented in the plant pollinator spectra were bumblebees, hoverflies and muscids (Fig. 1). Surprisingly, even the two often neglected groups of pollinators, nitidulids and other Diptera on average showed higher relative proportions in pollinator spectra than honeybees. The main axis (PC1) of phyloPCA (i.e., the main gradient of variation of pollinator spectra) was determined by a gradient between bumblebee- and diptera+nitidulid-dominated plant pollinator spectra (Table 1, Fig. 2A). The application of the broken stick rule identified another two principal components in phyloPCA to be of relevance. While plants with spectra dominated by bumblebees did not vary much along these two other principal components, pollinator spectra with a low proportion of bumblebees were further structured into those dominated by flies, hoverflies or nitidulid beetles (Fig. 2A and B; Supplementary material 1, Figs. S1 and S2). Finally, a substantial fraction of plant species had generalised pollinator spectra (Supplementary material 1, Fig. S1). The overall influence of plant phylogeny on pollinator spectra composition was only moderate (Pagel's lambda = 0.32).

Pollinator spectrum composition and its overall degree of generalisation The positions of plant species along the main gradient of pollinator spectrum composition (i.e., score on PC1) depended unimodally on ESI (plant's niche width) as well as it was strongly influenced by plant

phylogeny (Pagel's $\lambda = 0.81$ table 2). Both niche generalists and niche specialists (but not the intermediate species) had pollinator spectra more frequently dominated by dipterans and/or nitidulids (Fig. 3A). Among the species with dipteran and/or nitidulid-dominated pollinator spectra, the position on PC2 was determined by both their niche width and local dominance, with species with narrow niches and/or local dominance having pollinator spectra more frequently dominated by muscids (Fig. 3B; Supplementary material 2, Fig. S5). There was no detectable signal of plant phylogeny on a plant's position on PC2 and PC3 (Table 2). The overall generalisation of pollinator spectra (Simpson diversity) increased with the width of plant niches (Table 2; Fig. 3C).

Table 1: Axis loadings of pollinator functional groups on the first three principal components of phylPCA. Variation explained by axes is listed in the headings, loadings greater than 0.25 are in bold for convenience. Pagel's lambda estimated to 0.32.

Pollinator group	PC1 26.7%	PC2 18.8%	PC3 15.9%
honeybees	-0.05	-0.05	0.09
bumblebees	-0.97	0.16	0.07
other bees	-0.05	-0.19	0.15
oth. hymenoptera	0.15	-0.04	-0.08
hoverflies	0.31	-0.52	0.69
hoverflies	0.31	-0.52	0.69
muscids	0.45	0.86	0.03
meat flies	0.24	0.01	0.08
oth. diptera	0.27	-0.01	-0.13
nitidulids	0.11	-0.39	-0.83
oth. beetles	0.12	-0.2	-0.08
butterflies	0	-0.03	-0.01
thrips	0.03	-0.01	-0.06
others	0.05	-0.01	-0.03
not- determined	-0.07	0.14	0.02

Table 2: Responses of plant pollinator spectrum and its generalisation to Ecological Specialisation Index (niche width) and local dominance. Regression coefficients and p-values of pGLS reported. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. $p > 0.05$.

	PC1	PC2	PC3	Simpson diversity
no. of obs.	251	251	251	251
Pagels lambda	0.81	0	0	0.31
(95% CI)	(0.69-0.89)	(0-0.15)	(0-0.18)	(0.06-0.57)
R2	0.037	0.073	—	0.041
ESI – lin.	+0.054	+4.385**		-0.043**
– quadratic	+4.305**			
dominance – lin.		+4.390**		
– quadratic				

Table 3: Responses of relative proportions of individual functional groups to Ecological Specialisation Index (niche width) and local dominance. Regression coefficients and p-values of pGLS reported. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, n.s. $p > 0.05$.

	Hymenoptera				Diptera				Beetles Butterflies		
	Honeybee	Bumblebees	Other bees	Others	Hoverflies	Muscids	Meat flies	Others	Nitidulids	Others	
no. of obs.	137	183	175	145	222	189	117	197	139	152	160
Page's I	0.32	0.81	0.13	0.38	0.22	0.5	0.6	0.49	0	0	0.64
(95% CI)	(0.06-0.67)	(0.65-0.90)	(0-0.42)	(0.09-0.70)	(0.05-0.47)	(0.20-0.74)	(0.20-0.85)	(0.28-0.69)	(0-0.62)	(0-0.25)	(0.39-0.81)
R ²	0.099	0.039	0.038	0.029	0.036	0.058	—	—	—	—	—
ESI – lin.		+0.007n.s.	-0.017n.s.	-0.015*							
– quadratic		-0.045**	-0.028*								
dominance – lin.	+0.060**				-0.039*	+0.056***					
– quadratic											

Relative proportions of pollinator functional groups Of the pollinator groups studied separately, only hymenopterans, muscids and hoverflies responded to plant local dominance in community (Table 3). The relative proportions of bumblebees and other bees showed a response to ESI (i.e., plant niche width), similar to the response of plant species' positions on PC1 (Fig. 4B; Supplementary material 2, Fig. S6). The proportion of other Hymenoptera decreased monotonically with decreasing plant niche width (Supplementary material 2, Fig. S7). The average local dominance of plant species increased the proportion of both honeybees and muscids in the plant pollinator spectrum (Fig. 4A; Supplementary material 2, Fig. S8), whereas it decreased the proportion of hoverflies (Fig. 4C).

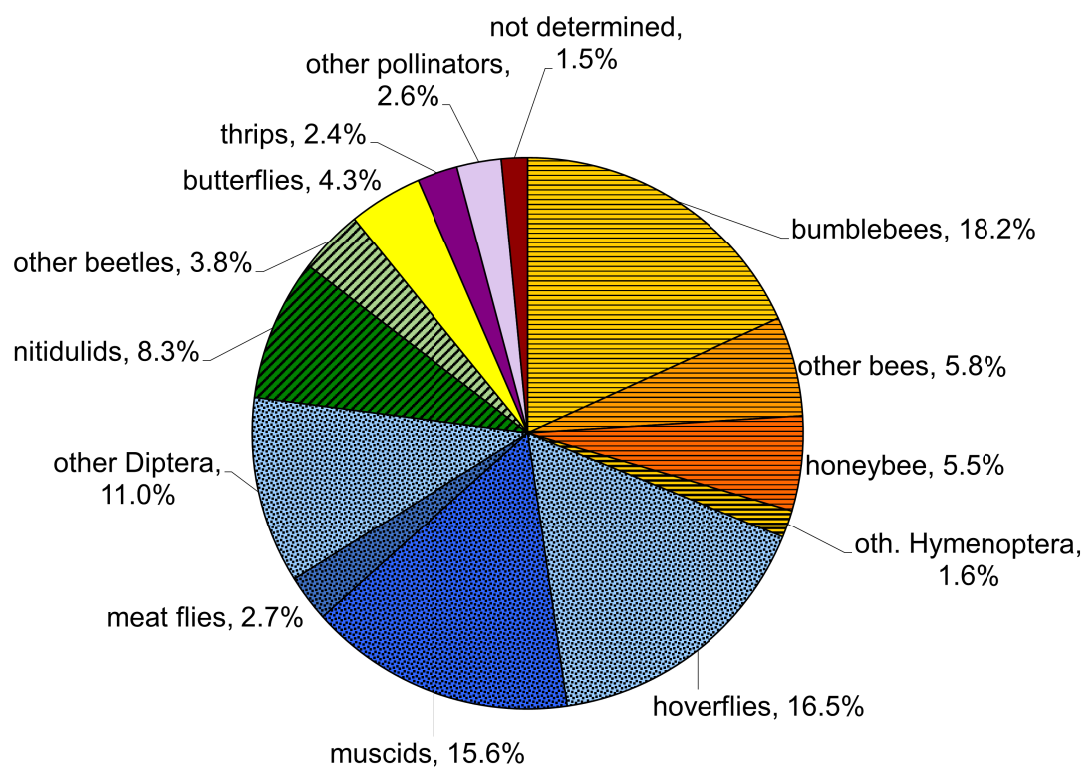


Fig. 1: Relative proportions of pollinator functional groups across the whole dataset. The relative proportions were obtained by averaging the relative proportions for individual species across the whole dataset. Different patterns represent different taxonomic group.

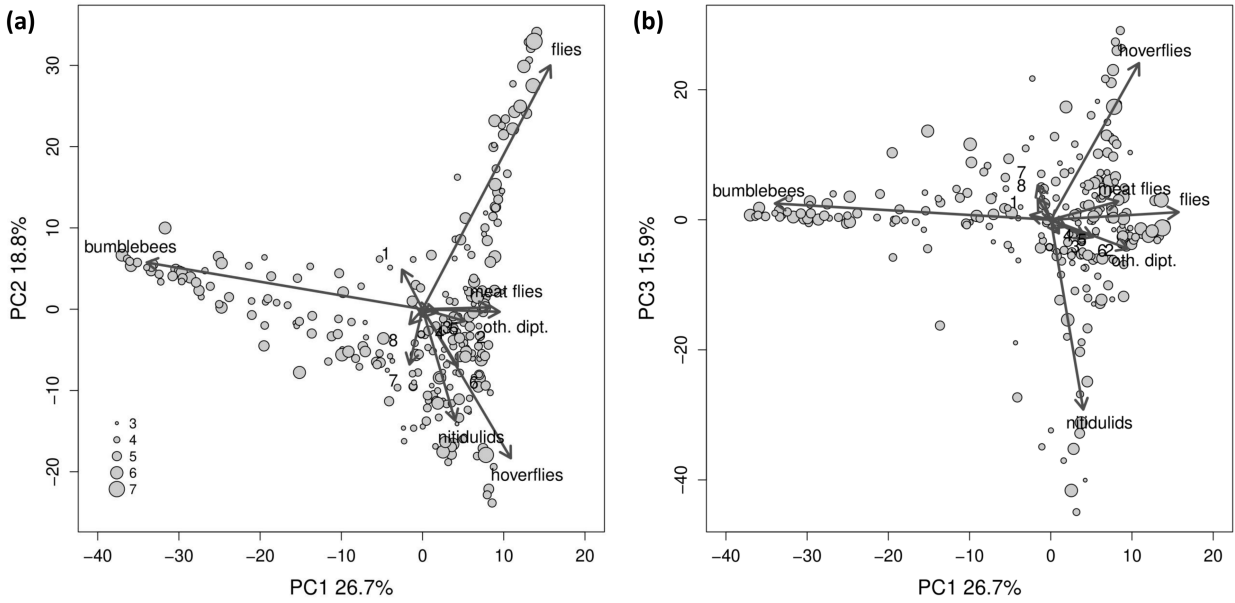


Fig. 2: Ordination diagrams of the phylogenetic PCA of plant–pollinator spectra, (a) 1st and 2nd principal components and (b) 1st and 3rd principal components. The size of the points is proportional to the plant species’ Ecological Specialisation Index (niche width). Pagel’s lambda: 0.32. Minority pollinator groups: 1—not determined; 2—oth. Hymenoptera; 3—thrips; 4—butterflies; 5—other pollinators; 6—other beetles; 7—other bees; 8—honeybee.

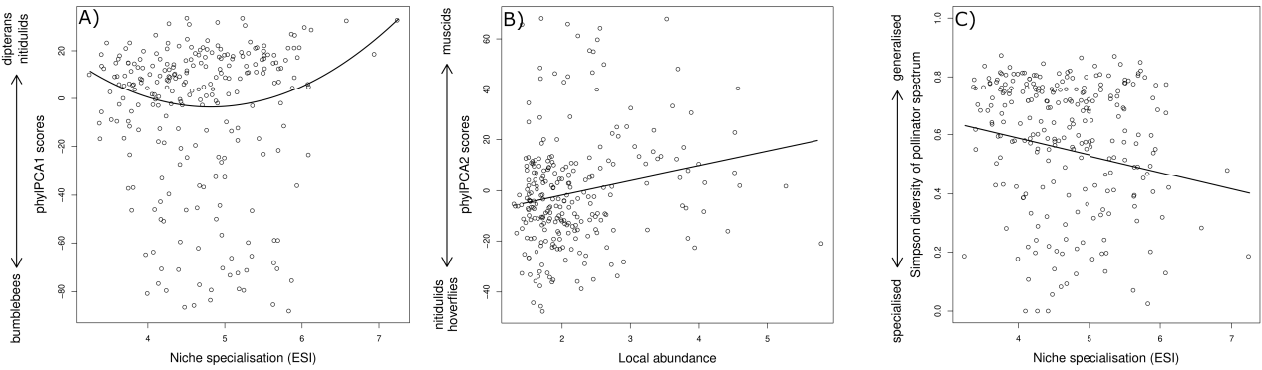


Fig 3: Relationship of plant–pollinator spectrum characteristics with the ESI (plant niche width) and local dominance: (a) plant species’ positions on the 1st phylPCA axis and ESI; (b) plant species’ positions on the 2nd phylPCA axis and local dominance; and (c) degree of generalisation of the plant–pollinator spectrum (Simpson diversity) and ESI. The fitted relationships are predictions from pGLS regressions listed in Table 2.

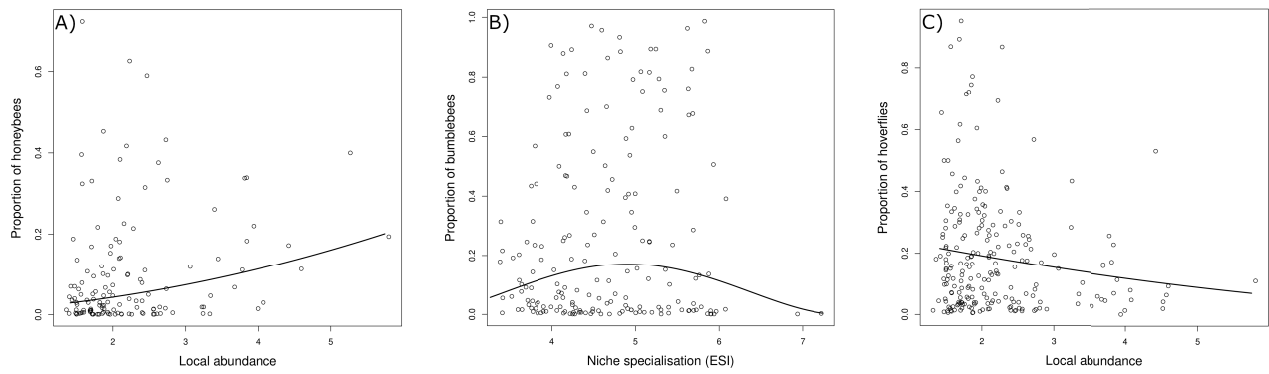


Fig 4 Relationship of relative proportions in plant–pollinator spectra of studied pollinator groups with ESI (plant niche width) and local dominance: (a) proportion of honeybees in pollinator spectrum and local dominance; (b) proportion of bumblebees and ESI; and (c) proportion of hoverflies and local dominance. The fitted relationships are predictions from pGLS regressions listed in Table 3.

8.1.1.0.6 Discussion The composition of plant pollinator spectra was aligned along three main axes. The first and most important axis showed a gradient of bumblebee domination of the plant pollinator spectrum. The species' position on the gradient was unimodally related to plant niche width and strongly correlated with plant phylogeny (bumblebee dominated plants were mainly from few plant families such as Fabaceae and Lamiaceae). Apart from plants dominated by bumblebees, the species with both narrower niches (low ESI) and simultaneously with high local dominance (i.e., specialised but abundant species occurring in only few habitats) were more likely to have their spectra dominated by muscids than by other groups. The relative proportion of honeybees and muscids in a plant's pollinator spectrum increased with the plant's local dominance, as was hypothesised based on plant's ability to be locally abundant.

Plant niche width and structure of the plant pollinator spectrum In general, the majority of studied plant species possessed a rather generalised pollinator spectrum (Fig. 1; Appendix A, Fig. S1), which is in line with other studies of pollination at the community or flora level (Ellis and Ellis-Adam, 1993; Herrera, 2020). Furthermore, our results indirectly support the theoretical considerations that generalisation of the pollinator spectrum should be more common in plants experiencing fluctuating pollinator composition across sites and/or seasons (Waser et al., 1996; Gómez and Zamora, 2006). However, the low proportion of explained variation (4%; Table 2) indicates that plants with generalised pollinator spectra due to having wide made up only a minority of our dataset. Interestingly, plant niche width was also unimodally related to the main gradient of phyPCA driven mainly by the proportion of bumblebees in the pollinator spectrum. The lack of plants with wide niches and bumblebee-dominated spectra could be the result of increased competition with other pollinator groups for these plants (e.g., Forup and Memmott, 2005) combined with often low bumblebee densities and vulnerability of their populations in agricultural landscapes (Lye et al., 2009; Le Féon et al., 2010). Similar reasons may apply to 'other bees' as well (Table 3). Conversely, the lack of plant species possessing both bumblebee-dominated spectra and narrow niches may be the result of over-representation of mountain plant species with narrow niches in our dataset. In temperate mountainous habitats, muscids often constitute a dominant pollinator functional group largely outnumbering bumblebees and hymenopterans in general due to strong presence of dipterans (WILLIS and BURKILL, 1895; e.g. Totland, 1993; Benadi et al., 2014).

Relationship between plant local dominance and plant pollinator spectra Plant local dominance showed weaker relationship with plant pollinator spectra than plant niche width. Contrary to predictions (Sargent and Otto, 2006), the plant's local dominance did not promote generalisation of its pollinator spectrum. However, local plant dominance increased proportion of muscids in pollinator spectra. This may suggest that plants possessing dipteran pollination syndrome may be able of local dominance, which attracts abundant dipterans by dominating by combined floral display of multiple individuals. For muscids, floral rewards constitute only a fraction of their diet, and they can utilise them only opportunistically [especially compared to most adult hoverflies, which fully rely on nectar and pollen; Gilbert (1981); Haslett (1989)]. Thus, it seems logical that muscids are more likely to concentrate on plant species constituting a dominant re-

source in the community. This is further corroborated by the fact that most plant species in our analysis with muscid-dominated pollinator spectra and low local dominance are often almost the most abundant floral resource during the time of their flowering in the flowering community (e.g., *Caltha palustris*, *Anemone nemorosa*, *Bellis perennis*; Supplementary material 1, Fig. S3). In addition muscids, honeybees showed the same positive response to plant local dominance reflecting their ability to concentrate on an abundant food source (Seeley and Visscher, 1988) combined with the large areas from which they gather resources (Steffan-Dewenter and Kühn, 2003). This effect was relatively strong (Table 3) and honeybee relative proportions were only weakly related to plant phylogeny, supporting the notion that local dominance was a more important driver of honeybee proportions than floral morphology and rewards offered (Goulson, 1994; Fowler et al., 2016). In contrast, the relative proportion of hoverflies in the pollinator spectrum surprisingly decreased with increasing plant local dominance. We hypothesise that this may be result of the relatively low hoverfly population densities and thus hoverflies being outnumbered on the dominant floral resources by more opportunistically behaving pollinator groups (e.g., honeybees or muscids). Additionally, hoverflies are known for being a very diverse pollinator functional groups with no clear shared preferences (Sommaggio, 1999; Branquart and Hemptinne, 2000). Therefore, they tend to be spread among a wide spectrum of plant species (e.g., Ssymank et al., 2008). The diversity of preferences of hoverflies can be further corroborated by the number of plant species where hoverflies were present in pollinator spectra, which was the highest among all groups (222 out of 251 plant species as compared to 183 and 189 for similarly abundant bumblebees and muscids respectively).

Patterns across plant phylogeny While butterflies and bumblebees show clear preferences for flowers with distinct morphology, such as long spurs or tubes (Faegri and Pijl, 1979), the relationship of relative proportions of dipterans (minus syrphids) with plant phylogeny may have multiple causes. First, dipterans typically have short proboscises (Kearns et al., 1998) and thus part of the phylogenetic pattern may be caused not by dipteran preference for flowers with particular traits but by their avoidance of morphologically intricate and inaccessible flowers. Second, at least one of our dipteran functional groups, namely, meat flies, seems to show consistent preferences for floral traits that mimic carrion or dung (Johnson and Jürgens, 2010; Chen et al., 2015). Third, we also cannot rule out that our results are shaped by the sample of plant taxa we have, since studies on a similarly numerous set of plant taxa from Spain (i.e., relatively related flora) have come to very different conclusions concerning the relationships of functional groups to phylogeny [see e.g. beetles and nitidulids; Herrera (2020); Herrera and Otero (2021)].

8.1.1.0.7 Conclusion In the present study, we showed that not only floral traits but also the seemingly unconnected aspects of plant niche – niche width and the ability to dominate communities – may contribute substantially to shaping of plant pollinator spectra. In particular, plant niche width seems to be an important trait predicting the possible vulnerability of plants to pollinator declines [similar to the plant mating system; Biesmeijer et al. (2006)] and may

be important factor in evaluating as the potential threat in nature conservation. Conversely, plant local dominance mainly promoted a higher proportion of opportunistic pollinator groups (honeybee, muscids) in the pollinator spectra. Finally, our results further emphasise that information on plant community structure is necessary for understanding the pollination ecology of most temperate plant species, which possess at least to some degree generalised pollinator spectra. In the future tests of pollinator syndromes, other ecological plant traits should be considered along traits directly connected with floral morphology as is colour, scent, or reward type.

8.1.1.0.8 Acknowledgement The authors are grateful to members of PEE and FEG workgroups in Prague for discussions of the topic and valuable suggestions to earlier versions of the manuscript and they would like to thank namely to Tomáš Herben and Martin Weiser for suggesting the use of ESI. Furthermore, the authors would like to thank to all the people (and especially to those involved in “Opylovači” project) who selflessly contributed their unpublished data to the FloViD database. ZJ has been supported by Charles University Research Centre Programme No. 204069. Authors are also grateful to anonymous reviewers who helped to improve the manuscript quality.

8.1.1.0.9 Conflict of interest statement The authors declare no conflicts of interest.

8.1.1.0.10 Authors’ contributions ZJ initiated the idea, collected data from the literature, ZJ and JŠ collected part of the data in the field, ZJ performed the analysis. ZJ and JŠ wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

8.1.1.0.11 Data availability statement Data used for the analysis are stored in Dryad repository DOI <https://doi.org/10.5061/dryad.fj6q5741b>.

8.2 Chapter II: Pollen presentation and diurnal structure of plant-pollinator interactions

8.2.1 Pollinator visitation closely tracks diurnal patterns in pollen release

Running title: Pollen presentation schedules

Jakub Štenc¹, Lukáš Jánošík¹, Eva Matoušková², Jiří Hadrava², Michael Mikát^{2,3,4}, Zdeněk Janovský¹

¹Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 41 Prague, Czech Republic

³Department of Biology, Faculty of Science, York University, 4700 Keele Street, 203D, M3J 1P3, Toronto, Canada

⁴Department of General Zoology, Faculty of Natural Sciences 1 - Biosciences, Martin Luther University, Hoher Weg 8 , 06100 Halle (Saale), Germany

*Corresponding author, e-mail address: jakubstenc@gmail.com.

8.2.1.1 Abstract

8.2.1.1.1 Premise of the study Animal-pollinated plants face a high risk of pollen loss during its transfer. To limit the negative effect of pollen losses by pollen consumption and heterospecific transfer, plant species may adjust and stratify their pollen availability during the day (i.e., form pollen presentation schedule) and attract pollinators in accurate time frames.

8.2.1.1.2 Methods We investigated diurnal patterns of pollen availability and pollinator visitation in three co-flowering plant species: *Succisa pratensis* with open flowers and accessible pollen, pollinated mainly by pollen-feeding hoverflies; *Centaurea jacea* with open flowers and less accessible pollen, pollinated mainly by pollen-collecting bees; and *Trifolium hybridum* with closed flowers and pollen accessible only after the active opening of the flower, pollinated exclusively by bees.

8.2.1.1.3 Key results The plant species that were studied differed in the pollen availability peak, tracked by the visitation activity of their pollinators. *S. pratensis* released all pollen in the morning, while pollinator activity was still low and peaked with a slight delay. In contrast, *C. jacea* and *T. hybridum* had distinct pollen presentation schedules, peaking in the early afternoon. The pollinator visitation of both of these species closely matched their pollen availability.

8.2.1.1.4 Conclusions Stratifying pollen availability to pollinators during the day may be one of several mechanisms allowing co-flowering plants to share their pollinators and decrease the probability of heterospecific pollen transfer.

8.2.1.2 Introduction Plants pollinated by insects suffer from high pollen loss during transfer, resulting in only a tiny fraction of produced pollen successfully reaching conspecific stigma (Harder and Thomson, 1989; Rademaker et al., 1997; Gong and Huang, 2014). Pollen loss promoted by pollen consumption (Parker et al., 2016), grooming behavior (Holmquist et al., 2012; Koch et al., 2017) or inaccurate transfer (Arceo-Gómez et al., 2016) can even lead to an almost complete depletion of pollen within a plant population (Stanghellini et al., 2002b; Parker et al., 2016; Koski et al., 2018) and consequently limit plant sexual reproduction. Facing such a threat, plants are under strong selective pressure to develop adaptations to prevent pollen losses by limiting the amount of pollen removed by a single pollen-wasting pollinator and increasing the number of pollen carriers (LeBuhn and Holsinger, 1998). In addition, co-flowering plants are threatened by the risk of heterospecific pollen transfer from and to other plant species (Arceo-Gómez et al., 2016). Therefore, plants should be driven to evolve different pollen presentation schedules to prevent pollen losses promoted by pollinators by pollen-wastage and heterospecific transfer.

According to pollen presentation theory (PPT), plants should be able to adjust the amount of pollen they offer pollinators to promote their transfer and increase male fitness (Harder and

Thomson, 1989; Harder and Wilson, 1994; Harder and Wilson, 1998; Thomson, 2006b). Theoretical models predict that partitioning and gradual pollen dispensing may be advantageous for plants with abundant but less efficient pollinators (Harder and Thomson, 1989; LeBuhn and Holsinger, 1998). In contrast, plants with less abundant but highly efficient pollinators (i.e. with a high ratio of the amount of pollen removed from a flower to pollen deposited on a conspecific flower) are supposed to benefit from placing as much pollen as possible on the pollinator during a single visit (Castellanos et al., 2006). Moreover, PPT predicts that gradual pollen presentation will likely increase the number of potential mating partners (Harder and Wilson, 1994).

Sharing pollinators affects plant fitness by direct competition for visits (Kunin, 1993) and by indirect effects through heterospecific pollen transfer (Hersch and Roy, 2007). Simultaneously, co-flowering species within the plant community often share pollinators, negatively affecting plant reproduction (Hegland et al., 2009). The negative effects on plant pollination may even be amplified in generalized systems, yet recent evidence shows a lower magnitude of heterospecific transfer than could be predicted based on pollinator visitation (Popic et al., 2013; Zhang et al., 2021). This surprisingly low prevalence of heterospecific pollen transfer in a co-flowering community may be promoted by plant adaptations, e.g., by placing pollen on different parts of the pollinator body (Huang and Shi, 2013), flower traits inducing constancy of individual pollinators (Waser, 1986a) or both (Huang et al., 2015). However, in generalized systems with pollinators simultaneously visiting more plant species and possessing only low flower constancy, temporal stratification of pollen presentation may be advantageous through temporal pollinator specialization (Brosi, 2016; Wang et al., 2021; Schwarz et al., 2021). Despite its possible importance, the diurnal level of temporal pollinator stratification gained only limited attention compared to the level of days, weeks or months when the interaction turnover is more expected. Moreover, to date, only a few studies have shown or mentioned the effect of diurnal pollinator stratification on pollen transfer (Stone et al., 1998; Huang and Shi, 2013; Queiroz et al., 2015; Ballantyne et al., 2017). Stone et al. (1998) showed an example of pollen release in the closely related co-flowering *Acacia* species, differing in their pollen presentation schedules and pollinator visitation activity tracking the pattern in pollen release. However, sufficient observations of pollen presentation schedules coupled with diurnal pollinator activity are still lacking.

To develop pollen presentation schedules, plants evolved various mechanisms allowing them to release pollen at different times and with different magnitudes (Lloyd and Yates, 1982). For example, some plants, such as Fabaceae, possess explosive pollen release mechanisms (reviewed in Aluri and Reddi (1995)), allowing the rapid release of a vast proportion of the pollen from flowers during the first pollinator visit. Other plants dispense pollen by gradual maturation of anthers within a flower and present pollen from only a fraction of anthers at each moment (Ren and Bu, 2014). A similar goal, i.e., gradual pollen release, can also be reached by the pump mechanism employed, e.g., by most Asteraceae, where pollen is pumped out by gradual pistil growth through the flower tube (Erbar and Leins, 1995; Galloni et al., 2007). Furthermore, plants often display flowers organized into inflorescences possessing more simultaneously

presented flowers. Plants forming inflorescences benefit from attracting more pollinators but may suffer a higher risk of geitonogamy (i.e., self-pollination; Ishii and Sakai (2001); Karron et al. (2004)), especially when the inflorescence is compact and forms one single unit from the point of view of the pollinator (e.g., capitulum of Asteraceae or Fabaceae) (Liao and Harder, 2014). Hence, floral and inflorescence traits may act together to affect pollen transfer and pollinator behavior (Harder et al., 2004; Kudo and Harder, 2005). Overall, the mechanisms of pollen release within flowers and flower development within inflorescences enable variation in the timing of pollen presentation among species and their populations (Stone et al., 1998; Nansen and Korie, 2000; Kudo and Harder, 2005; Eisen et al., 2017), resulting in structured pollen presentation schedules.

However, pollinators utilizing various floral resources are optimizing their behavior based on the distribution of floral rewards to maximize their intake (Charnov, 1976; Pyke, 1978b, 2019). According to Optimal Foraging Theory and the Marginal Value Theorem (Charnov, 1976), pollinators facing a decrease in reward gain are more likely to leave a patch of flowers after reward depletion (Heinrich, 1976; Waddington, 1980) and to switch to another floral source (Chittka et al., 1997), indicating that pollinators can adjust their behavior to the current spatial distribution of rewards (Dukas and Real, 1993; Chittka and Brockmann, 2005; Baude et al., 2011). Similarly, the amount of available reward also changes over time, based on its release by plants and consequent depletion by pollinators. Therefore, according to the gathered reward, pollinator behavior may also change over time. Consequently, pollinator flower visitation may closely track diurnal reward availability. Moreover, the timing of their visitation activity could vary among co-flowering species, depending on the schedule of reward availability, allowing plant stratification of shared pollinators over time (Stone et al., 1998). Diurnal stratification of plant-pollinator interactions may consequently affect the interaction network structure between plants and pollinators with increasing specialization in particular time frames (Schwarz et al., 2021; Wang et al., 2021).

In this study, we investigated the pollen presentation schedule of three co-flowering species and the diurnal visitation pattern of their pollinators, aiming to explore how closely pollinator activity tracks pollen availability. For our study, we selected three plant species exhibiting differences in their pollen release mechanism, flower and inflorescence organization and partial overlap in their pollinator spectra. Notably, we address the following questions: I. Does pollen availability show a pattern during the day? II. Does pollinator visitation activity track pollen availability? We expect that the studied plants will differ in the pattern of pollen presentation.

8.2.1.3 Methods

8.2.1.3.1 Study system To achieve our aims, we conducted an experiment with three plant species: *Trifolium hybridum* L. (Fabaceae), *Centaurea jacea* L. (Asteraceae) and *Succisa pratensis* Moench (Caprifoliaceae), which differ in their pollen dispensing mechanisms: *T. hybridum* flowers are closed, with explosive release of pollen toward the front part of the pollina-

tor body as in other Fabaceae species (Galloni et al., 2007); *C. jacea* presents pollen gradually throughout the day using a so-called pump mechanism, where pollen is extruded from a closed tube by the growing style (Erbar and Leins (1995)); and *S. pratensis* releases pollen openly on four anthers sticking out from the flower and the pollen is easily accessible to pollinators. The individual flowers of all studied species are organized into inflorescences (capitula - *T. hybridum*, *S. pratensis* and flowerheads - *C. jacea*, Fig. 1).

The study was conducted at the “K Handrkovu” meadow near Vernýřov village, Central Bohemia region, Czech Republic (N 49.8466, E 15.1498). A more detailed description of the locality can be found in Janovský et al. (2013). We carried out our experiment under stable weather conditions with no or only moderate wind and sunny weather. We checked this by measuring solar radiation, relative air humidity and temperature using Minikin RTHi/QTHi (EMS BRNO, Brno, Czech Republic) automatic dataloggers. Furthermore, we measured the average wind speed at 30-minute intervals (Windmaster 2, Kaindl electronics, Rohrbach, Germany). Weather conditions were comparable during the whole experimental run, with moderate increases in temperature and radiation and decreases in relative humidity on the second day (see Supplemental Data with this article Appendix S1, Figs.S1 and S2).

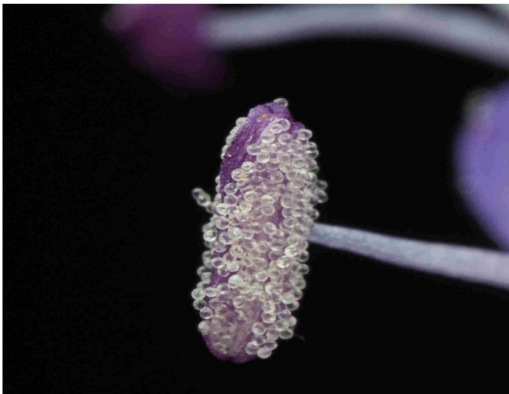
A) *Trifolium hybridum*



B) *Centaurea jacea*



C) *Succisa pratensis*



8.2.1.3.2 Pollen availability Flowers of all three species were collected during 15th and 16th August 2021 (Day 1 and Day 2, respectively). Sampling was carried out in hourly intervals from 8:00 until 17:00 to cover the span of main pollinator activity. At each sampling event, we collected flowers into 5 vials per species (hereafter called samples). The vials contained 2 ml of a water solution of detergent and basic fuchsin to stain pollen grains. Flowers for each sample were collected from 5 randomly selected inflorescences within a 50 m circle around the weather condition measuring tools to avoid the effect of specific microclimatic conditions. Flowers for each sample were collected from 5 randomly selected inflorescences within approximately 50 m of the devices measuring weather conditions. From each of the 5 inflorescences per sample, we randomly collected 3 (*T. hybridum*, *C. jacea*) or 5 individual flowers (*S. pratensis*). The collected flowers offered pollen (from at least one anther) and had to be actively visited by pollinators.

The collected samples were transported to a field laboratory on the same day. They were first sonicated by ultrasound for 60 seconds (using Ultrasonic Laboratory 2, 40 kHz, Ultrazvuk Hradec Králové, Czech Republic). Then, they were vortexed, and 5 µl of homogenized solution was pipetted onto a microscopic slide immediately afterward. The slides were covered by cover glass and sealed by nail polish. Slides were labeled and stored before pollen counting. To minimize subsampling error, we made three subsamples per vial, resulting in 900 subsamples that were further analyzed.

Three months later, the slides were scanned by automatic slide scanner (Axioscan 7 Microscope Slide Scanner, Zeiss, Oberkochen, Germany), and combined pixel and object classification in Ilastic software (Berg et al., 2019) was applied to the resulting scans to automatically recognize pollen grains. Objects identified as pollen were then counted by FIJI software (Schindelin et al., 2012). The numbers of counted pollen grains from subsamples of a sample were then averaged and used in further analysis.

To estimate total pollen production, we collected flower buds with developed anthers prior to their active flowering stage. Collected buds were manually opened by tweezers inside a vial to avoid pollen losses. Samples were then processed according to the same protocol as samples for pollen presentation, except extended sonication was performed for 10 minutes to ensure anther fission and pollen release. Later, the anthers were inspected under a light microscope and pollen grains remaining within the anther tissue were counted and summed up with the amount of pollen counted by image analysis.

We cannot completely prevent potential contamination of our samples by pollen of species with similar pollen morphology, even though it seems unlikely to happen. However, pollen grains of *S. pratensis* and *C. jacea* are distinctive from other co-flowering species due their size and morphological appearance, whereas *T. hybridum* possess closed flowers which may decrease potential contamination by other co-flowering *T. hybridum* species (i.e., *T. repens* and *T. pratense*).

8.2.1.3.3 Flowering schedule On the following day (17 August 2021, thereafter Day 3), we investigated the proportion of pollen presenting flowers within the inflorescence of each species during the day. Flowers per inflorescence were counted and classified into 3 categories: flower buds, active flowers and old flowers. Active flowers refer to a stage in which flowers were visibly offering pollen and actively visited by pollinators, i.e., those that could be sampled for pollen presentation. Flowers were counted on 20 randomly chosen inflorescences per species every hour from 8:00 to 17:00 to match the pollen presentation schedule.

8.2.1.3.4 Pollinator diurnal visitation activity To compare the pollen presentation schedule with pollinator activity, we used aggregated data on plant-pollinator interactions from the same site and time of the year (approximately 6-day collection campaigns between 13th and 26th August, matched through flowering of *S. pratensis*) for the last 10 seasons (2011-2021). These data were collected following the protocol described in Janovský et al. (2013).

In brief, plants and pollinators were counted in 4 m×4 m plots, arranged in a regular 20 m×20 m grid covering the whole meadow giving a total of 93 plots. Each plot was censused for pollinators at least 20 times each year. During a census, all pollinators visiting flowers and touching reproductive structures of studied plant species were recorded. The censuses were conducted from 7:00 to 19:00 with observations randomized over plots, days and daytime. An overview of the recorded pollinators is provided in Appendix S1, Table S1.

Plants were counted twice (with the exception of 2011 with only one census) during each year's period of pollinator censuses. A lattice dividing the plot into 64 subplots of 0.5 m×0.5 m was strung over the plot. The presence or absence of actively flowering inflorescences (both in the male and female phases) of the studied species was recorded in each subplot, thus, a semiquantitative assessment of inflorescence density in each plot was obtained which further served to standardize the observed counts of pollinators on the studied species.

Since pollinator occurrences on studied plants in plots were rather stochastic, we needed to average observations across plots for each plant species to obtain reasonable estimates of pollinator density at a given time interval. For *S. pratensis* and *C. jacea*, we averaged observations from all plots observed in 10-minute intervals, whereas for *T. hybridum* with lower pollinator densities the observations were averaged over 20-minute time intervals. The averaging considered inflorescence densities by dividing the sum of all pollinator observations from a given plant species and time interval by the sum of inflorescence densities of the given plant species from all plots that were observed in that time interval. We refer to this quantity as the pollinator density hereafter.

8.2.1.3.5 Data analysis To answer Question I, we conducted a set of two analyses for each of the studied species. First, we modeled the amount of available pollen in individual flowers using the average number of pollen grains per sample as a response. The predictors were time of the day, sampling day and their interaction as there were tangible differences between the days

in terms of available pollen, but not in the shape of the relationship to the time of day. Second, we modeled the proportion of actively flowering flowers within an inflorescence with respect to the time of the day. We considered both linear and quadratic terms (available pollen per flower) or even cubic terms (proportion of actively flowering flowers within the inflorescence) for time of the day, since unimodal patterns during the day could be expected. In both analyses, we used a linear model, and to meet its assumptions (namely homogeneity of variance), we had to transform the response variables by taking their logarithm (available pollen per flower) or square-root (proportion of actively flowering flowers). We considered the cubic term only in case a quadratic term turned out to be significant. Since the studied plant species differed in their overall pollen production per flower (Table S1), we standardized for the purpose of data visualization (but not analysis) the data by total pollen production per flower of the species. Furthermore, we combined the two models to provide an estimate of the available amount of pollen per inflorescence for the three species under study to compare it with pollinator densities (Fig. 2A).

To answer Question II, we analyzed pollinator densities with respect to time of day. Similar to models aimed at answering Question I, we considered a unimodal pattern of pollinator densities with respect to time of day. In models with a significant quadratic term, we considered the cubic term as well. The response variable was transformed by square-root in order to meet the assumption of homogeneity of variance. Since we were interested mainly in the timing of maxima of pollinator density during the day, we standardized pollinator densities by their maximum for the purpose of data visualization (Fig. 2B, non-standardized pollinator densities can be found in Appendix S1, Table S1, Fig. 3)

All analyses were performed in R version 4.2.0 under base installation (R Core Team (2022b), available at www.r-project.org)

8.2.1.4 Results

8.2.1.4.1 Pollen presentation The amount of available pollen showed a significant relationship with the time in all plant species under study (Table 1). In *S. pratensis* and *T. hybridum*, the amount of available pollen per flower decreased steadily from morning onward. While the flowers were almost depleted by 13:00 in *S. pratensis*, the decrease was less steep in *T. hybridum*. However, *C. jacea* followed a unimodal pattern during the day with a peak at approximately 13:00 (Fig. 1).

C. jacea and *T. hybridum* also differed significantly in the amount of available pollen between the two days of our observation (Table 1, Fig. 1), showing increased pollen release on the second day of sampling. However, the pattern of release during the day remained the same; there was only a difference in magnitude.

Plant species also differed in their total pollen production: *T. hybridum* produced on average 5 162 pollen grains (\pm standard deviation (SD) 350), *C. jacea* produced 6 008 pollen grains

(\pm SD 785) and *S. pratensis* produced 991 (\pm SD 232) (Appendix S1, Table S2).

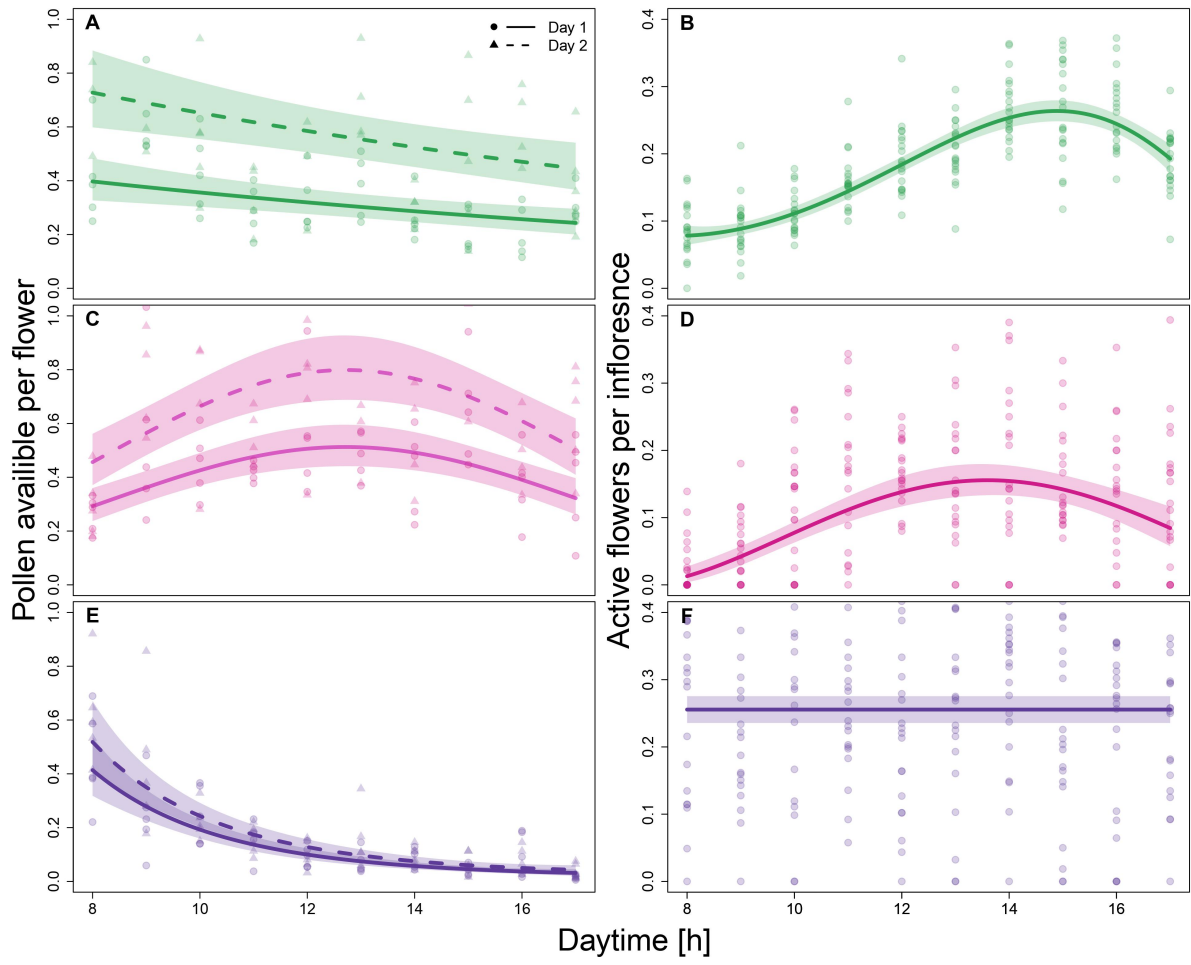
8.2.1.4.2 Flowering schedule The proportion of active flowers of both *C. jacea* and *T. hybridum* changed significantly with time (Table 2), showing a unimodal pattern with peaks at approximately 13:00 (*C. jacea*) and 15:00 (*T. hybridum*). In contrast, *S. pratensis* showed almost no change in inflorescence development with approximately 25% of flowers active from early morning until the end of our observation and without a distinctive peak (Fig. 1 C).

Table 1: Linear model of amount of available pollen (log-transformed) depending on time of day and day: of sampling. SS = sum of squares, Coef. = coefficients, R2 = coefficient of determination. Coefficients for statistically nonsignificant predictors are not shown (represented by a dash).

Predictor	<i>T. hybridum</i>				<i>C. jacea</i>				<i>S. pratensis</i>			
	Df	Coef.SE	R ²	P-value	Df	Coef.SE	R ²	P-value	Df	Coef.SE	R ²	P-value
day	1	9.151	0.226	<0.001	1	4.852	0.175	<0.001	1	1.185	0.016	<0.05
time	1	5.419	0.134	<0.001	1	0.464	0.017	0.13	1	47.674	0.635	<0.001
time ²	1	0.323	0.008	0.28	1	3.442	0.124	<0.001	1	1.134	0.015	<0.05
day x time	1	0.017	0.000	0.8	1	0.195	0.007	0.33	1	0.013	0.000	0.83
day x time ²	1	0.001	0.000	0.96	1	0.014	0.001	0.79	1	0.150	0.002	0.45
Residuals	94	25.554	0.632		94	18.812	0.677		94	24.870	0.331	

Table 2: Linear model of proportion of active flowers within an inflorescence (square-root transformed) depending on time of day.

Predictor	<i>T. hybridum</i>				<i>C. jacea</i>				<i>S. pratensis</i>			
	Df	Sum sq.	R ²	P-value	Df	Sum sq.	R ²	P-value	Df	Sum sq.	R ²	P-value
time	1	47.399	0.478	<0.001	1	25.937	0.107	<0.001	1	2.548	0.011	0.14
time ²	1	11.687	0.118	<0.001	1	35.883	0.148	<0.001				
time ³	1	5.188	0.052	<0.001								
Residuals	194	34.953	0.352		198	180.642	0.745		198	235.235	0.989	



8.2.1.4.3 Pollinator activity We recorded 439, 5855, 7300 and pollinators visiting *T. hybridum*, *C. jacea* and *S. pratensis* respectively, throughout the eleven years of our pollinator observations. Pollinator spectrum of *T. hybridum* was strongly dominated by honeybee (61.5%; bumblebees 25.7% and hoverflies 3.6%). The spectrum of *C. jacea* was dominated by honeybee (43.7%), bumblebees (32.6%) and hoverflies (14.6%). On the other hand, hoverflies dominated the pollinator spectrum of *S. pratensis* making up 75.6%, while the proportion of honeybee and bumblebees was much lower (2.3% and 12.6% respectively) (Appendix S1, Table S1, Fig. 3).

Data for *T. hybridum* are based on 404 plot \times year combinations (min = 2, median = 43, max = 66 plots per year) each censused for pollinators at least 20 times). *C. jacea* pollinators was censused on 676 plot \times year combinations (min = 41, median = 60, max 82 plots per year) and *S. pratensis* on 182 plot \times year combinations (min = 13, median = 17, max = 22 plots per year).

Mean inflorescence densities per occupied plot were 5.1, 7.0 and 7.9 occupied subplots per plot for *T. hybridum*, *C. jacea*, and *S. pratensis* respectively.

Pollinators of all three studied plant species showed a unimodal diurnal pattern of visits (Table 3, Fig. 3), but they differed in timing of their peak density of visits. These occurred at ca 15:45, 13:30 and 11:45 for *T. hybridum*, *C. jacea* and *S. pratensis* respectively matching thus the pattern of the peaks of pollen availability in inflorescences.

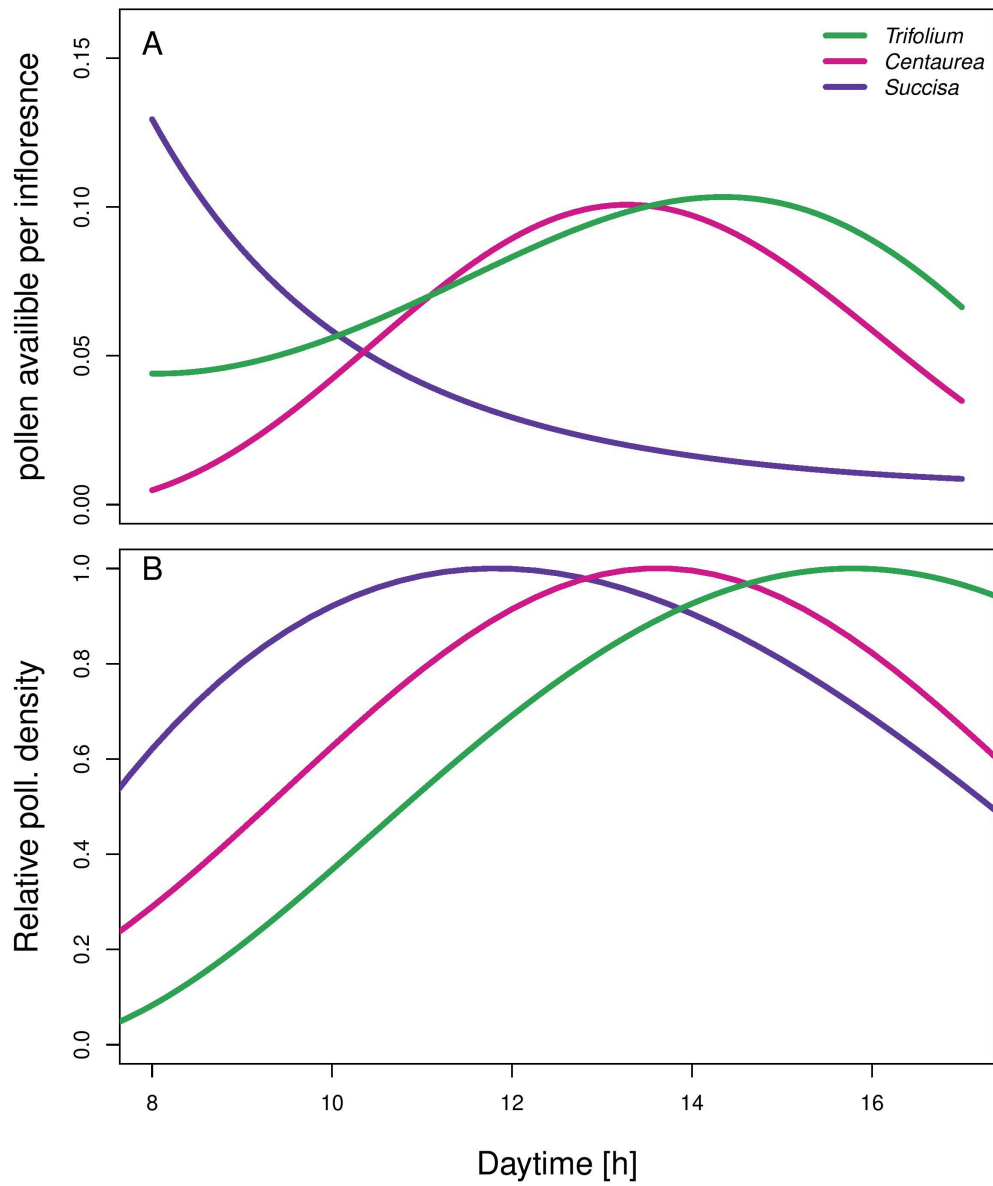


Table 3: Linear model of pollinator density (square-root transformed) depending on the time of day.

Predictor	<i>T. hybridum</i>				<i>C. jacea</i>				<i>S. pratensis</i>			
	Df	Sum sq.	R ²	P-value	Df	Sum sq.	R ²	P-value	Df	Sum sq.	R ²	P-value
time	1	5.223	0.522	<0.001	1	2.369	0.061	<0.001	1	2.850	0.180	<0.001
time ²	1	1.568	0.157	<0.001	1	28.599	0.741	<0.001	1	7.612	0.480	<0.001
time ³									1	0.215	0.014	0.1
Residuals	33	3.217	0.321		69	7.614	0.197		68	5.177	0.327	

8.2.1.5 Discussion Our results showed a strong diurnal pattern in pollen availability of three co-flowering plant species, closely matched by the peaks in visitation by their pollinators. However, the mechanisms of the timing of the peaks of pollen availability differed between the studied plant species. The afternoon peak of *Trifolium hybridum* pollen availability (Fig. 2 A) was caused mainly by the pattern of opening of flowers within an inflorescences, while the average amount of pollen in flowers showed only a moderate decrease during the day. This is in line with the explosive release of pollen from anthers, which is expected to lead into removal of the majority of pollen from flowers after a single visit (Galloni et al., 2007).

On the other hand, *Centaurea jacea* released pollen gradually from 09:00, with a peak at approximately 13:00 and a decrease in the late afternoon (Fig. 2 A). Gradual pollen presentation was caused by combination of gradual release of pollen from flowers and gradual opening of individual flowers within inflorescences. Pollen in *C. jacea* flowers was present on the top of a narrow flower tubes (Fig. 1 B), which are accessible to pollinators in only limited amounts at a time.

Succisa pratensis released pollen in the early morning in easily accessible anthers (Fig. 1 C), and since that moment the amount of available pollen decreased rapidly, whereas majority of pollen was already removed from flowers by pollinators before 12:00. The number of open flowers remained constant throughout the day, suggesting a single cohort of flowers develops every day in the morning throughout the lifetime of an inflorescence.

Several studies reported diurnal pattern in pollen presentation in various plant species (Percival, 1950; Young and Stanton, 1990; Gregg, 1991; LeBuhn and Anderson, 1994; Stone et al., 1998; Nansen and Korie, 2000; Castellanos et al., 2006; Eisen et al., 2017). Theoretical models by Harder and Thomson (1989) and LeBuhn and Holsinger (1998) showed that gradual pollen release and pollen presentation may strongly increase male fitness. Consequently LeBuhn and Holsinger (1998) predicts strong evolutionary pressure on development of pollen presentation schedules in plants (but see Eisen et al. (2017)). However, the particular presentation schedules may differ in the amount of pollen available at a moment, depending on the pollinator effectiveness: the presentation schedule should be more gradual with less effective pollinators and vice versa (Harder and Thomson, 1989; Castellanos et al., 2006). This is in the line with our observation, because studied plant species differed in their pollen presentation strategy: *C. jacea* and *T. hybridum* released pollen more gradually (Fig. 2 A, B), possibly because they are visited mainly by pollen-collecting bees (i.e., majority of pollen collected during a visit is eaten), whereas *S. pratensis* is visited mainly by hoverflies (Appendix S1, Table S1), which are more abundant and possibly remove lower amount of pollen per visit. However, detailed comparison of pollinator effectiveness is necessary to confirm this conclusion.

Our results also show different contribution of inflorescence development and architecture to pollen presentation: Whereas *T. hybridum* and *C. jacea* opened flowers within inflorescences gradually, *Succisa* did not, which resulted into rapid decline in the amount of available pollen. This fact may highlight the importance to include observations of inflorescence development to

other studies of pollen presentation. Moreover, scarce evidence suggests changes in number of active flowers within inflorescence per day during the inflorescence development (Percival, 1950; Thomson et al., 1989; Young and Stanton, 1990). It would be therefore interesting to include this observation into the next experiments.

Pollinator visitation activity tracked the pattern of pollen availability. *S. pratensis* was the only species where the maximum of pollinator visits did not match the actual peak in pollen availability (Fig. 3). We hypothesize that this is due to the overall diurnal pattern of activity of pollinators since we observed generally only few pollinators before 8:30 and a steady increase in pollinator activity was observed over all of the study site since that time until approximately 11:00. Between 8:00 and 9:00 a rapid change in microclimatic conditions typically occurs at the site – there is an increase in temperature and radiation and decrease in relative air humidity (Appendix S1, Figs. 1 and 2, A; B and C) connected also with evaporation of the morning dew. Thus, we suggest that the actual peak of pollinator visits to *S. pratensis* is a result of an equilibrium of an overall increase in pollinator activity with daytime and a concurrent decrease in the amount of available pollen on *S. pratensis*. *S. pratensis* also differs from the other two species under study by being an important source of nectar as well, which may be the reason for a less pronounced decrease in pollinator visits following the decrease in available pollen (as compared to *C. jacea*). If such shift in the sought-out reward indeed occurs, then the afternoon pollinator visits might contribute less to *S. pratensis* pollination than it could be expected based on their still relatively high densities (Young et al., 2007). However, we neither measured the amount of available nectar nor observed types of pollinator foraging behaviour in our study.

Furthermore, the matching patterns of the pollen release and pollinator visitation during day may be an important factor affecting the structure of community interaction network. E.g., Schwarz et al. (2021) experimentally proved the pollinator response to changing patterns in flower accessibility and revealed that diurnal reward availability is possibly an important driver affecting the structure of plant-pollinator interactions. Hence, overlooking the temporal structure in plant-pollinator interactions may possibly lead to overestimates of pollinator generalization (Brosi, 2016), because plant-pollinator interactions are usually analyzed at the species level (e.g., Ballantyne et al. (2017) but see e.g., Dupont et al. (2011); Tur et al. (2014)) without considering differences in visitation on diurnal scale. Our results indirectly suggest a strong temporal structure in the plant-pollinator interactions driven by pollen availability. A detailed investigation conducted on a finer scale may reveal that generalized systems are temporarily structured and more specialized than is expected (e.g., Lucas et al. (2018)).

Differences in the timing of pollen availability and subsequent pollinator visitation between co-flowering species may be advantageous for plant species sharing pollinators. While the temporal separation of pollen presentation is predicted to decrease heterospecific pollen transport and limit its negative effects on plant fitness, there is surprisingly little evidence for it to take place. Stone et al. (1998) showed differences in pollen presentation matching pollinator activity in

several species sharing pollinators. Investigation of heterospecific pollen transfer in pollinator sharing plant communities showed lower deposition of heterospecific pollen deposited on stigmas that could be expected from the pollinator visitation network (Zhang et al., 2021). Whereas floral morphology is usually considered an important factor limiting heterospecific pollen transfer (Moreira-Hernández and Muchhala, 2019; Peuker et al., 2020; Zhang et al., 2021), the effect of differences in pollen presentation schedules is usually not investigated. Moreover, only a limited number of studies have compared pollen presentation schedules of several co-flowering species (e.g., Stone et al. (1998)), and to our knowledge nobody has directly linked pollen presentation schedules in co-flowering community with pollinator pollen carry-over or pollen deposition. Ballantyne et al. (2017) found minor differences in the peaks of pollen deposition in several plants in the co-flowering community, suggesting differences in pollen presentation schedules; however, the experiment was not designed primarily to investigate this relationship. Similarly, some studies (Herrera, 1990; Stanghellini et al., 2002a; Rader et al., 2012; Willmer and Finlayson, 2014) showed diurnal variation in pollinator effectiveness in plant species, but without further investigation of causal mechanisms. Therefore, further research may reveal the relative importance of pollen presentation schedules in the co-flowering plant communities in comparison to other mechanisms allowing avoidance of heterospecific pollen transfer (such as pollen placement on different parts of the pollinator body, see Huang and Shi (2013)).

8.2.1.6 Conclusions Our findings show differences in the pollen presentation schedules of three co-flowering species and the response of the pollinator visitation activity in common grassland. In conclusion, pollinators closely track the pollen availability of the studied plant species, which may possibly decrease the competition for pollinators and allow co-flowering plants to share their pollinators. This highlights the need to consider temporal variation in plant-pollinator interactions even at the diurnal level.

8.2.1.7 Acknowledgements The authors thanks to members of Flower Ecology Group in Prague for discussions of the topic and valuable suggestions to earlier versions of the manuscript. Furthermore, the authors would like to thank to all the people involved in “Opylovači” project who selflessly participated in more than a decade of fieldwork. JŠ and LJ acknowledge support by the project “Grant Schemes at CU” (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935). ZJ has been supported by Charles University Research Centre Programme No. 204069. Microscopy was performed in the Laboratory of Confocal and Fluorescence Microscopy co-financed by the European Regional Development Fund and the state budget of the Czech Republic, projects no. CZ.1.05/4.1.00/16.0347 and CZ.2.16/3.1.00/21515, and supported by the Czech-BioImaging large RI project LM2018129. Computational resources were supplied by the project “e-Infrastruktura CZ” (e-INFRA LM2018140) provided within the program Projects of Large Research, Development and Innovations Infrastructures.

8.2.1.8 Author contribution ZJ and JŠ conceived the idea of research. JŠ and LJ designed the method for sampling pollen and collected the pollen and inflorescence data. EM, JH,

MM and ZJ collected the data on plant and pollinator abundances. ZJ and JŠ analysed the data. JŠ and ZJ wrote the manuscript with edits and comments from the rest of the authors.

8.2.1.9 Data availability The data for this manuscript are available at the FigShare repository: <https://figshare.com/account/home#/projects/149173>

8.2.1.10 Supporting information Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Supporting information:

- Figure S1: Microclimatic measurements 1
- Figure S2: Microclimatic measurements 2
- Table S1: Overview of pollinator visitors
- Table S2: Pollen production and average number of flowers per inflorescence
- Figure S3: Pollinator densities
- Figure S4: Absolute pollen availability during day

8.2.2 Pollen presentation mitigate competition for pollinators due to diurnal stratification of pollen transfer

Running title: Diurnal stratification of pollen transfer.

Jakub Štenc^{1*}, Lukáš Janošík¹, Martin Freudenfeld¹, Eva Matoušková², Jiří Hadrava², Michael Mikát², Klára Daňková², Tereza Hadravová², Tadeáš Ryšan², Jasna Simonová², Klára Koupilová³, Alice Haveldová¹, Eliška Konečná¹, Jan Martínek⁴, Barbora Jelínková⁴, Stanislav Vosolobě⁴, Karolína Dobešová⁴, Marie Smyčková^{1,5}, Jan Smyčka^{1,6}, Jitka Smyčková⁷, Lucie Studená⁷, Josef Nováček⁷, Štěpán Káně⁷, Kateřina Knotková⁸, Tomáš Figura^{4,9,10}, Jan Simon-Pražák^{2,11}, Kateřina Čermáková¹², Jiří Janoušek¹², Václav Bočan¹², Vojtěch Brož¹³, Anna Suchá¹⁴, Eva Horčíčková¹⁵, Tomáš Dvořák^{2,16}, Tomáš Jor^{2,16}, Doubravka Požárová¹⁷, Karel Plavec¹⁸, Zdeněk Janovský¹⁹

¹ Department of Botany, Faculty of Science, Charles University, Benátská 2, 128 41 Prague, Czech Republic

² Department of Zoology, Faculty of Science, Charles University, Viničná 7, 128 41 Prague, Czech Republic

³ Rovná 1333, Sulice, 25168, Czech Republic

⁴ Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 128 44 Prague, Czech Republic

⁵ Department of Forest Ecology, Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague, Czech Republic

⁶ Center for Theoretical Study, Charles University and the Academy of Sciences of the Czech Republic, 110 00, Prague, Czech Republic

⁷ Faculty of Science, Charles University, Albertov 6, 128 00 Prague 2, Czech Republic

⁸ Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, Brno 611 37, Czech Republic

⁹ Department of Mycorrhizal Symbioses, Institute of Botany, Czech Academy of Sciences, Lesní 322, 25243 Průhonice, Czech Republic

¹⁰ Naturalis Biodiversity Center, Darwinweg 2, 2333 CR, Leiden, The Netherlands

¹¹ Museum of Eastern Bohemia in Hradec Králové, Eliščíno nábřeží 465, 500 03 Hradec Králové 3, Czech Republic

¹² Department of Cell Biology, Faculty of Science, Charles University, Viničná 7, 128 41 Prague, Czech Republic

¹³ Department of Ecology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic

¹⁴ Faculty of Education, Charles University, Magdalény Rettigové 4, Prague 1, 116 39, Czech Republic

¹⁵ Department of Environment, Faculty of Environment, Jan Evangelista Purkyně University in Ústí nad Labem, Pasteurova 3632/15, 400 96, Ústí nad Labem, Czech Republic

¹⁶ Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences, Kamýcká 129, 165 00, Prague , Czech Republic

¹⁷ Lhota pod Džbánem 23, 27007 Mutějovice, Czech Republic

¹⁸ Gymnázium AD Fontes s.r.o, Jánská 14, 586 01, Jihlava, Czech Republic

¹⁹ Svatý Jan t. Krsovice 1, 285 04 Uhlířské Janovice, Czech Republic

*Contact and corresponding author, e-mail address: jakubstenc@gmail.com.

8.2.2.1 Open Research statement All the data are available online on FigShare repository: The data are under temporal embargo until acceptance of the manuscript in the journal or available upon request.

https://figshare.com/projects/Strategies_of_pollen_presentation_mitigate_competition_for_pollinators_due_to_partial_diurnal_stratification_of_pollen_transfer/184342

8.2.2.2 Key words *Centaurea jacea*, plant sexual reproduction, pollen release schedule, pollen transfer, pollinator diurnal activity, pollinator sharing, *Succisa pratensis*, *Trifolium hybridum*

8.2.2.3 Abstract *Premise:*

Pollen presentation and release strategies are believed to affect the timing of plant-pollen interactions and therefore play a crucial role in pollen transfer. However, only limited evidence links pollen release and temporal changes in the pollen load carried by pollinators. In the present study, we aim to investigate how different pollen presentation strategies affect both the quantity and quality of pollen transferred by plant pollinators and discuss the potential for structuring plant-pollinator networks.

Methods:

We investigated pollen load on pollinators collected from three co-flowering species during the daytime with a known pollen presentation strategy: *Succisa pratensis* releasing pollen early in the morning, *Centaurea jacea* which employs a gradual pollen presentation and *Trifolium hybridum* with explosive pollen release during pollinator visits. We compared the patterns in the amount of conspecific pollen grains on the pollinator body (pollen of visited plant species, i.e., pollen quantity) and the proportions of conspecific pollen grains from the total pollen load (pollen quality) of different pollinators of the studied plant species. Then we linked pollen transfer to the pollinator visitation pattern during the day.

Results:

The three plant species differed in the pattern of pollen transfer by their pollinators. Pollinators of *Succisa pratensis* carried the majority of the pollen in the morning after the pollen was released followed by a drop in both pollen quantity and quality. Pollinators of *C. jacea* and *T. hybridum* carried more stable pollen loads over time, which we explain by plant spatial density and flower morphology combined with pollen release strategy, respectively.

Conclusion:

By linking the diurnal pattern of pollen transfer with the pollinator visitation pattern, our results demonstrate the diurnal structuring of pollen transfer and the plant's ability to mitigate the negative effect of pollinator sharing.

8.2.2.4 Introduction Pollen transfer mediated by animal vectors represents one of the most important biological interactions worldwide, and is responsible for the sexual reproduction of the vast majority of flowering plants (Ollerton *et al.*, 2011). However, pollen transfer mediated by animal pollinators is surprisingly inefficient, resulting in only a small proportion of successfully transferred pollen (e.g., Gong and Huang (2014)). In particular, in generalized plant-pollinator systems, where plants often share their pollinators, low pollination efficiency is often caused by competition for pollinators and/or heterospecific pollen transfer mediated by less flower constant pollinators (Arceo-Gómez *et al.*, 2016).

Competition for pollinators between co-flowering plants potentially reduces visitation (Mesgaran *et al.*, 2017), it may reduce the quantity (i.e., the amount of conspecific pollen) and quality (i.e., the proportion of conspecific pollen) of pollen transferred (Campbell, 1985; Feinsinger and Tiebout, 1991) and plant fitness and hypothetically destabilize the coexistence of co-flowering species (Johnson *et al.*, 2022; Bruninga-Socolar *et al.*, 2023). Similarly, heterospecific pollen transfer may have negative direct and indirect consequences for both male and female reproductive success by pollen depletion from the population (Parker *et al.*, 2016), reducing the deposition of conspecific pollen (Morales and Traveset, 2008; Fang and Huang, 2016; Moreira-Hernández and Muchhala, 2019) and, as a result directly, reducing plant fitness (Briggs *et al.*, 2016; Lanuza *et al.*, 2021). On the other hand, co-flowering species may play crucial roles in facilitating pollinator visitation, especially in the case of less attractive plant species (Feldman *et al.*, 2004; Mesgaran *et al.*, 2017; Ye *et al.*, 2020). Moreover, pollinator sharing may have a lower negative impact on pollen transfer and plant reproduction than can be expected from the flower visitation pattern (e.g., Montgomery, 2009; Moreira-Hernández *et al.*, 2023), indicating that plant can (to some extent) mitigate the negative effects of pollinator sharing (i.e., competition and heterospecific pollen transfer). To mitigate the negative effects of pollinator sharing, plants have developed several mechanisms to limit heterospecific pollen transfer and competition for shared pollinators. These mechanisms include varying pollen placement on the pollinator’s body (Huang *et al.*, 2015), pollinator partitioning in time (Stone *et al.*, 1998) and space (Kipling and Warren, 2014) and promoting pollinator flower constancy (Huang *et al.*, 2015; Us Van Der Niet *et al.*, 2020).

Flower constancy (i.e., pollinator tendency to visit consequently flowers of the same species during the foraging Waser (1986a)) has been widely discussed as a potential mechanism decreasing heterospecific pollen transfer (Kunin and Iwasa, 1996; Montgomery, 2009), and it has been hypothesized that flower constancy may allow “temporal specialization” of individual pollinators during foraging among flowers (Brosi, 2016) enabling the coexistence of plant species sharing pollinators (Huang *et al.*, 2015). However, the level of flower constancy depends on the quantity and quality of the reward (Chittka, 1997), and with decrease in available rewards, pollinators may switch to visit other species (Chittka, 1997). Therefore, plants may benefit from a temporal increase in reward availability, resulting in an increase in conspecific pollen transfer. For example, plants may develop a pollen presentation schedule, i.e., a pattern of pollen availability tracked by pollinator visitation (Stone *et al.*, 1998; Štenc *et al.*, 2023).

It has been hypothesized that the displacement of pollen presentation schedules serves resource partitioning to avoid the negative effect of plant competition for pollinators (e.g., Stone et al., 1998; Štenc et al., 2023). However, further investigation of mechanisms allowing plants to temporally increase the amount of pollen presented to pollinators corroborated with pollen transfer quantity and quality data is scarce, leaving a gap in our further understanding of plant-pollinator interactions.

In a previous study, we demonstrated that the studied species differ in the timing of pollen release, which is closely followed by pollinator visitation activity on the flowers (Štenc et al., 2023). In the current study, we aimed to investigate how the diurnal dynamics of pollen release affect the pollen load on pollinator bodies collected from the same plant species. For the study, we used three co-flowering plant species with partially overlapping pollinator spectra, but differing pollen release strategies and local densities, resulting in distinct pollinator visitation patterns. We hypothesize that the timing of pollen presentation allows some plant species to establish “temporal dominance”, which translates to a higher proportion of conspecific pollen transferred by even less flower constant pollinators. We predict that the studied species utilize different mechanisms to mitigate the negative effect of pollinator sharing, which combined may decrease the probability of heterospecific pollen transfer.

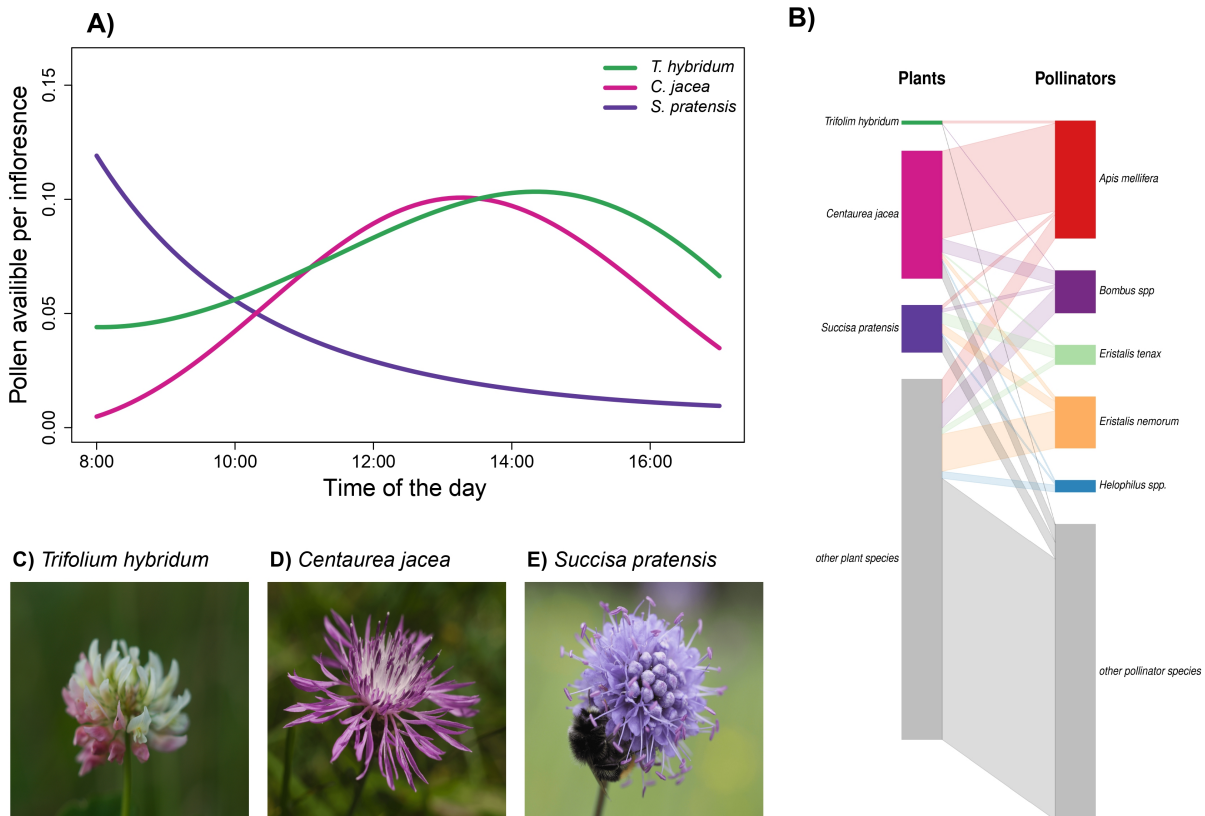


Fig. 1: A) The pollen availability changes during the day and differs among species: while *S. pratensis* releases pollen in the morning hours, *C. jacea* and *T. hybridum* peak in the afternoon, @Stenc2023. B) Plant-pollinator network from the study site. The studied species and their interactions are indicated. C), D) and E) Inflorescence of *T. hybridum*, *C. jacea* and *S. pratensis*, respectively.

8.2.2.5 Methods

8.2.2.5.1 Study system To conduct our experiment, we used study system of three plant species: *Trifolium hybridum* L. (Fabaceae), *Centaurea jacea* L. (Asteraceae) and *Succisa pratensis* Moench (Caprifoliaceae) from co-flowering meadow plant community. All three species co-occur together and partly share their pollinators (Fig. 1 B); however, they differ in the timing of pollen release (Fig. 1 A), flowering plant density and flower morphology (Fig. 1 C).

The study site was located at the meadow “K Handrkovu” close to village Vernýřov in Central Bohemia Region, Czech Republic (N 49.8466, E 15.1498). The study site was closely described in Janovský et al. (2013), and the study system was described in Štenc et al. (2023). The study was conducted under sunny weather with no or only moderate wind conditions. Moreover, we directly measured solar radiation, relative air humidity and temperature at the study site with two Minikin RTHi/QTHi dataloggers (EMS BRNO, Brno, Czech Republic) approximately 50 meters apart. The wind conditions were measured as average wind speed at 30-minute intervals with two anemometers (Windmaster 2, Kaindl electronics, Rohrbach, Germany) placed next to the Minikin dataloggers. An overview of the climatic conditions at the site can be found in Appendix 1, Fig. S1 and Fig. S2.

8.2.2.5.2 Pollinator pollen load To estimate the diurnal pattern of the number of pollen grains carried by different pollinators, we collected insects while foraging on flowers of the studied species during the time window of pollinator activity (from 8:00 to 18:00 Central European Summer Time). The sampling of pollen loads occurred at the study site during 2021 (10-13th, and 16th of August).

Freely foraging pollinators were captured by an insect net immediately after a legitimate visit (i.e., visit that included touching the reproductive parts of the flower) with a duration longer than 5 seconds to ensure the individual had enough time to collect pollen. As we did not closely track previous foraging of pollinators, there was no control for previously visited flowers by individual pollinators.

After capture, pollinators were identified and killed immediately with tweezers and while held in clean tweezers by one half of the body, the other half of the pollinator body was swabbed with a 5 x 5 x 2 mm block of fuchsin-stained jelly held in another tweezers cleaned regularly between samples following Kearns and Inouye (1993). In the case of *Apis mellifera* and *Bombus* spp., we avoided swabbing pollen baskets, as the pollen in them most likely does not reach the stigma (Thorp, 2000). The jelly blocks were then moved to the sterile microtube and labeled, the insect specimens were stored in 70% ethanol. The jelly blocks were melted on microscopic slides in the field laboratory, covered with a slide cover and sealed with nail polish to avoid contamination.

The slides were examined at 400x magnification. The number of conspecific and heterospecific pollen grains was counted with respect to the plant species after which visit the pollinator was captured. The pollen grains were identified visually within the 0.5 x 0.5 mm grid printed on transparent foil and placed over the sample. Within the grid, every second cell was inspected,

therefore, counted pollen from the sample represents approximately 1/4 of pollen from the whole pollinator body (1/2 of the body swabbed x 1/2 of the sample). The counts representing 1/4 of the pollinator body were then used in the analyses and we present the data multiplied by 4 to show the estimates of the realistic amount of carried pollen.

8.2.2.5.3 Pollinator density To estimate pollinator diurnal activity and density, we used aggregate data on plant-pollinator interactions from the same site and time of the year for 11 seasons (2011-2021). The time of data collection during the season corresponded to the flowering time of *Succisa pratensis* (usually middle August). These data were collected following the same protocol as described in Janovský et al. (2013) at the same study site and brief description of the method follows.

Pollinator censuses were conducted over 93 plots regularly arranged in a 20 × 20 m grid over the whole study site. Each plot is a 4 × 4 m square with a permanently marked position within the grid and is censused every year at least 20 times during the collection campaign spanning approximately the 13th and 26th August, roughly matching the peak of flowering of *S. pratensis*. The censuses were conducted at the time of main pollinator activity from 7:00 to 19:00 (Central European Summer Time). During the census, all pollinators legitimately visiting (i.e., touching reproductive structures for more than 2 seconds) flowers within the plot were recorded with the plant species identity and time.

To control for flower density, we counted flowering plant stalks in 64 cells within the lattice of 0.5 × 0.5 m subplots evenly dividing the plot. For *Trifolium hybridum* we recorded only presence or absence data in the subplots, resulting in a semiquantitative assessment of inflorescence density in each plot.

To account for potential phenology shifts within the sampling period, the plant censuses were conducted twice during each sampling campaign (with an exception of 2011 with only one census).

8.2.2.5.4 Statistical analysis Conspecific pollen load (pollen quantity)

To investigate the diurnal pattern in the amount of conspecific pollen, we applied linear models with time of day and pollinator species and their interactions as predictors for individual plant species. In the case of time of day, we considered both linear and quadratic terms in expectation of unimodal patterns. To achieve homogeneity of variance, we applied logarithmic transformation to the response variable (amount of conspecific pollen). For *T. hybridum* we had only one pollinator species available; hence, we did not test the interaction between pollinator species and time.

Percentage of conspecific pollen (pollen quality)

We computed the percentage of conspecific pollen derived from the sum of all pollen counted on the pollinator body. Then, we fitted a generalized linear model (GLM) for each plant species

separately with a quasibinomial distribution. We utilized the quasibinomial distribution to avoid the effect of overdispersion which did not allow for the use of a binomial distribution. In all models, we used time of day as a predictor, and in the models of *C. jacea* and *S. pratensis*, we also tested the effect of pollinator species and the interaction of time with pollinator species (except the case of *T. hybridum*).

Pollinator density

Pollinator density was computed from plant-pollinator interaction data as the number of pollinators visiting plant species in a 10-minutes long time interval during the day standardized by plant species density.

First, we counted the number of pollinator visitors in plots per plant species in 10-minute time intervals during the day. As we possessed reliable data for the period from 7:00 to 18:30, this resulted in 70 individual 10-minute time intervals per census. Then we summed the recorded interactions originating from a given time interval, which we standardized the number of visitors by the sum of plant flowering stalk density in the plots, obtained from the plant censuses. For the standardization by plant density, we used the average value of flowering stalk density from two censuses per year. Therefore, we estimated the number of pollinator visits per stalk for 70 intervals during the day.

Then, we constructed separate models for each combination of pollinator species and plant species with polynomial regression since unimodal patterns of pollinator visitation activity during the day were expected. The cubic term was considered only in the case that a quadratic term was significant. The use of polynomial terms serves in our study solely to track the general shape of the relationship between pollinator density and time by using linear model, but we do not assume a polynomial functional relationship between pollinator density and time. To meet the assumptions of the linear model, we square-root transformed the response variable (pollinator density).

To graphically present the relationship between pollinator density and time, we constructed bar plots showing the estimated density of pollinator species per plant in 10-minute intervals represented by each bar (Fig. 3). The raw values and prediction with confidence intervals can be seen in the supplements (Fig. S3).

Pollinator performance over time

To illustrate the potential pollinator contribution to pollen transfer during the day, we multiplied model predictions for the amount of conspecific pollen and pollinator density. Based on the proposal of Ne'eman et al. (2010), we call this combination of pollinator density and pollen transfer “pollinator performance”. The pollinator performance during the day then represents the number of conspecific pollen grains transferred by pollinators in a given time per flowering stalk by pollinator species.

All analyses were performed in R version 4.2.0 under the base installation (R Core Team, 2022b).

To present the plant-pollinator network we used the Bipartite package (Dormann et al., 2008).

8.2.2.6 Results In total, we collected pollen loads from 472 individual pollinators (80,130 and 261 from *Trifolium hybridum*, *Centaurea jacea* and *Succisa pratensis* respectively). Pollinators belonged to 5 groups following the lowest taxonomic resolution possible with sufficient observations: honeybee (*Apis mellifera* Linnaeus, 1758), bumblebees (*Bombus* spp.), *Eristalis nemorum* (Linnaeus, 1758), *Eristalis tenax* (Linnaeus, 1758) and *Helophilus* spp. (see Table S1). In total, we identified 163,139 pollen grains into two groups: conspecific and heterospecific pollen, according to the species identity of the plant from which the pollinator was sampled.

8.2.2.6.1 Amount of conspecific pollen in pollen load The amount of conspecific pollen varied significantly in all plant species over time (Table 2). The pollen load of *A. mellifera* on *T. hybridum* showed a unimodal response to time, with a peak at 13:00 (Fig. 2 A). Pollen loads on both *A. mellifera* and *Bombus* species visiting *C. jacea* increased with time, with differences in magnitude: generally, *Bombus* species carried lower amounts of *C. jacea* pollen grains than did *A. mellifera* (Fig. 2 C). Pollen loads carried by all the pollinators of *S. pratensis* decreased rapidly with time. The decrease was less steep in the case of *A. mellifera*, however, with a very similar pattern as that in the case of *E. tenax*, *E. interupta* and *Helophilus* species (Fig. 2 E).

Table 1: Summary of pollinator visitation by plant species. The left part of the Table shows the summary for 2021 when the pollen transfer experiment was conducted. The right part of the Table shows the sum of recorded interactions for the years 2011-2021.

Pollinator	2021				2011-2021			
	<i>Trifolium hybridum</i>	<i>Centaurea jacea</i>	<i>Succisa pratensis</i>	Other species	<i>Trifolium hybridum</i>	<i>Centaurea jacea</i>	<i>Succisa pratensis</i>	Other species
<i>Apis mellifera</i>	13	513	22	139	297	2,592	230	960
<i>Bombus spp.</i>	2	80	19	147	120	1,946	1,071	965
<i>Eristalis tenax</i>	0	9	68	36	0	226	2,752	616
<i>Eristalis nemorum</i>	0	28	55	217	0	64	964	713
<i>Helophilus spp.</i>	0	14	14	41	7	410	1,661	456
Other pollinators	4	101	98	1,529	74	1,173	1,912	33,691

Table 2: Summary of linear models for individual plant species of the amount of conspecific pollen (log-transformed) on the pollinator body depending on time of day and pollinator species and their interactions. CI stands for 95% confidence interval. Significant predictors are in bold. (level of alpha value is 0.05) The intercept represents value the for *Apis mellifera*.

Predictors	<i>Trifolium hybridum</i>			<i>Centaurea jacea</i>			<i>Succisa pratensis</i>		
	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p
Intercept	3.34	2.67 – 4.02	<0.001	4.82	4.14 – 5.51	<0.001	4.50	4.06 – 4.94	<0.001
time	0.01	0.01 – 0.02	<0.001	0.01	0.00 – 0.01	0.004	-0.00	-0.01 – -0.00	<0.001
time ²	-0.00	-0.00 – -0.00	<0.001	-0.00	-0.00 – -0.00	0.028	-	-	-
pollinator (<i>Bombus</i> spp.)	-	-	-	-2.28	-2.90 – -1.67	<0.001	-	-	-
pollinator (<i>Helophilus</i> spp.)	-	-	-	-	-	-	0.83	0.21 – 1.46	0.009
pollinator (<i>Helophilus</i> spp.)	-	-	-	-	-	-	-	-	-
pollinator (<i>Eristalis tenax</i>)	-	-	-	-	-	-	1.22	0.64 – 1.80	<0.001
time × pollinator (<i>Eristalis nemorum</i>)	-	-	-	-	-	-	-	-	-
time × pollinator (<i>Eristalis tenax</i>)	-	-	-	-	-	-	-0.00	-0.00 – -0.00	0.003
time × pollinator (<i>Helophilus</i> spp.)	-	-	-	-	-	-	-0.00	-0.00 – -0.00	0.003
Observations	80			130			261		
R ² /R ² adjusted	0.195 / 0.174			0.446 / 0.428			0.653 / 0.643		

8.2.2.6.2 Proportion of conspecific pollen in pollen load The proportion of conspecific pollen did not change significantly with time in the case of *T. hybridum* (Table 3 A). The average proportion of *T. hybridum* pollen was approximately 70%, with higher variation in the morning hours. The pollen loads of *A. mellifera* from *C. jacea* showed a constant pattern over time with approximately 95% conspecific pollen but differed from pollen loads of *Bombus* species, in which the proportion of *C. jacea* pollen increased by a factor of three with time. *S. pratensis* showed a rapid decrease in the proportion of conspecific pollen from morning hours, with less than 40% of conspecific pollen carried by pollinators after 13:00. Moreover, individual pollinator species varied in the proportion of conspecific pollen, with *E. tenax* carrying, on average, the highest proportion of conspecific pollen and *E. interrupta* the least. However, the variability in the pollen load of individual pollinators was considerable (see Fig. 2).

Table 3: Summary of quasibinomial generalized linear models for individual plant species of the percentage of conspecific pollen (pollen quality) on the pollinator body depending on time of day and pollinator species and their interactions. CI stands for 95% confidence interval. Significant predictors are in bold (level of alpha value is 0.05). The intercept represents value for *Apis mellifera*.

Predictors	<i>Trifolium hybridum</i>			<i>Centaurea jacea</i>			<i>Succisa pratensis</i>		
	Odds Ratios	CI	p	Odds Ratios	CI	p	Odds Ratios	CI	p
Intercept	2.15	2.00 – 2.31	<0.001	12.73	5.04 – 36.62	<0.001	7.60	3.34 – 18.67	<0.001
time	1.00	1.00 – 1.00	0.125	1.00	1.00 – 1.00	0.654	0.99	0.98 – 0.99	<0.001
pollinator (<i>Bombus</i> spp.)	-	-	-	0.02	0.00 – 0.08	<0.001	-	-	-
pollinator (<i>Helophilus</i> spp.)	-	-	-	-	-	-	0.25	0.09 – 0.70	0.009
pollinator (<i>Eristalis nemorum</i>)	-	-	-	-	-	-	-	-	-
pollinator (<i>Eristalis tenax</i>)	-	-	-	-	-	-	0.59	0.21 – 1.57	0.297
time × pollinator (<i>Eristalis nemorum</i>)	-	-	-	-	-	-	-	-	-
time × pollinator (<i>Eristalis tenax</i>)	-	-	-	-	-	-	1.00	1.00 – 1.01	0.060
time × pollinator (<i>Helophilus</i> spp.)	-	-	-	-	-	-	1.01	1.00 – 1.01	0.012
Observations	80			130			261		

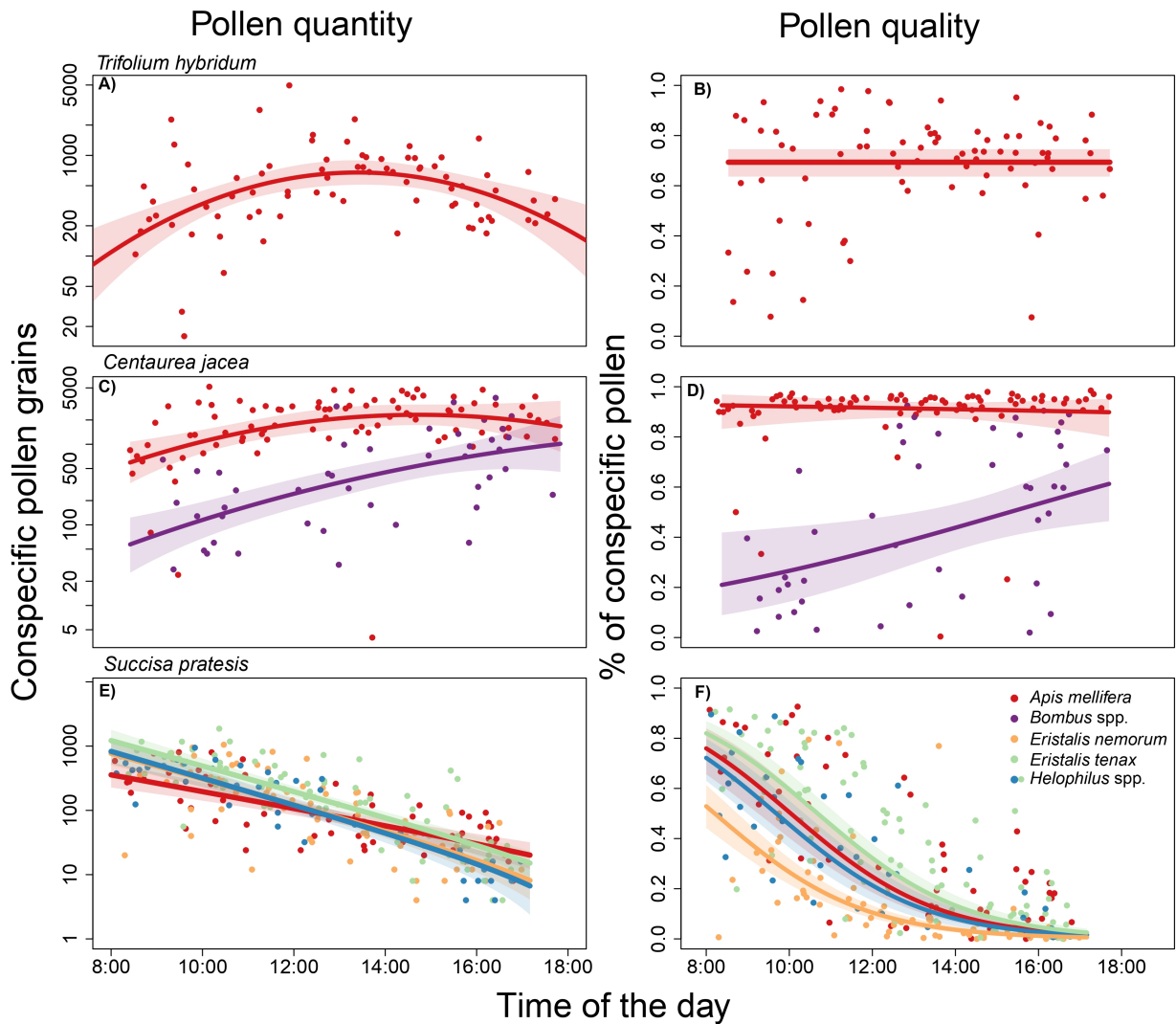


Fig. 2: Pollen load by plant species by time of day. Left panel (A,C and E) shows the pollen load quantity as the number of conspecific pollen grains on the pollinator body and the right panels (B, D and F) show the pollen load quality as the % of conspecific pollen grains from all the pollen grains on the pollinator body. Graphs A) and B) are for *T. hybridum*, C) and D) for *C. jacea* and E) and F) for *S. pratensis*.

Table 4: The summary of linear models of pollinator density (square root-transformed) of *Trifolium hybridum* depending on time of day. CI stands for 95% confidence interval. Significant predictors are in bold (level of alpha value is 0.05). The intercept represents the value for *Apis mellifera*.

Predictors	<i>Apis mellifera</i>			<i>Bombus spp.</i>		
	Estimates	CI	p	Estimates	CI	p
Intercept	0.06	0.05 – 0.07	<0.001	0.04	0.03 – 0.05	<0.001
time	0.16	0.10 – 0.22	<0.001	0.08	0.03 – 0.12	0.001
time ²	-0.06	-0.12 – -0.00	0.044	-0.08	-0.13 – -0.04	0.001
Observations	64			58		
R ² /R ² adjusted	0.363 / 0.343			0.320 / 0.295		

8.2.2.6.3 Pollinator visitation *T. hybridum* was predominantly visited by *A. mellifera* and partially by *Bombus* species (Fig. 1. B, Table 1). The number of visits increased with time and peaked between 15:00 and 16:00 (Fig. 3 A). Flowers of *C. jacea* were dominated by *A. mellifera* and *Bombus* spp. with occasional visits by *Helophilus* spp. (Table 1). The visitation peaked between 14:00 and 15:00; however, this was caused mainly by the gradual increase and decrease in the number of visiting *A. mellifera* while bumblebee visits slowly increased until 17:00. In the case of *S. pratensis*, pollinator visits increased suddenly the morning hours and peaked in between 9:00 and 10:00. The composition of *S. pratensis* pollinators was more diverse and dominated mainly by hoverflies. The most frequent visitors were *E. tenax*, *Helophilus* spp., *Bombus* spp., *E. nemorum* and *A. mellifera* (Table 1.).

Table 5: The summary of linear models of pollinator density (square root-transformed) of *Centaurea jacea* depending on time of day. CI stands for 95% confidence interval. Significant predictors are in bold (level of alpha value is 0.05). The intercept represents value for *Apis mellifera*.

Predictors	<i>Apis mellifera</i>			<i>Bombus</i> spp.			<i>Helophilus</i> spp.		
	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p
Intercept	0.12	0.12 – 0.13	<0.001	0.11	0.11 – 0.12	<0.001	0.05	0.05 – 0.06	<0.001
time	0.19	0.16 – 0.23	<0.001	0.12	0.09 – 0.15	<0.001	-0.01	-0.05 – 0.02	0.413
time ²	-0.31	-0.35 – -0.27	<0.001	-0.06	-0.09 – -0.03	<0.001	-0.08	-0.12 – -0.05	<0.001
Observations	70			70			70		
R ² /R ² adjusted	0.861 / 0.857			0.551 / 0.538			0.253 / 0.231		

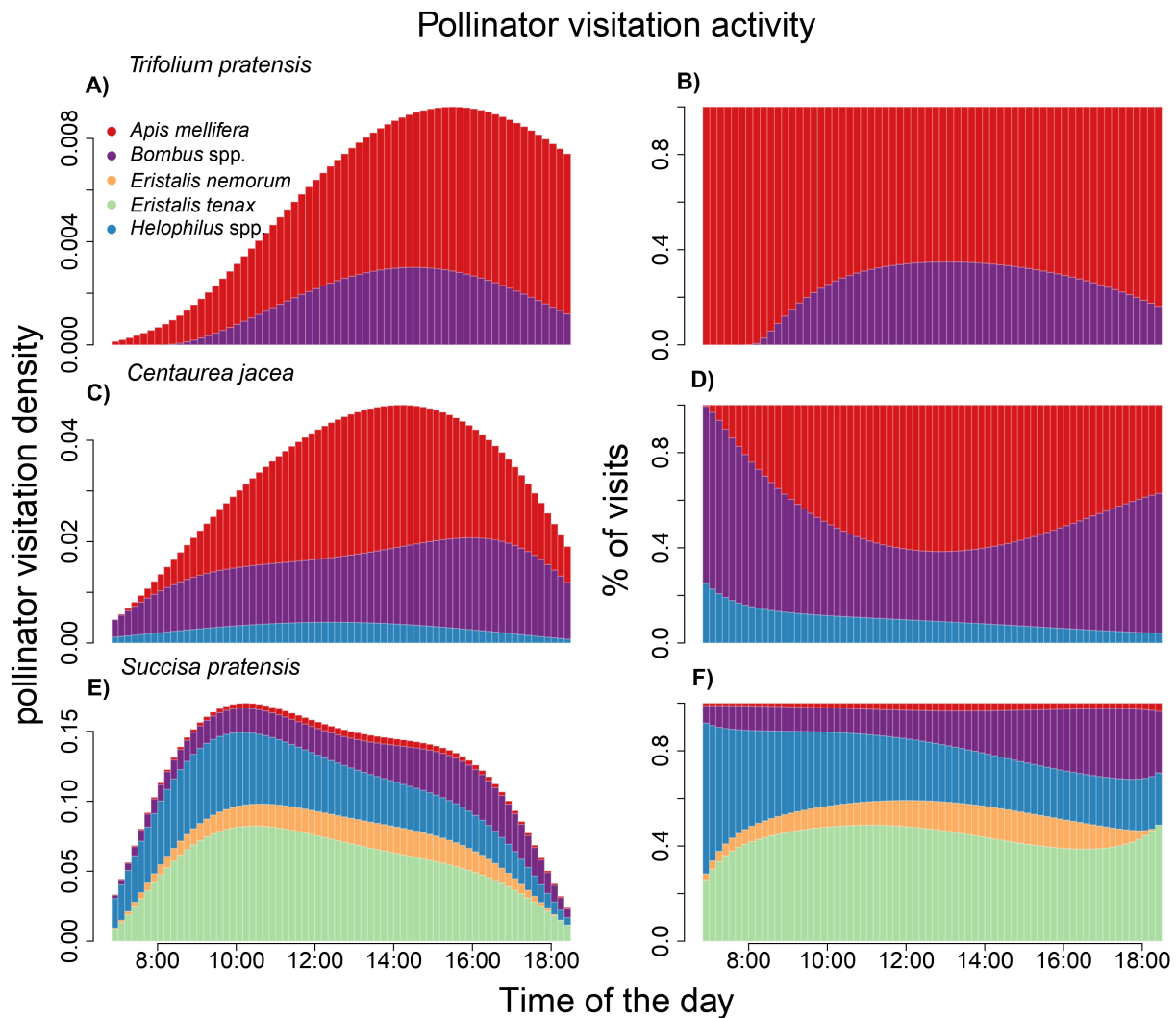


Fig. 3: Pollinator density by time of day. Every bar represents predicted pollinator density for 10-minute time frame. The left panel (A, C and E) shows the predicted number of pollinator visitors per flowering stalk, and the right panels (B, D and F) show their relative proportions. Graphs A) and B) are for *T. hybridum*, C) and D) for *C. jacea* and E) and F) for *S. pratensis*. Other groups of pollinators visiting plant species are not shown as they represent a wide group sparsely interacting with the plant species.

Table 6: Summary of linear models of pollinator density (square root-transformed) of *Succisa pratensis* depending on time of day. CI stands for 95% confidence interval. Significant predictors are in bold (level of alpha value is 0.05). The intercept represents value for *Apis mellifera*.

Predictors	<i>Apis mellifera</i>			<i>Bombus spp.</i>			<i>Helophilus spp.</i>			<i>Eristalis tenax</i>			<i>Eristalis nemorum</i>		
	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p	Estimates	CI	p
Intercept	0.05	0.05 – 0.06	<0.001	0.14	0.13 – 0.15	<0.001	0.18	0.18 – 0.19	<0.001	0.23	0.22 – 0.24	<0.001	0.11	0.10 – 0.11	<0.001
time	0.02	-0.04 – 0.09	0.455	0.16	0.08 – 0.23	<0.001	-0.25	-0.31 – -0.18	<0.001	-0.11	-0.19 – -0.03	0.009	-0.01	-0.06 – 0.05	0.846
time ²	-0.12	-0.18 – -0.05	<0.001	-0.15	-0.23 – -0.07	<0.001	-0.17	-0.24 – -0.11	<0.001	-0.38	-0.46 – -0.29	<0.001	-0.27	-0.33 – -0.21	<0.001
time ³	-	-	-	-	-	-	-	-	-	0.10	0.02 – 0.19	0.013	-0.05	-0.10 – 0.01	0.130
time ⁴	-	-	-	-	-	-	-	-	-	-0.12	-0.20 – -0.03	0.006	-0.08	-0.14 – -0.02	0.011
Observations	70			70			70			70			70		
R ² /R ² adjusted	0.173 / 0.148			0.329 / 0.309			0.563 / 0.549			0.622 / 0.599			0.590 / 0.565		

8.2.2.6.4 Pollinator performance over time Based on pollen carried by pollinators and pollinator visitation density, the performance varied over time for each plant species. The pollinator performance of *A. mellifera* in *T. hybridum* pollen transfer was the lowest (Fig. 4 A) due to the relatively lower amount of pollen transferred by the pollinators and the low visitation density. In *C. jacea*, *A. mellifera* contributed to the majority of pollen transfer while *Bombus* species' contribution was limited (Fig. 4 B) mainly by their lower visitation density (Fig. 3 C, D). Similarly, in the case of *S. pratensis*, the differences in pollinator performance were driven mainly by the pollinator density, resulting into limited performance of *Apis mellifera* and *E. interrupta* who both form only small proportion of visits to *S. pratensis* (Figs. 1 B, 3 E, F and Table 1). *E. tenax* and *Helophilus* contributed to pollen transfer of *S. pratensis* mainly in the early morning hours as both pollinator visitation and pollen load peaked in similar times (Fig. 4 C).

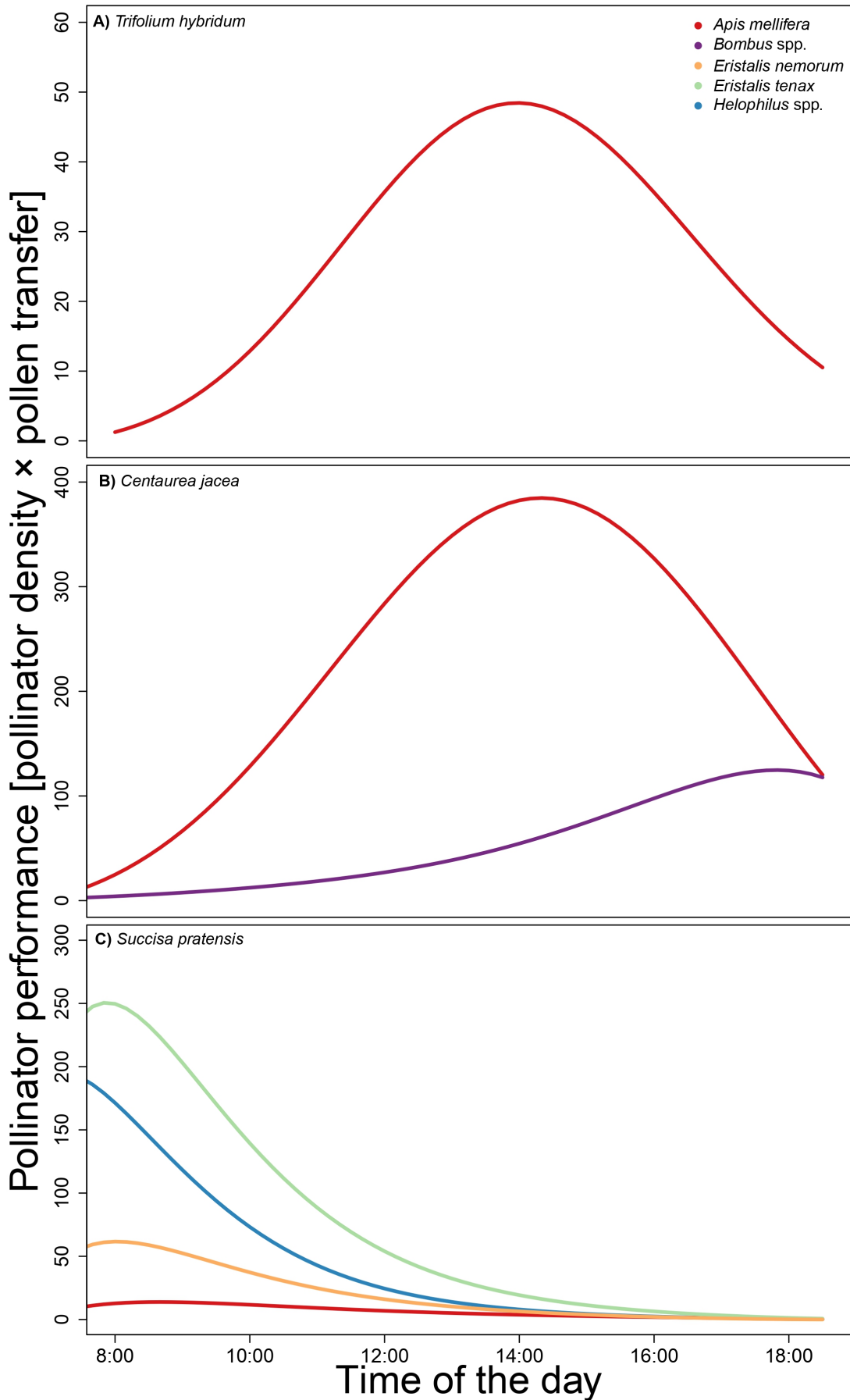


Fig. 4: Pollinator performance over time per plant species. Pollinator performance represents the combination (multiplication) of predictions for pollinator density and the transfer of conspecific pollen grains at a given time. Graphs A), B) and C) are for *Trifolium hybridum*, *Centaurea*

8.2.2.7 Discussion Our results show diurnal dynamics in the amount of conspecific pollen load (pollen quantity) carried by individual pollinators, with distinctive patterns among the three studied plant species and similar changes in the proportion of conspecific pollen (pollen quality). During the day, the quantity and quality of the carried pollen load vary significantly, with distinctive patterns for each study plant species (Fig. 2, Tables 2 and 3). Concurrently, the pollinator visitation pattern differed among plant species, resulting in plant-pollinator specific patterns in the number of interactions and their proportions during the day (Fig. 3, Tables 4, 5 and 6). Finally, the combination of pollen transfer and pollinator density illustrates pollinator performance during the day (Fig. 4). In the following text, we further discuss the mechanisms leading to particular patterns of pollen transfer by individual plant species and then summarize the results.

Trifolium hybridum

The quantity of conspecific pollen loads carried by *T. hybridum* pollinators peaked at approximately 13:00, approximately one hour before the peak of available pollen in the inflorescence and peak of pollinator visits. On the other hand, time did not have significant effect on the pollen quality, which was highly variable throughout the day and considerably more variable in the morning hours. The peak in pollen load quantity preceding the peak in pollen availability may be caused by the fact that our estimate is also driven by the number of open flowers within the inflorescence (Štenc et al., 2023), which mainly contributed to the pattern in pollen presentation dynamics. Furthermore, the higher stochasticity in the pollen quality of *T. hybridum* may be explained by the pollen release mechanism: *Trifolium* species release pollen via explosive mechanism, resulting in the removing of the majority of pollen from individual flowers during a single pollinator visit (Galloni et al., 2007). Therefore, pollinators visiting individual flowers may encounter flowers that were already visited and depleted by previous visits. Indeed, a study from Kudo and Harder (2005) reported high pollen removal per single visit in several Fabaceae species (approximately 40-80% of all pollen produced per flower, depending on the species), resulting in more stochastic pattern of flowers with and without pollen.

The explosive pollen release may also lead to more specific pollen placement, predominantly on the upper-front of the pollinator body (Galloni et al., 2007), allowing partitioning of pollinator body among co-flowering species and finally decreasing the probability of heterospecific pollen transfer. This may be especially advantageous as *T. hybridum* has low pollination visitation density (Fig 3. A), resulting in lower amount of pollen transferred per flowering stalk (i.e., pollinator performance, Fig. 4) and a greater need to maximize successful pollen transfer per visit (Harder and Thomson, 1989). Unfortunately, we aggregated the pollen from the whole pollinator body into one sample, without further possibility of comparing the pollen composition from individual parts of pollinator body. Furthermore, *Trifolium hybridum* may compete for pollinators with other *Trifolium* or Fabaceae species with similar pollen release mechanisms and placement.

Centaurea jacea

Surprisingly, neither pollen load quantity nor quality followed the pattern of pollen release in *C. jacea*. While pollen quality appears to be relatively stable throughout the day, pollen quantity increased with time. Furthermore, both pollen load quality and quantity differ significantly between the *A. mellifera* and *Bombus* spp., with *Bombus* spp. possessing lower quantity and quality of the pollen. We explain the described pattern by plant conspecific density and pollinator behavior: First, flowering stalks of *C. jacea* are one among the most abundant from the set of plant species visited by both *A. mellifera* and *Bombus* spp., leading to preferential visits to *C. jacea* (Fig. 1 B, Fig. S4), resulting in high quality of the transferred pollen, especially by *A. mellifera* (Fig. 2 C, D). Second, the difference of the pollen quality of carried pollen between *A. mellifera* and *Bombus* species may be explained by differences in foraging behavior. Experiments frequently report high flower constancy (Waser, 1986b) of both *A. mellifera* (Free, 1963; Us Van Der Niet et al., 2020) and *Bombus* species (Huang et al., 2015), however *Bombus* species are known for specializing on one major plant species during foraging but occasionally visiting flowers of alternative (minor) plant species (Brian, 1952; Heinrich, 1979; Somme et al., 2015). The foraging pattern including occasional flower visits of other plant species, leads to lower flower constancy and more mixed pollen load (Somme et al., 2015). On the other hand, this behavior does not necessarily have to be translated to heterospecific pollen deposition to the stigma (Stanley and Stout, 2014), as deposition depends on several factors (Minnaar et al., 2019), e.g., pollen placement on the pollinator body, pollen layering and pollen carry-over. Furthermore, pollen layering and consequent accumulation on pollinator body may explain the gradual increase in the amount of conspecific pollen over time. Foraging individual may accumulate pollen during consequential visits of the same species during the day, as not all the pollen grains from the pollen load are deposited on the plant stigma during single visit (Delmas et al., 2016). In particular, pollen grains placed on the ungroomed parts of the pollinator body (Koch et al., 2017) are most likely to accumulate with an increasing number of visits by an individual pollinator and their subsequent fate is unclear.

Succisa pratensis

In *S. pratensis*, the pollen loads of pollinators closely track the pollen presentation schedule (compare Fig. 1 A and Fig. 2 E), resulting in a rapid decrease in both pollen quantity and quality during the day. Similarly, pollinator density decreases rapidly after the decrease of available pollen (Fig. 3 E, F). These results suggest short “temporal specialization” of *S. pratensis* pollinators, possibly caused by the short term dominance in the amount of available rewards in the early morning hours before anthesis of other co-flowering species visited by the same pollinators. The temporal dominance may cause temporal flower constancy of generalist pollinators (*Helophilus* and *Eristalis* hoverflies), resulting in higher conspecific pollen transfer. Temporal dominance may be analogous situation to local dominance presented by Levin and Anderson (1970). According to their model, increase of the plant species local dominance leads to decrease of the interspecific pollinator visits even of less constant pollinator species. We argue that achieving temporal dominance is similar in the effect to achieving local dominance and it allows to *S. pratensis* to increase the specificity of pollinator visits. This can be illustrated by

the estimate of pollinator performance (Fig. 4), which shows clear peak in the morning hours, suggesting that the majority of the pollen is transferred in the early morning hours.

Surprisingly, the patterns in both pollen quantity and quality are very similar for all investigated pollinators, indicating that pollen presentation has a strong influence on the pollen transfer in the case of *S. pratensis*. However, the similarity in the amount of pollen transferred may also be caused by the similar pollen load capacity of *S. pratensis* pollinators due their similar size and hairiness.

Synthesis

In a recent article, Brosi (2016) hypothesized that pollinator temporal specialization may help to mitigate heterospecific pollen transfer between co-flowering species and allow plant co-existence (Bruninga-Socolar et al., 2023). In a previous article (Štenc et al., 2023), we presented data showing how pollinator visitation closely tracks diurnal patterns in pollen release in the same study system. We hypothesized that further investigation may reveal that the pollen release schedule plays crucial role in the mitigation of heterospecific pollen transfer. Indeed, based on our current results, we conclude that several mechanisms may simultaneously contribute to the mitigation of heterospecific pollen transfer and act together: While in the case of *S. pratensis* we detected a direct link between the pattern of pollen release and pollen transfer (similar to Graças Vidal et al. (2010) and Parker et al. (2016)), in the case of *C. jacea* and *T. hybridum* we speculate that mainly flower constancy and specific pollen placement help to facilitate conspecific pollen transfer, respectively.

Our results thus reveal that multiple mechanisms (e.g., pollen release, flower constancy and specific pollen placement) allow long-term coexistence of co-flowering species sharing their pollinators. While in play, such mechanisms may explain why the pattern of heterospecific pollen deposition within the plant community does not match the pattern of pollinator heterospecific visits (Fang and Huang, 2016) and pattern of pollinator sharing (e.g., Zhang et al. (2021); Fang et al. (2022)). Similarly, (Peucker et al., 2020) and Cullen et al. (2021) found that flower traits and plant community structure do not well explain the composition of pollen load on the pollinator body. We argue, that these discrepancies may be a crucial gap in our understanding of the plant-pollinator network which is likely to be more than just the sum of plant-pollinator interactions at the species level. For example, in a recent study, Wang et al. (2021) proved that pollinator specialization within networks may vary on a diurnal scale and in some species cause higher temporal specialization which is expected to increase the specificity of pollen transfer (Brosi, 2016). Concurrently, Schwarz et al. (2021) experimentally demonstrated that by affecting the timing of flower opening, the structure of the plant-pollinator interactions may be altered in response. However, the evidence further linking the diurnal structure of the plant-pollinator network, pollinator behavior and the efficiency of pollen transfer is sparse. Based on our results, we suggest further focus on the investigation of fine-scale plant-pollinator interactions (e.g., diurnal scale) combined with observations of pollinator behavior and pollen transfer efficiency to disentangle the mechanisms structuring the interactions within the plant-pollinator

community.

8.2.2.8 Conclusions Our data show how different strategies of pollen release shape pollen transfer and plant-pollinator interactions: The pollen release mechanism of *Trifolium hybridum* leads to specific placement of pollen on the pollinator body and possibly more precise pollen transfer even with lower density of visits. On the other hand, high spatial density of *Centaurea jacea* stimulates flower constancy and specificity of pollen transfer and *Succisa pratensis* has a strict pollen release schedule, followed by a close pollinator visitation pattern, resulting in high specificity of pollen transfer for a short period of time. Combined, these mechanisms mitigate the effect of pollen sharing and limit the effect of competition for pollinators in the generalized plant-pollinator community.

8.2.2.9 Author contributions The research idea: Jakub Štenc, Zdeněk Janovský, Lukáš Janošík; Data collection: Jakub Štenc, Lukáš Janošík, Martin Freudenfeld, Eva Matoušková, Jiří Hadrava, Michael Mikát, Klára Daňková, Tereza Hadravová, Tadeáš Ryšan, Jasna Simonová, Klára Koupilová, Alice Havelková, Eliška Konečná, Jan Martínek, Barbora Jelínková, Stanislav Vosolsobě, Karolína Dobešová, Marie Smyčková, Jan Smyčka, Jitka Smyčková, Lucie Studená, Josef Nováček, Štěpán Káně, Kateřina Knotková, Tomáš Figura, Jan Simon-Pražák, Kateřina Čermáková, Jiří Janoušek, Václav Bočan, Vojtěch Brož, Anna Suchá, Eva Horčíčková, Tomáš Dvořák, Tomáš Jor, Doubravka Požárová, Karel Plavec, Zdeněk Janovský; Samples processing: Jakub Štenc, Lukáš Janošík, Martin Freudenfeld; Analysis: Zdeněk Janovský, Jakub Štenc; Funding: Jakub Štenc, Lukáš Janošík, Zdeněk Janovský; Jakub Štenc led writing of the manuscript, other coauthors commented on the text. All authors agreed with the final version of the manuscript.

8.2.2.10 Acknowledgment The authors are grateful to all members of Flower Ecology Group in Prague and Plant Experimental Ecology for discussions of the topic and valuable suggestions regarding earlier versions of the manuscript, particularly to Barbora Lepková and Tomáš Herben. Furthermore, the authors would like thank to all the people involved in the “Opylovači” project who selflessly participated in more than a decade of fieldwork. Jakub Štenc and Lukáš Janošík acknowledge support by the project “Grant Schemes at CU” (reg. no. CZ.02.2.69/0.0/0.0/19_073/0016935). Jiří Hadrava and Michael Mikát has been supported by Charles University Research Centre Programme No. 204069.

8.2.2.11 Conflict of interest statement The authors declare no conflicts of interest.

8.2.2.12 Supporting Information All supporting information can be found online in Appendix which contains:

Appendix 1:

- Fig. S1: Measured microclimatic variables on the 1 close to *Succisa pratensis* microsite. Details about measurement tools in Methods. Each line represents one day. In plots for A) Relative air humidity; B) Temperature and C) Photosynthetic active radiation show measurements for every 30 seconds as points and average for every 10 minutes as lines. D) Average wind speed shows average values for continuous measurement of wind speed during cca 30-minute-long period.
- Fig. S2: Measured microclimatic variables on the position 2 close to *Centaurea jacea* and *Trifolium hybridum* microsites. Details about measurement tools in Methods. Day 1 and 2 (14th and 15th August 2021, respectively) are days of pollen presentation sampling and Day 3 (16th August 2021) is the day of consequent flowering schedule sampling. In plots for A) Relative air humidity; B) Temperature and C) Solar irradiance show measurements for every 30 seconds as points and average for every 10 minutes as lines. D) Average wind speed shows average values for continuous measurement of wind speed during cca 30-minute-long periods. Unfortunately, due to machine malfunction, data for solar irradiance and wind speed are limited to 13th and 16th August.
- Fig. S3: Pollinator density in time of the day. Every point represents averaged pollinator density for 10-minute time frame. Graphs A), B) and C) stands for *T. hybridum*, *C. jacea* and *S. pratensis*, respectively. Other groups of pollinators visiting plant species are not shown as they represent wide group sparsely interacting with the plant species.
- Table S1: The overview of sampled individual pollinators per plant species.
- Fig. S4: Relative flowering stalk density in observed plots in 2021. Density is an average value obtained from two censuses during the observation period. Maximal value 1 refers to the all flowering stalks counted within the plot.

8.3 Chapter III: Pollinator foraging behaviour

8.3.1 Innate preferences of *Eristalis tenax* L. (Syrphidae) for flower colour, size and symmetry

Running title: Innate preferences of *Eristalis tenax*

Eva Matoušková¹, Jakub Štenc², Zdeněk Janovský³

¹Department of Zoology, Faculty of Science, Charles University, Viničná 7, CZ-128 41 Prague, Czech Republic

²Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 41 Prague, Czech Republic

³Svatý Jan t. Krsovice 1, 285 04 Uhlířské Janovice, Czech Republic

*Corresponding author, e-mail address: jakubstenc@gmail.com.

8.3.1.1 Abstract Plants developed complex flowers that differ in visual traits such as colour, size and shape to attract pollinators. However, pollinators seem to respond differently to individual traits. Our objective was to unravel the effect of individual traits and their interplay in forming pollinator preferences. We measured the preferences of naive drone flies *Eristalis tenax* (Syrphidae) for artificial flowers differing in colour, size and flower symmetry under controlled conditions to untangle the effects of individual traits on pollinator behaviour. *E. tenax* showed the highest selectivity toward colour, followed by size, and only moderate selectivity in relation to flower symmetry. Flower colour interacted with flower size: Yellow colour was generally preferred, particularly when the flowers were large in comparison to other forms. Our results suggest that preferences for flower traits are structured, which may suggest that there have been different evolutionary pressures on individual floral traits.

8.3.1.2 Key words Artificial flowers; *Eristalis tenax*; Innate preferences; Pollination; Syrphidae

8.3.1.3 Introduction Pollination of the majority of flowering plants depends on animal pollinators (Ollerton et al., 2011), often resulting in limits to their sexual reproduction if plants fail to attract sufficient numbers of pollinators (Larsson and H., 2000; Ashman et al., 2004; Panique and Caruso, 2020). To attract pollinators, plants developed visually complex flowers varying in traits such as colour, size and shape, forming a “multi-sensory billboard” for pollinators (Raguso, 2004). Different pollinator functional groups are thought to be attracted by different combinations of floral traits, which in turn causes selection pressures on floral traits and forms pollination syndromes, i.e., sets of of flower traits associated with particular pollinator groups (Fenster et al., 2004; but see Ollerton et al., 2009). There are numerous cases showing evidence for pollinator selection of individual floral traits (e.g., Pérez-Barrales et al. (2007); Lavi and Sapir (2015); Gervasi and Schiestl (2017)), but much less is known about how pollinators perceive combinations of floral traits and how these combinations are translated into observed pollinator floral preferences (Leonard et al., 2011).

The search image hypothesis assumes that the main purpose of a distinct combination of floral traits is to help pollinators easily recognize flowers from their background and to help pollinators associate flowers with reward (Chittka et al., 1999; Goulson, 2000). However, insect pollinators also have strong sensory and memory capacity limitations (e.g. Waser, 1986a; Goulson and Cory, 1993; Chittka et al., 1999; Spaethe et al., 2001; Riedel and Lunau, 2015; An et al., 2018), and they discern stimuli and incorporate them into their search image only if the stimulus exceeds the threshold of the pollinator’s recognition ability (Schiestl and Johnson, 2013; Riedel and Lunau, 2015). These limitations could also partly explain why the effects of floral traits on pollinator preference largely depend on the framing of the choice, i.e., on the diversity and complexity of other flowers a pollinator can choose from (Austin et al., 2019). Based on these limitations of pollinator perception, an alternative explanation can be drawn: pollinators in fact optimize their foraging behaviour by following simple, easily detectable floral traits (Gegear

and Lavery, 2005), and flowers possess complex sets of traits as a bet hedging strategy, i.e., to enable them to attract different groups of pollinators (Kaczorowski et al., 2012).

Flower colour and size are often considered to be easily detected flower traits, and they greatly affect pollinator choices. It is hypothesized that this effect is due their role in pollinator recognition of flowers from the background (Chittka, 1997; Kelber, 1997) and from other co-flowering plant species (Chittka and Raine, 2006; Reverté et al., 2016). Additionally, flower colour and size can be easily associated with the amount of reward within the flower (Giurfa et al., 1995; Blarer et al., 2002). The majority of diurnal pollinators show preferences towards a specific flower colour (Lunau and Maier, 1995) and they tend to prefer larger flowers (Lunau, 1991; Conner and Rush, 1996; Blarer et al., 2002; Lavi and Sapir, 2015; but see Kelber, 1997). However, the effects of flower colour and size are likely to interact since flower size affects the ability of pollinators to detect flower colour (Ishii and Masuda, 2014).

The effect of floral symmetry on pollinator preferences has been relatively understudied compared to flower colour and size despite its possible importance: Flowers with bilateral symmetry have evolved multiple times from flowers with radial symmetry (Jabbour et al., 2009). Some authors have indicated the importance of flower symmetry as a cue for attracting pollinators (Giurfa et al., 1999; Rodríguez et al., 2004). Moreover, it has been hypothesized that bilateral symmetry should increase the precision of pollen placement on the reproductive structures within the flower (Ushimaru et al., 2009; Culbert and Forrest, 2016). However, radial flowers may be visited by a wider spectrum of pollinator groups (Fenster et al., 2004), providing them with a higher visitation frequency. Bilateral flowers have also been connected with a higher degree of specialization toward plant-pollinator interactions (Fenster et al., 2004) as well as plant species vulnerability to extinction (Stefanaki et al., 2015). However, the existing studies show bee preferences toward both radial (Free, 1970; Wignall et al., 2006; Culbert and Forrest, 2016) and bilateral (West and Lavery, 1998; Rodríguez et al., 2004) floral symmetry, while the preferences of other pollinators are largely unknown.

Flowers typically combine multiple visual traits together, and it remains unclear how exactly the combination of floral traits affects pollinator preferences and decisions. The available evidence shows that bees increase their selectivity when offered flowers that differ in more than one visual trait (Gegeer and Lavery, 1998; Goulson and Wright, 1998). However, studies combining multiple traits in a manipulative way typically do not combine floral traits factorially (e.g., Gegeer and Lavery, 2005); thus, it is impossible to disentangle the main effects of individual floral traits from their interactions.

In our study, we tested the combined effects of three floral traits on the visitation choices (preferences) of naive *Eristalis tenax* hoverflies, which are known for their preference for yellow flowers (An et al., 2018) but have unknown preferences for the majority of other floral traits. We aimed to investigate the preferences of *E. tenax* for individual flower traits to disentangle the main effect and interactions of the traits. Specifically, we asked the following questions: 1) Do flower traits (colour, size and shape) differ in their relative importance for *E. tenax* visitation

behaviour? 2) Are there any important interactions between the effects of individual floral traits? To achieve these goals, we employed artificial flowers that differed in colour (yellow \times purple), size (small \times large) and symmetry (radially \times bilaterally symmetrical) combined in a full-factorial design. Laboratory-reared *E. tenax* individuals were then observed in arrays always containing two floral forms.

8.3.1.4 Materials and Methods

8.3.1.4.1 Experimental setup We tested the preferences of naive *Eristalis tenax* L. (Syrphidae) individuals toward flower colour, size and symmetry in terms of three different floral traits, namely, colour (yellow \times purple), size (large \times small) and shape (radial \times bilateral). For this purpose, we set up arenas containing a 4 \times 4 array of artificial flowers. Every arena contained two forms combining the tested traits and always differed in at least one trait. This arena was set up for all possible combinations of flower forms. The proportion of *E. tenax* visits to individual flower forms during the experimental trials was employed as a measure of *E. tenax* preference for individual trait combinations.

8.3.1.4.2 Artificial flowers We designed 3D representations of radial and bilateral artificial flowers in two sizes using the online tool OnShape (<https://www.onshape.com>). Both bilateral and radial flowers had five petals and did not differ in their surface area within their size category. The large forms had a 50 mm diameter and a surface area of 10 cm², while the small forms had a diameter of 30 mm and a surface area of approximately 4.5 cm². Bilateral flower forms had their petals aggregated into two groups (3+2) at opposite poles of the main axis of symmetry (see Supplemental Material of this article in Appendix S1, Figs. S1 and S2 for a more detailed description of the artificial flowers). The artificial flowers were printed from white PLA filament (FLM-PLA-175-WHT, Prusa Research, a.s., Prague, Czech Republic) on a Prusa i3 MK3S+ 3D-printer (Prusa Research, a.s., Prague, Czech Republic) and were then painted yellow and purple using acrylic colours (the reflectance spectra are presented in Fig. 1). Each flower was also combined with a vial to provide it with a steady supply of 30% sucrose solution as the reward (following the methodology of Ishii and Masuda (2014); see Appendix S1, Fig. S2). The 3D models can be found at Sketchfab: <https://sketchfab.com/jakubstenc>. All flowers were presented horizontally (facing up) in the same plane without any difference in height nor angle in which they were presented to hoverflies.

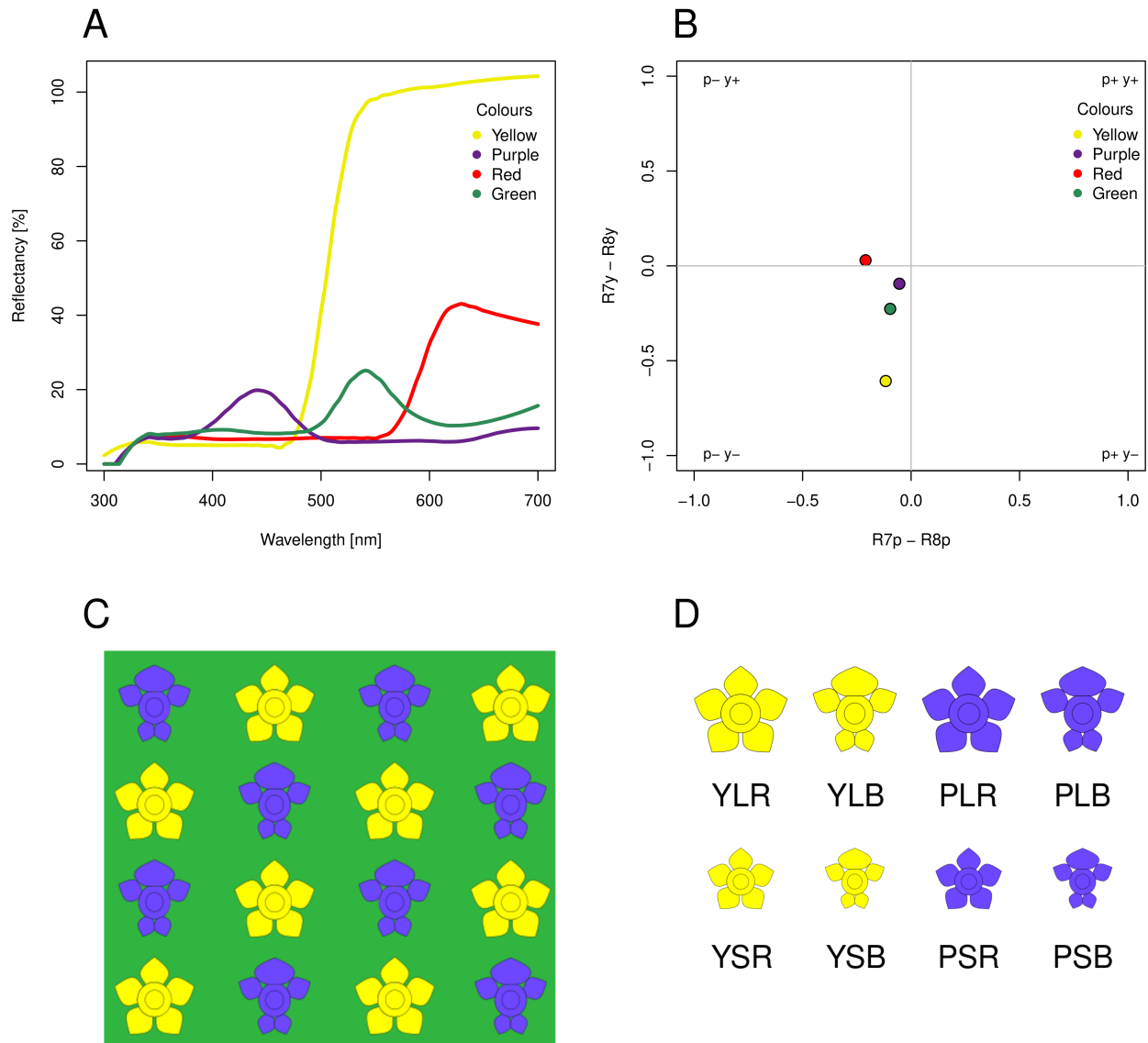


Fig. 1: Reflection spectra (A) and projection onto the dipteran visual model (B) based on Troje (1993) about artificial flowers presented to *E. tenax* (yellow and purple), red training flowers (red) and the material of the green background (green). Flowers were placed into arrays in fixed spatial positions and with a green background (C). The flower forms differed in colour (yellow and purple), size (large and small) and symmetry (radial and bilateral), resulting in 8 flower forms in total (D).

8.3.1.4.3 Rearing of naive *Eristalis tenax* Gravid *Eristalis tenax* females were acquired at their overwintering sites in the Alkazar Quarry, Central Bohemia, Czech Republic (N 49°57.03762', E 14°7.43738'). Following acclimation, the *E. tenax* females laid eggs, which were then transferred to buckets containing rabbit dung diluted with water at a ratio of 3:1, in which the larvae were reared according to a standard protocol (Nicholas et al., 2018). The reared adults were kept in rearing cages under an SunLux UV 150 W PAR38 UV-Vis lamp (SunLux, s.r.o., Brno, Czech Republic) and had unlimited access to water, sugar and pollen.

Five days prior to the tests, the adults were provided with red artificial flowers to learn to use them as a food source without being preconditioned to any of the flower colours used in the experimental trials. The red flowers supplied with nectar stayed in the rearing cages throughout the whole experiment. We used adults reared from the two largest clutches (135 and 140 individuals) for the experimental trials to control for any possible effects of clutch identity.

8.3.1.4.4 Experimental trials The experimental trials were performed out in two 80×80×80 cm arenas made from a thin transparent net strung within a wooden frame. The bottom of the cage was covered with a green non-woven cloth simulating a green background. On the green cloth, a 4×4 array of artificial flowers was placed on 20 cm-high stands. The two flower forms tested against each other were organized in a chessboard pattern to control for any spatial effects (Fig. 1).

During each experimental trial, 10 *E. tenax* males and 10 females originating from the same clutch were allowed to enter the arena and were left for five minutes to acclimate to the environment. After getting accustomed to the arena, the individuals who visited artificial flowers were censused every 3 minutes for the next 30 minutes, rendering 11 censuses in total per trial. We deemed 3 minutes sufficient to avoid the pseudo-replication of censuses since most *E. tenax* visits lasted much less than a minute, and thus the hoverflies had sufficient time to reshuffle. Moreover, only a fraction of the individuals visited the flowers at a given point in time (typically less than half).

Every tested flower combination entered into at least four trials (two from each of the two clutches) and if fewer than 150 visits were recorded, additional trials were added (see S1 Table S1 for total sampling effort). The allocation of individuals from the two clutches between the arenas was switched between the rounds of experimental trials. Typically, eight rounds of trials (maximum 10) in the two arenas were run on the same experimental day. We strove to avoid using the same *E. tenax* individuals for more than one experimental trial per day and individuals from previous trials were kept separately to avoid mixing with non-used individuals. Nevertheless, we typically had to use some of those from the first trial for the second time during the last trials of the day (typically to balance the sex ratio).

A total of 179 experimental trials were conducted between 12 and 24 January 2021 in an experimental room under a constant temperature (24 °C). The windows of the experimental room had drawn drapes to diffuse any direct sunlight, so the main light sources were the two UV-vis lamps of the same type as those used for the rearing cages.

8.3.1.4.5 Data analysis The analysis employed the data from individual experimental trials as separate data points. Since we lacked eight sets of experimental trials with the two identical flower morphs offered, we used simulated data. Since these data should follow a fully random pattern, we simulated four experimental trials with a binomial probability of success equal to 0.5 for each of these combinations (facing the two identical flower forms, a hoverfly cannot choose otherwise than randomly). The number of visits per simulated trial was set to

the mean number of all recorded experimental trials, i.e., to 35. Furthermore, the binomial nature of the response variable required to designate which of the two flower forms present in the experimental trial is a ‘success’ (i.e., focal form) and which is a ‘failure’ (i.e., antagonist form). We randomly assigned half of the experimental trials from a particular flower morph combination to each of the two participating flower forms.

The main analysis was conducted by means of a binomial generalized linear model (hereafter GLM) with the number of visits to the focal morph out of the total number of visits recorded within the experimental trial being as the response variable. The predictors were the colour, size, and shape of the focal flower morph, the presence of differences in the antagonist flower morph in terms of colour, size and shape and all second- and third-order interactions of these six predictors. For a preliminary analysis, we also added the clutch identity to these predictors, but since neither this predictor nor its interaction terms were significant (data not shown), we did not include the clutch identity in the final analysis.

After fitting the model, we detected considerable overdispersion in our binomial data (i.e., residual deviance greatly exceeding the residual number of degrees of freedom); therefore, we refitted the binomial GLM as quasibinomial with subsequent changes in the tests of significance for individual model terms (see Crawley (2012) for more details). All the computations were conducted in the R statistical environment (available at www.r-project.org) version 4.2.0 under base installation (R Core Team, 2022b).

8.3.1.5 Results In total, we recorded 2250 visits of *E. tenax* individuals to flowers during 179 experimental trials. The strength of the detected *E. tenax* preferences ranged from no detected preferences (primarily flower form combinations involving only purple flower forms) to very strong preferences (up to 93%) for combinations involving large yellow flowers as the focal flower forms (Table 1).

The binomial GLM indicated a very strong effect of the colour of the focal flower forms, with a strong *E. tenax* preference for yellow over purple flowers (Table 2). An order of magnitude weaker effect was observed for flower size, in which large flowers were preferred over small flowers (Fig. 2). The effect of flower size was greatly reduced if the flower forms in the array differed in both size and colour (Fig. 2), showing an interaction between size and colour. The effect of flower shape on *E. tenax* preference was only marginally significant (Table 1).

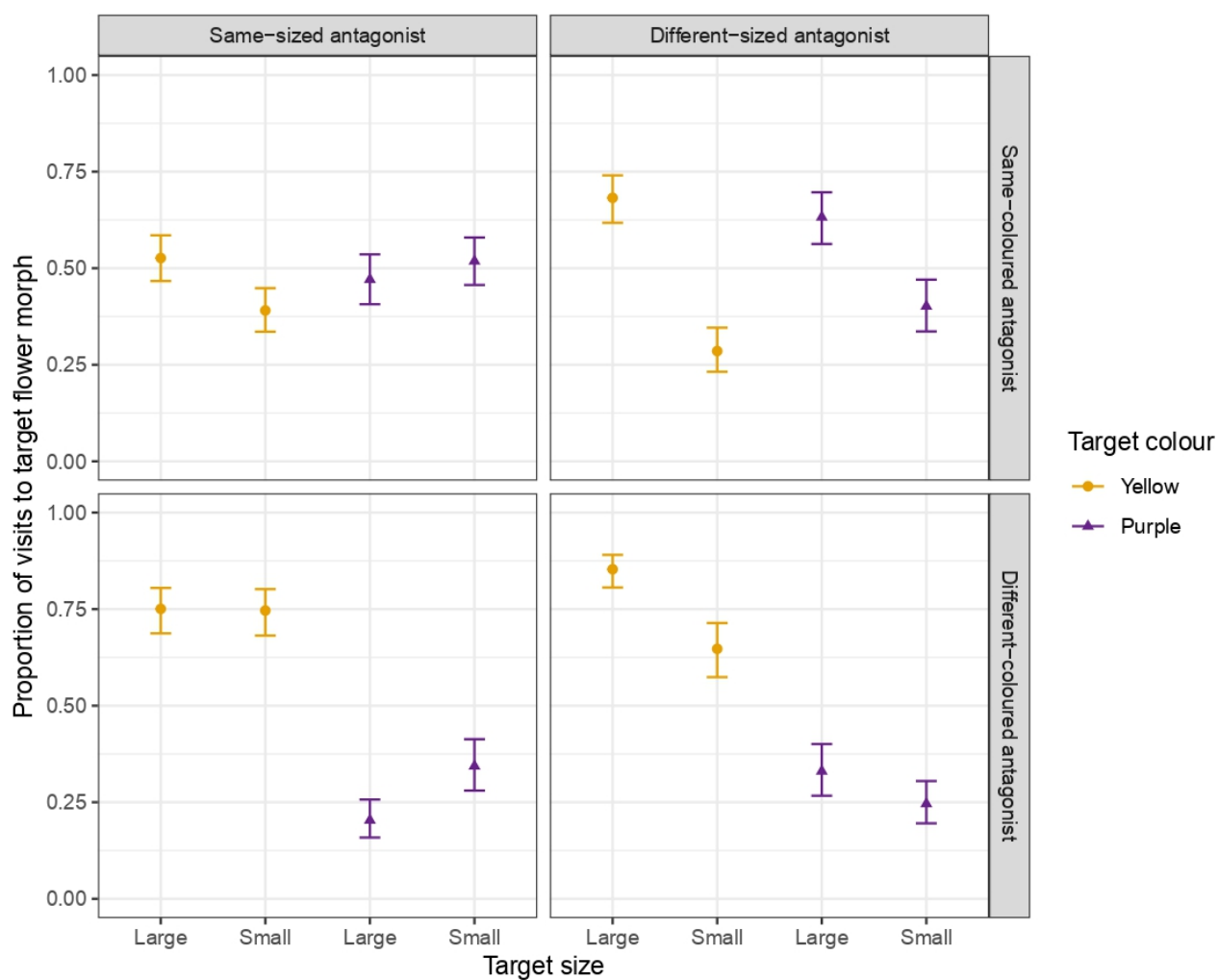


Fig. 2: Proportion of *E. tenax* visits to focal flower morph (preference) in comparison to antagonist forms during the experimental trial. The data are divided into quadrants with respect to differences in colour and size of the antagonist forms. Data from floral morph combinations differing in shape are grouped together.

Table 1: The results for all the predictors used for Generalised linear model with quasi-binomial distribution, the significant predictors are bold. Predictors col, size, sym stand for colour, size, symmetry and diff. col, diff. size and diff. sym stand for difference in colour, size and symmetry respectively. Link function: logit, NULL deviance = 2062.33, Df = 226, Residual deviance = 783.34, Dispersion parameter = 3.772546.

Predictor	Deviance	Resid.Df	Resid.DevP-value
col	285.64	225	1,776.69<0.001
size	98.60	224	1,678.09<0.001
sym	13.84	223	1,664.260.06
diff. col	5.62	222	1,658.640.22
diff. size	6.34	221	1,652.290.2
diff. sym	12.54	220	1,639.750.07
col:size	68.19	219	1,571.56<0.001
col:sym	0.16	218	1,571.400.84
size:sym	1.62	217	1,569.780.51
col:diff. col	539.27	216	1,030.51<0.001
col:diff. size	10.69	215	1,019.820.09
col:diff. sym	3.20	214	1,016.630.36
size:diff. col	15.39	213	1,001.24<0.05
size:diff. size	136.33	212	864.91<0.001
size:diff. sym	10.65	211	854.260.1
sym:diff. col	6.87	210	847.390.18
sym:diff. size	0.95	209	846.440.62
sym:diff. sym	1.14	208	845.300.58
col:size:sym	4.89	207	840.410.26
col:size:diff. col	0.92	206	839.490.62
col:size:diff. size	15.19	205	824.30<0.05
col:size:diff. sym	0.20	204	824.100.82

Table 1: The results for all the predictors used for Generalised linear model with quasi-binomial distribution, the significant predictors are bold. Predictors col, size, sym stand for colour, size, symmetry and diff. col, diff. size and diff. sym stand for difference in colour, size and symmetry respectively. Link function: logit, NULL deviance = 2062.33, Df = 226, Residual deviance = 783.34, Dispersion parameter = 3.772546.

Predictor	Deviance	Resid.Df	Resid.DevP-value
col:sym:diff. col	12.03	203	812.070.08
col:sym:diff. size	0.00	202	812.070.97
col:sym:diff. sym	0.05	201	812.020.91
size:sym:diff. col	1.10	200	810.910.59
size:sym:diff. size	1.02	199	809.890.6
size:sym:diff. sym	8.73	198	801.160.13
col:size:sym:diff. col	7.61	197	793.550.16
col:size:sym:diff. size	0.49	196	793.060.72
col:size:sym:diff. sym	9.72	195	783.340.11

Table 2: Proportions of visits to focal flower forms aggregated over experimental trials with the same combination of focal and antagonist flower forms. Numbers in bold indicate a combination in which *E. tenax* significantly ($\alpha = 0.05$) deviated from 50% (binomial test; H_0 : *E. tenax* does not show preference for either of the two flower forms presented, i.e., the probability of success equals 0.5).

	YLR	YLB	YSR	YSB	PLR	PLB	PSR	PSB
YLR		31%	17%	22%	8%	20%	29%	24%
YLB	56%		6%	25%	24%	1%	24%	22%
YSR	91%	71%		41%	37%	37%	38%	32%
YSB	80%	70%	39%		39%	38%	38%	31%
PLR	79%	86%	74%	68%		51%	52%	39%
PLB	86%	88%	58%	63%	59%		53%	48%
PSR	70%	86%	72%	76%	57%	58%		36%
PSB	73%	77%	62%	80%	58%	60%	44%	

8.3.1.6 Discussion *E. tenax* showed the expected strong innate preference for yellow colour as was expected from previous studies (Lunau and Wacht, 1994; An et al., 2018), followed by a relatively weaker preference for larger flower size. Additionally, if the focal and antagonist flower forms differed both in colour and size, the effect of the difference in colour largely overrode the effect of the difference in flower size (Fig. 2). Lastly, flower symmetry did not have any biologically relevant effect on *E. tenax* preference (Table 2).

The detected interaction of the effects of flower colour and flower size seems consistent with the concept that flower colour is the main attractant causing pollinator preference, while a larger flower size primarily increases the perception intensity of the preferred colour (Ishii and Masuda, 2014). Since we did not observe any effect of flower size when the focal flower was of the non-preferred purple colour (comparison of yellow and small flowers versus purple and large), we hypothesize that flower size increased flower detectability for *E. tenax* rather than actively stimulated preference for size itself.

Flower symmetry does not have a significant effect on *E. tenax* preferences when flowers differed only in symmetry or more traits. This observation may suggest that naive *E. tenax* does not possess any preference toward flower symmetry and that all observed preferences by wild hoverflies are caused by learned preferences based on association with rewards (e.g., Gómez and Perfecttiy, 2010). However, to our knowledge, there is no direct evidence supporting the ability of hoverflies to recognize and form learned preferences based on flower symmetry. Furthermore, various experiments found mixed evidence for bee preference towards flower symmetry, resulting only in moderate preferences for both radial (Free, 1970; Wignall et al., 2006; Culbert and Forrest, 2016) and bilateral (West and Lavery, 1998; Rodríguez et al., 2004) flower symmetry. Consequently, several studies indicate that variations in flower symmetry do not affect plant reproductive output (Frey et al., 2005; Weeks and Frey, 2007) or pollinator visitation to flowers (Frey and Bukoski, 2014). It could be expected to be less important in pollinator visitation decisions, despite being considered an important visual cue by some researchers (reviewed in Giurfa et al., 1999). However, flower symmetry may play an important role in processes other than pollinator attraction: According to the precision hypothesis, it may affect pollen placement on stigmas and increase pollination effectiveness (Culbert and Forrest, 2016; Naghiloo et al., 2020). Flower symmetry is also often connected with the angle in which are flowers presented to pollinators: bilaterally symmetrical flowers are often oriented horizontally and therefore possibly increase the visual angle in which they can be seen by pollinators Ishii and Masuda (2014). This may affect pollinator landing frequency and pollen transfer Ushimaru et al. (2009). In our study, we oriented all flowers vertically, which may result in possibly weaker response of *E. tenax* to the flower symmetry, but currently we lack further evidence to prove this hypothesis. The combined effect of flower symmetry and orientation should be further studied in fully factorial design to disentangle the relative importance of both factors to pollinator behaviour.

E. tenax is probably one of the most studied dipteran model organisms with well-known preferences with respect to colour (Lunau and Maier, 1995; Wacht et al., 1996; Riedel and Lunau,

2015; An et al., 2018; Hannah et al., 2019), UV patterns (Dinkel and Lunau, 2001) and chemical stimuli (Wacht et al., 1996); however, its preferences for other traits are largely unknown. Similarly to our study, several studies confirmed the strong preference of *E. tenax* towards yellow colour (Lunau and Wacht, 1994; Riedel and Lunau, 2015; An et al., 2018; Hannah et al., 2019), possibly associated with the colour of yellow pollen (Lunau and Wacht, 1994). Nonetheless, to the best of our knowledge, we present the first study testing hoverfly preference regarding flower symmetry. In contrast, numerous studies have focused on bees, finding a mechanism of forming both innate and learned preferences, suggesting bees have the ability to develop preferences towards flowers that differ in symmetry based on the amount of reward (Plowright et al., 2017), but no evidence is known for hoverflies.

What do our results reveal about *E. tenax* preferences for flowers based on a combination of multiple visual traits? We argue that based on our results, preferences for floral traits of naive *E. tenax* are primarily driven by flower colour, are slightly increased by size and are not affected by flower symmetry. In conclusion, our experiment did show increased selectivity, when flowers differed in two specific traits (colour and size) but no increase in selectivity was found when a third trait was added. These results are similar to results from studies conducted on bees. For bees, preference based on colour was also the most important, and their selectivity also increased when the preferred colour was coupled with larger flower size (Gegear and Laverty, 2005). Other studies showed increasing selectivity of pollinators when flowers differed in more than one trait (e.g., Gross (1992); Gegear and Laverty (1998); Goulson and Wright (1998)) and with an increased number of possible choices (Austin et al., 2019). However, the majority of previous studies lack the ability to evaluate the relative importance of individual traits directly because they used (either) non-manipulative or non-factorial experimental designs. Hence, we would like to stress the importance of fully factorial experiments to untangle the structure of interactions among individual traits affecting pollinator behaviour.

8.3.1.7 Conclusions Our study shows the different degrees of importance of individual flower traits in forming the innate preferences of hoverflies. Flower colour played the most important role, followed by flower size, but flower symmetry did not show any effect on the preferences of *E. tenax*. Moreover, individual traits interacted together and amplified their effect on pollinator preferences. Our results suggest that individual traits may play different roles in forming pollinator preferences, which may partially explain the extensive diversity of flower complexity.

8.3.1.8 Acknowledgments We thank the members of the Flower Ecology Group in Prague for discussions on the topic and valuable suggestions to earlier versions of the manuscript. We also thank the Youth Tourist Association for allowing us to use their facility and American Journal Editors for language editing of the manuscript. We are also grateful to Alice Haveldová for the illustration of the artificial flower. This work was supported by the Charles University Grant Agency [Grant No. 842120].

8.3.1.9 Authors contribution EM, ZJ and JŠ conceived the research idea. EM and ZJ designed the method for testing *E. tenax* preferences. Em and JŠ designed and produced artificial flowers. EM collected the data on plant and pollinator abundances. EM and ZŠ analysed the data. EM, JŠ and ZJ wrote the manuscript.

8.3.1.10 Data availability statement The data underlying this article are available in figshare digital repository, at <https://doi.org/10.6084/m9.figshare.21674255.v1>

8.3.1.11 Supporting Information Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Supporting information:

- Figure S1: Technical drawing of different forms of artificial flowers
- Figure S2: Part of the artificial flowers
- Table S1: Sampling effort per experimental trials

8.3.2 Learning to be unfaithful - Flower constancy of *Eristalis tenax* L. (Syrphidae) decreases with experience

Running title: Flower constancy of *Eristalis tenax*

Alice Haveldová¹, Jakub Štenc¹, Eva Matoušková², Zdeněk Janovský³

¹Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 41 Prague, Czech Republic

²Department of Zoology, Faculty of Science, Charles University, Viničná 7, CZ-128 41 Prague, Czech Republic

³Svatý Jan t. Krsovice 1, CZ-285 04 Uhlířské Janovice, Czech Republic

*Corresponding author, e-mail address: jakubstenc@gmail.com.

8.3.2.1 Abstract Pollinators are known to exhibit preferences and flower constancy (i.e., the proportion of the visits to the same plant species) toward particular floral traits, which are driving their decisions while foraging between flowers. Furthermore, pollinators are adjusting their preferences and flower constancy based on their experience with the flower reward. However, while evidence of the pollinator learning on their foraging is known for several pollinator taxa, evidence for hoverflies is limited despite the fact that they represent one of the most abundant pollinator groups in temperate zones. We compared the preferences and flower constancy of naive and experienced individuals of *Eristalis tenax* in controlled, full-factorial experiments using artificial flowers differing in flower color and size. Our results show that previous experience with flowers in an environment rich on flowering species decreased flower constancy and changed preferences of *E. tenax* and we discuss the effect of changes in preferences and constancy on the *E. tenax* foraging among flowering plants.

8.3.2.2 Key words Artificial flowers; *Eristalis tenax*; Flower constancy; Innate preferences; Pollination; Syrphidae

8.3.2.3 Introduction Pollinators foraging between flowers of the same species are crucial for pollen transfer and consequently sexual reproduction of majority vascular plant species (Ollerton et al., 2011) with direct implication for plant evolution and diversification (Niet and Johnson, 2012; Niet et al., 2014). Yet, from pollinator perspective, it is beneficial to optimize for nutrient and energy intake obtained during foraging (Pyke, 1978a, 2019) which may lead to visit numerous plant species during foraging bout (Kunin and Iwasa, 1996) and consequently to heterospecific pollen transfer (Briggs et al., 2016). However, for pollinators may be beneficial to choose flowers based on their traits signaling the presence of reward (Wright and Schiestl, 2009; Krishna and Keasar, 2018). Plants may then benefit from developing such traits which are attracting pollinators, hence increasing the probability of visits (Jersáková et al., 2012; Kooi et al., 2019) and deposition of conspecific pollen (Montgomery, 2009; Us Van Der Niet et al., 2020). This may be even more beneficial in the species rich community with predominantly generalist plant-pollinator interactions (Waser et al., 1996) where pollinator temporal specialization is hypothesized be crucial for successful pollen delivery to stigma (Brosi, 2016; Bruninga-Socolar et al., 2023).

Pollinators foraging among flowers are deciding to which flowers to visit based on two main components: pollinator preference and flower constancy (Waser, 1986a). Preferences are supposedly beneficial to pollinator by decreasing the time necessary to find rewarding flower in complex environment (Austin et al., 2019) and may be one of the primary drivers in flower traits evolution (Fenster et al., 2004; Reverté et al., 2016; Trunschke et al., 2021). Consequently, several taxa of pollinators are known to exhibit flower constancy: behaviour when pollinator tends to visit flowers of the same species (or type) in consequential visits during they foraging bout even in presence of other plant species flowers (Wells and Wells, 1986; Waser, 1986a; Hill et al., 1997). While the benefit of flower constancy for pollen transfer is straightforward (Brosi, 2016), from pollinator perspective it may be sub-optimal as they may ignore flowers with greater reward which is in contrast with optimal foraging theory (Pyke, 1978a, 1984; Waser, 1986a; Wells and Wells, 1986; Latty and Trueblood, 2020). This provoked formulation of several hypothesis explaining why should be pollinators flower constant, usually based on the pollinator sensory (Goulson, 2000) and memory (Raine and Chittka, 2007) limitations, assuming that by being flower constant, pollinators may increase their energy intake by shortening the time necessary for obtaining reward (handling time, e.g., Chittka, 1997). Therefore, it has been hypothesized that flower constancy may be primarily beneficial to pollinator in complex environment (Austin et al., 2019), where is the reward availability variable and can be associated with the advertising signal (Raguso, 2004).

It has been demonstrated, that some pollinators are able to change their preferences and modify flower constancy based on previous experience. For example, individual bees increased their constancy after finding flowers with higher reward level (Chittka et al., 1999; Grüter et al., 2011; but see Hill et al., 1997). Pollinators are suppose to be born with set of innate preferences, navigating them toward flowers from which the reward is expected (Lunau and Maier, 1995; Gumbert, 2000), however, based on the experience with previously obtained reward, pollinators

change they preference to some extent (Kelber, 2003; Weiss and Papaj, 2003). The learned preferences then can persist for days or weeks (Goulson and Cory, 1993) and further affect pollinator foraging behavior (Gumbert, 2000). Experimental evidence also shows that the increasing complexity (i.e., number of possible choices) slows down bees decision and response to reward level changes (Austin et al., 2019). This may be important in flower rich environment with variable reward level, where the cost of deciding among many choices may be higher and especially bees may benefit from increased constancy toward to already proven reward sources. On the other hand, if the reward intake and handling time does not differ significantly among the flowers, it is beneficial to pollinator to lower flower constancy (Gegeer and Thomson, 2004). However, our knowledge about the effect of learning on flower constancy is limited to few pollinator taxa, with majority of the research focused primarily on bees (e.g., Chittka et al., 1999; Austin et al., 2019), to a lesser extend on butterflies (e.g., Goulson and Cory, 1993) and birds (e.g., Schmid et al., 2016).

Hoverflies are one of the most common pollinators in Central Europe (Janovský and Štenc, 2023), with often generalized foraging strategy (Klecka et al., 2018; Lucas et al., 2018; Doyle et al., 2020; Horiuchi et al., 2022) and exhibiting various level of flower constancy to different plant species (Goulson and Wright, 1998; Janovský et al., 2017). Particularly drone fly *Eristalis tenax* (L.) proved to be an ideal model for manipulative studies with hoverflies due its' abundance and relatively easy rearing process (Nicholas et al., 2018). Moreover, during last decades, experiments proved innate preference of *E. tenax* toward bright yellow (Lunau and Wacht, 1997; Dinkel and Lunau, 2001; An et al., 2018; Matoušková et al., 2023) and larger flowers (Matoušková et al., 2023), nonetheless comparing to bees, the number of studies involving *E. tenax* is limited, especially studies involving learning and flower constancy o *E. tenax* (e.g., Lunau and Wacht, 1997).

In this study, we tested the difference in foraging behaviour of naive and experienced individuals *Eristalis tenax* by using artificial flowers. We observed individual pollinator choices during foraging between flowers of two floral morphs at the time in fully factorial experiment and compared their preferences and flower constancy toward two traits: flower color and size, aiming to investigate the effect of experience on the foraging behaviour. We hypothesize that experience with flowers differing in visual traits and the amount of available reward may shape preferences and flower constancy of *E. tenax*, which shows known innate preferences toward both tested traits, but unknown flower constancy. Specifically, we asked the following questions: 1) How does colour and size shape preferences of naive and experienced individuals of *E. tenax*? 2) Does experienced individuals of *E. tenax* exhibits different preferences and flower constancy?

8.3.2.4 Materials and Methods

8.3.2.4.1 Experimental setup To compare the flower preferences and constancy of naive and experienced *Eristalis tenax* L. (Syrphidae) we used reared naive individuals and experienced hoverflies collected in their native environment to which we presented artificial flowers offering

steady supply of sugar solution and differing in flower color (yellow vs. white) and size (large vs. small). Both naive and experienced hoverflies were then offered the same combinations of treatments, resulting in fully factorial experimental design for each hoverfly group allowing to us compare the results.

8.3.2.4.2 Artificial flowers We designed 3D models of artificial flowers in the online tool OnShape (<https://www.onshape.com>). Flower models had five petals and central disc which carried vial with 3% sucrose providing steady reward, following protocol from Ishii and Masuda (2014). Flower models were designed in two size categories with the large variant with 50 mm diameter and a surface area of 10 cm² and small variant with the diameter of 30 mm and a surface area of approximately 4.5 cm² (see Supplemental Material of this article in Appendix S1, Figs. S1 and S2 for a more detailed description of the artificial flowers, the model can be found online <https://sketchfab.com/jakubstenc>). The models were then printed from PLA filament of three colors: white, yellow and purple (FLM-PLA-175-WHT, PLA- Pineapple Yellow and PLA Azure Blue, respectively, Prusa Research, a.s., Prague, Czech Republic) on a Prusa i3 MK3S+ 3D-printer (Prusa Research, a.s., Prague, Czech Republic, the reflectance spectra are presented in Fig. 1). To test the preferences and flower constancy of *E. tenax* was used 4 morphs combining the difference in colour and size: Yellow-Large (YL), Yellow-Small (YS), White-Large (WL) and White-Small (WS).

8.3.2.4.3 Experimental arena For both experiments with naive and experienced hoverflies, artificial flowers were placed into experimental arena in the cage from thin transparent net material (80 × 80 × 80 cm for naive and 140 × 140 × 140 cm for experienced hoverflies). Combination of two flower forms (i.e., treatment) were arranged in chessboard pattern of 4 × 4 individual flowers spaced 15 cm apart and thus not allowing hoverflies to crawl from one flower to another without active flying (Fig 1. A, B). All the flowers were mounted on wooden sticks in 25 cm height from the ground covered with green to simulate green background (see the position in dipteran visual space in Fig. 1 E). All flowers were presented in the same horizontal plane in the same height and facing up to allow landing and feeding.

Pollinator foraging activity was recorded by using recording camera (Lamax W9.1, elem6 s.r.o., Prague, Czech Republic). The camera was placed above the flowers with clear view on the flowers and surrounding arena, allowing to visually track foraging bout of an individual hoverfly. The video records were analysed visually, with recording the sequence of pollinator visit and time. Unfortunately, we were not able to clearly distinguish individual sexes, therefore we do not analysed the effect of sex on the pollinator behaviour.

8.3.2.4.4 Experiment with naive *Eristalis tenax* Similarly to Matoušková et al. (2023), we acquired overwintering gravid females of *Eristalis tenax* at the Alkazar Quarry, Central Bohemia, Czech Republic (49.9506N, 14.1240E, WGS 1984) during winter months of 2021. Females of *E. tenax* were acclimated to room temperature in small cage and consequently

laid eggs. Following the standard protocol from Nicholas et al. (2018), eggs were transferred to buckets with diluted rabbit dung with water (3:1). After development from eggs to larvae and to adults, individuals of *E. tenax* were kept in rearing cage in constant environment under an SunLux UV 150 W PAR38 UV-Vis lamp (SunLux, s.r.o., Brno, Czech Republic), with access to water, sugar and pollen. In the end, 115 individuals from 2 clutches were successfully reared. Adult individuals were kept for approximately 2 weeks in cages prior to the experiments in a room with stable temperature (cca 24 °C) and light conditions, the same conditions we then used for the experiments.

To train the hoverflies to feed from the artificial flowers, the adults were provided with blue flowers 2 days prior to the experiment. After pre-training phase, 15 randomly chosen individuals was released to the experimental arena with particular combination of floral forms (treatment). After the experimental run, the individuals were removed from the arena and placed to the rearing cage with supply of water, sugar and pollen. No individual was used in treatments twice per day, but all individuals were used in the following day, again, randomly selected for the treatment. During the day, 6 experimental runs were conducted in 3 time intervals in two experimental arenas simultaneously (8:30 - 10:00, 10:30 - 12:00 and 12:30 - 14:00). Each treatment was repeated 3 times during 4 experimental days (29.12.2021 – 1.1.2022) with one replication per each time interval. Exceptionally, treatment WL vs. WS was repeated in two additional runs due to low foraging activity in the previous runs.

8.3.2.4.5 Experiment with experienced *Eristalis tenax* During August of 2022, we collected foraging individuals of *E. tenax* from species rich plant-pollinator community in Central Bohemia near to Vernýřov village (49.8466N, 15.1498E WGS 1984). The locality consist predominantly from agriculturally managed (mowed twice per year) grassland community, rich on flowering plant species (see table SXY). Closer description of the locality can be found in Janovský et al. (2013). Adult individuals were carefully caught with insect net during foraging and then placed to small transport cage at the afternoon (16:00 - 17:00, prior the decrease of pollinator activity at the locality, see Štenc et al. (2023)) prior to the experiment. The next morning, collected individuals were released to the 2 experimental arenas placed on the locality and their behaviour was recorded on cameras since the morning hours (cca 9:00) until the end of pollinator activity (cca 17:00). In the arena, hoverflies had access to additional water supply and after the experiment they were released on different locality to avoid pseudo-replication. All the experimental runs were conducted under sunny weather and no or moderate wind for 6 days in total (11.8.2022 – 16.8.2022).

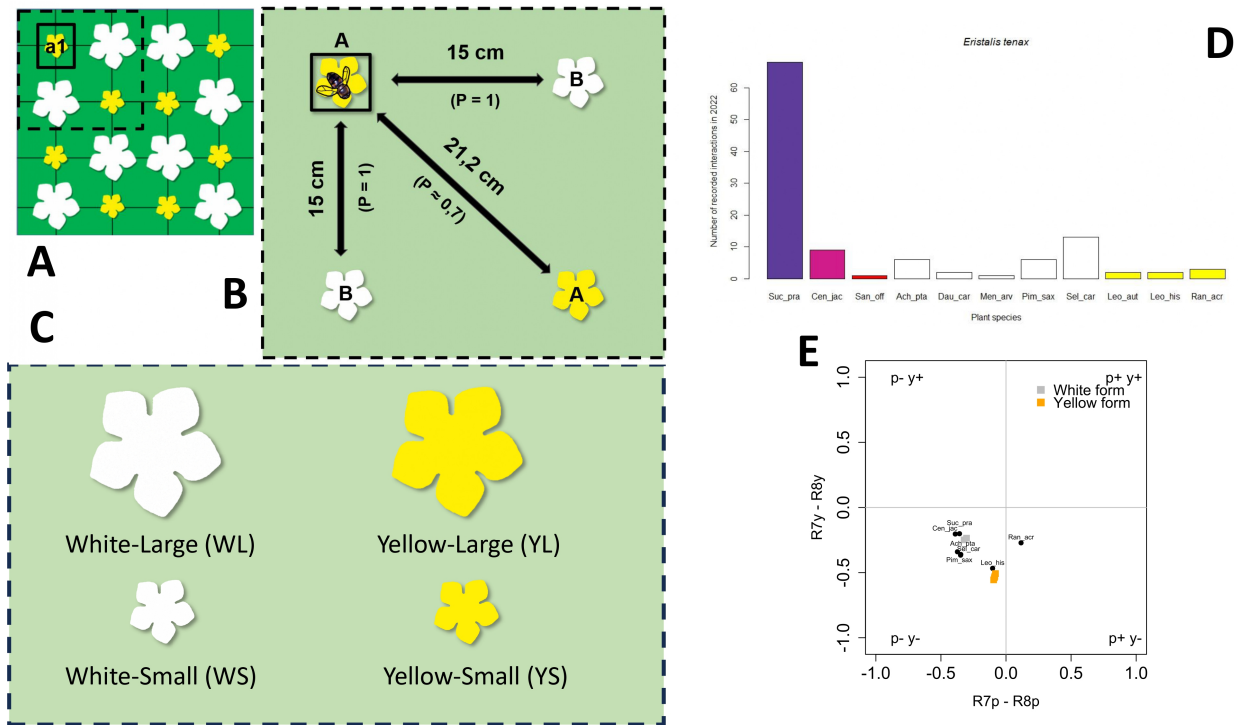
8.3.2.5 Data analysis The visual analyses of pollinator foraging behaviour from video records resulted into distinction of “visits”, “flights” and “sequences” during the foraging bouts of *E. tenax*. The visit was defined as a clear landing on the upper part of the artificial flower, the flight was then the connecting movement between two consequent visits and sequence consists from all the visits and connecting flights between visits. In all the visits, we recorded the

identity of the visited flower and its' position in the arena, resulting into distinctions of the flights between the same or different flower forms. The sequence than was characterized by the time of start, end length (both in time and in the number of visited flowers). The sequence ended if the hoverfly exited from the view for time longer than 5 second. Multiple sequences from one individual were most likely recorded, however, we were not able to control for identity of individuals and thus we treat sequences as independent

All analyses were conducted in R language and environment for statistical computing (R Core Team, 2022a). The visual model was rendered with pavo package (Maia et al., 2019).

8.3.2.5.1 Pollinator preference We measured pollinator preference as the proportion of visited flower forms from the flowering sequences. The preferences were tested by binomial test ($H_0 = E. tenax$ does not prefer any of the two tested flower forms within the treatment) with one tested flower form in the treatment coded as “success” and the other one as “failure”. Then we used quasi-binomial Generalized Linear Model (GLM) to test the effect of colour, size and the number of traits the flower forms differed in, i.e., difference in colour was coded as 1 and difference in colour and size was coded as 2. To achieve full factorial test design, we simulated pollinator decision in the treatments with the same flower forms in the experimental arena, i.e., situations when pollinators cannot distinguish between forms. This simulation was based on the mean sequence number and length with 50% probability of “success” which is equal to neutral expectation. The simulated data were generated by the RandVec function from Surrogate package (Van Der Elst et al., 2023). We performed the same analysis for both naive and experienced hoverflies separately, but by using the same methodology.

8.3.2.5.2 Flower constancy To test flower constancy of hoverflies toward individual flower forms we we employed a GLM with binomial distribution. Our response variable, flower constancy, was measured as the ratio of choices made by hoverflies during flight with respect to the previous visit, hence, visit to the next flower form was considered as constant if it was the same form as the previously visited one. As the predictors we included flower colour, size and difference in traits (same as in the case of preferences). Furthermore, to control for the effect of spatial distribution of the flowers in the arena, we computed angular diameter of flowers in the arena based on the position of individual hoverfly in the arena and assuming the flowers as a circles. For the computation we assumed the hoverfly to be able to see clearly all flowers within the arena from position placed 5 cm above the flower currently visiting. Then we used in the model as predictor the ratio of sum of angular diameters of currently visited flower form and sum of angular diameters of the other form present in the arena, i.e., the variable describes how much of the visual field of the hoverfly can be covered by the visited form versus the other form depending on hoverfly position in the arena. Consequently, we draw predictions based on the model for two possible situations: In the model we considered 3-way interactions of the predictors as the 4-way interaction was not significant.



{Fig.

1: A and B) illustration of the experimental arena and arrangement of the artificial flowers. C) Illustration of tested flower forms. D) Number of recorded interactions of *E. tenax* with flowering plants at the locality where were the experienced hoverflies collected. E) Position of white and yellow forms of artificial flowers and selected flowering species on the locality in the visual model for dipteran (based on *Musca domestica*).=“ “}

8.3.2.6 Results In total, we recorded 5 270 and 31 973 visits of *E. tenax* individuals to flowers exhibited during 429 and 4 129 foraging sequences for naive and experienced individuals respectively. Both visits and foraging sequences were not distributed evenly among treatments: In general, naive individuals of *E. tenax* visited more flowers per sequence (in average 12 and 8, respectively, Table S 1). Similarly, individual flower forms differed in the number of visits by hoverflies, with slightly more visits toward yellow forms by naive hoverflies (YL: 1 829, YS: 1 682, WL: 693, WS: 637), but quite balanced in the case of experienced hoverflies (YL: 8 000, YS: 7 855, WL: 8 857, WS: 7 261).

8.3.2.6.1 Preferences Both naive and experienced hoverflies showed significant preferences (Table 1), however differed in their magnitude and preferences to particular floral form (Fig. 2): Naive individuals strongly preferred yellow flowers (both large and small), especially, when compared yellow-large (YL) form with white-small form (WS). Flower size itself affected preferences only mildly (Fig. 2, Table 2). On the other hand, experienced hoverflies showed overall lesser (but still significant) preferences when compared to naive hoverflies (Fig. 2, Table 2). Interestingly, experienced hoverflies showed preferences toward white-small form (WS) in treatment comparing to yellow-large form (YL), which is in contrast with the preferences of naive hoverflies.



	 Naive hoverflies				 Experienced hoverflies			
Predictor	Deviance	Res. d.f.	Res. deviance	P-value	Deviance	Res.d.f.	Res. deviance	P-value
colour	133.39	548	1,509.88	<0.001	71.90	5,621	9,039.18	<0.001
size	10.36	547	1,499.53	<0.001	15.16	5,620	9,024.02	<0.001
diff.	10.76	544	1,488.76	0.09	1.66	5,617	9,022.36	0.734
colour:size	14.22	543	1,474.54	<0.001	22.70	5,616	8,999.66	<0.001
colour:diff.	434.80	540	1,039.74	<0.001	98.77	5,613	8,900.89	<0.001
size:diff.	18.97	537	1,020.77	<0.001	150.90	5,610	8,749.99	<0.001
colour:size:diff	17.20	534	1,003.57	<0.001	49.67	5,607	8,700.32	<0.001

Table 1: The results of Generalised linear models with quasibinomial distribution for naive (left part) and experienced hoverflies (right part) testing the effect of flower colour, size and difference in traits on hoverfly preferences, the significant predictors are in bold. Predictor diff. stands for difference in traits. Link function: logit, NULL deviance for naive hoverflies = 1643.3, Df = 549, Residual deviance for naive hoverflies = 1003.6 and NULL deviance for experienced hoverflies = 9111.1, Df = 5622, Residual deviance for experienced hoverflies = 8700.3.

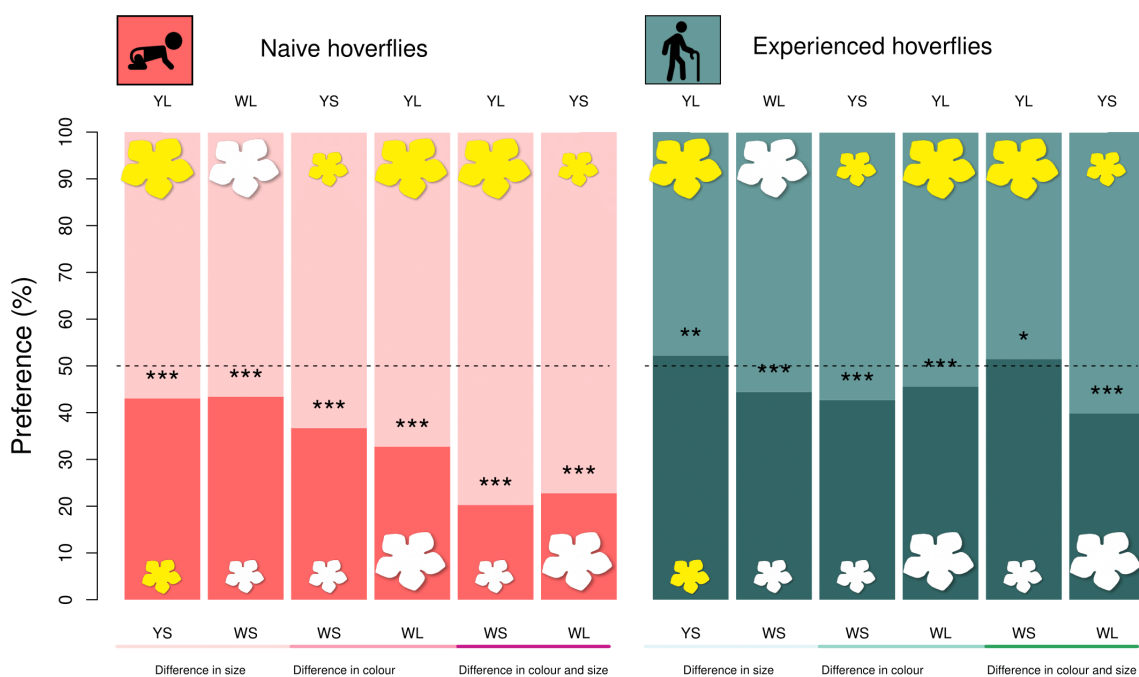


Fig. 2: The preferences of naive (left) and experienced (right) individuals of *E. tenax* derived from binomial tests (Table 1). Significant preferences are marked as follows: *** $p < 0,001$; ** $p < 0,01$; * $p < 0,05$



















									
	x	63 % ***	57 % ***	80 % ***		x	57 % ***	56 % ***	49 %*
	37 %	x	23 % ***	57 % ***		43 %	x	40 % ***	48 % **
	43 %	77 %	x	67 % ***		44 %	60 %	x	54 % ***
	20 %	43 %	33 %	x		51 %	52 %	46 %	x

Table 2: Results of the binomial tests for each pair of flower forms for naive (left) and experienced (right) hoverflies. Significant preferences are marked as follows: *** $p < 0,001$; ** $p < 0,01$; * $p < 0,05$.

8.3.2.6.2 Constancy Naive individuals of *E. tenax* were significantly constant only when the floral forms differed in color (Fig. 3, Table 2). When the flower forms differed only in size pollinators did not show significant constancy to any offered flower form. Consequently, difference in both flower color and size had only limited additive effect on flower constancy, suggesting that main driver of flower constancy of naive *E. tenax* is flower color (Table 2). Generally, hoverflies exhibited stronger flower constancy toward yellow flower forms. Flower constancy of experienced hoverflies, similarly to naive hoverflies, was the highest when flower forms differed in colors. In contrast to naive hoverflies, flower form size had a significant effect on flower constancy of experienced individuals. However, overall the flower constancy of experienced hoverflies was about 10-15% lower than the flower constancy of naive individuals (Fig. 3).

The angular diameter ratio, i.e., the ratio of visible flower forms from hoverfly position, had significant effect in both naive and experienced individuals of *E. tenax*. Hence, hoverflies were affected by their position in the arena, lowering the constancy in situation where in their visual field was more visible flowers of the other form. In the Fig. 3, we presented two extreme scenarios derived from the GLM (Table 3): predictions based on the minimal and maximal ratio of angular diameters of visited and non-visited flower forms. The difference between the extreme cases then presents the approximate effect of the spatial distribution of the flower forms: in the minimal scenario, hoverflies tended to lower their constancy and they more likely visited the other flower morph than in the situation where they were closer to the same form. The difference between minimal and maximal scenario was higher for the experienced hoverflies, suggesting the higher effect of the angular diameter ratio on hoverfly flower constancy.



Predictor	 Naive hoverflies				 Experienced hoverflies			
	Deviance	Res. d.f.	Res. deviance	P-value	Deviance	Res.d.f.	Res. deviance	P-value
colour	169.39	4,839	6,541.55	<0.001	394.04	27,843	37,537.20	<0.001
size	16.82	4,838	6,524.72	<0.001	21.42	27,842	37,515.77	<0.001
diff.	563.65	4,836	5,961.08	<0.001	845.57	27,840	36,670.21	<0.001
ang. dist. ratio	42.90	4,835	5,918.18	<0.001	201.29	27,839	36,468.92	<0.001
colour:size	5.49	4,834	5,912.69	<0.001	39.51	27,838	36,429.41	<0.001
colour:diff.	172.61	4,832	5,740.08	<0.001	8.00	27,836	36,421.41	<0.001
colour:ang. dist. ratio	0.41	4,831	5,739.66	0.52	2.64	27,835	36,418.77	0.104
size:diff.	51.90	4,829	5,687.76	<0.001	690.28	27,833	35,728.49	<0.001
size:ang. dist. ratio	0.15	4,828	5,687.61	0.697	67.09	27,832	35,661.39	<0.001
diff.:ang. dist. ratio	41.35	4,826	5,646.26	<0.001	68.63	27,830	35,592.77	<0.001
colour:size:diff	6.78	4,824	5,639.48	<0.001	137.05	27,828	35,455.72	<0.001
colour:size:ang. dist. ratio	0.30	4,823	5,639.19	0.585	7.44	27,827	35,448.28	<0.001
colour:diff.:ang. dist. ratio	6.04	4,821	5,633.14	0.05	4.73	27,825	35,443.55	0.09
size:diff.:ang. dist. ratio	12.09	4,819	5,621.06	<0.001	10.53	27,823	35,433.02	<0.001

Table 3: The results of Generalised linear models with binomial distribution for naive (left part) and experienced hoverflies (right part), the significant predictors are in bold. Predictors diff. and ang. dist. ratio stands for difference in traits and angular distance ratio, respectively. Link function: logit, NULL deviance for naive hoverflies = 6710.9, Df = 4840, Residual deviance for naive hoverflies = 5621.1 and NULL deviance for experienced hoverflies = 37931, Df = 27844, Residual deviance for experienced hoverflies = 35433.

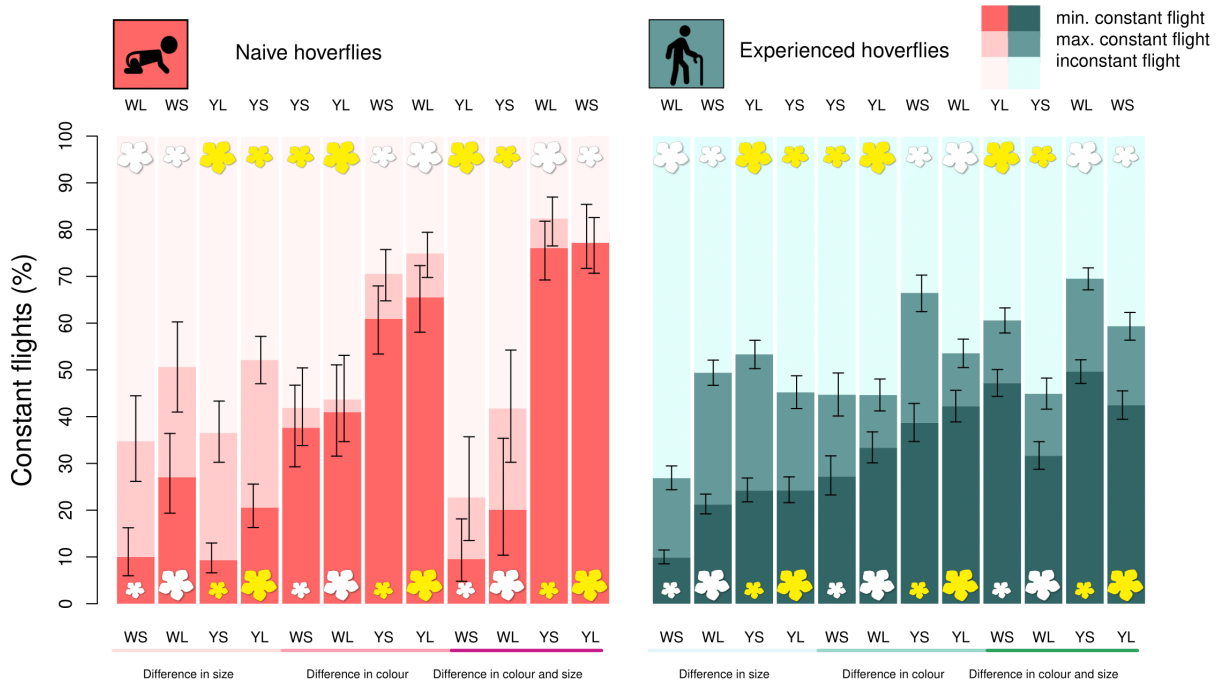


Fig. 3: Constancy of naive (left) and experienced (right) hoverflies. The letters and images refer to the flower forms compared to each other with the starting form in the lower row and compared form in the upper row. The lower and upper part of the barplot refers to the constancy in the scenario with lowest and highest ratio of angular diameters of the flower forms (the same vs the other) in the arena, respectively. Hence the difference between minimal and maximal value can be seen as rough estimate of the effect of the flowers spatial distribution on *E. tenax* constancy.

8.3.2.7 Discussion Our results revealed expected preference of *Eristalis tenax* toward yellow colored artificial flowers, increased by the large flower size. However, we found that experienced individuals of *E. tenax* showed different level of preferences (Fig. 2) and lower flower constancy (Fig. 3) than naive individuals. To our knowledge, this is the first study showing such a result in *E. tenax*. Therefore, here we further discuss possible mechanisms leading to the changes in pollinator preferences and flower constancy during foraging.

Strong preferences of naive *E. tenax* toward yellow colour are well known and documented (An et al., 2018; Matoušková et al., 2023) and it has been hypothesized that this is caused by the association of yellow colour with pollen (Lunau and Wacht, 1994; Wacht et al., 1996). *Eristalis tenax* possess strong innate preferences when compared to preferences detected in similar experiments with bees (Gegear and Laverly, 2005), butterflies (Goulson and Cory, 1993) and even individuals of closely related species *E. cerealis* behave similarly (Kandori et al., 2021). Interestingly, experienced *E. tenax* showed generally lower preferences and particularly lower preferences toward yellow colour compared to the naive individuals. This may be explained by the previous foraging experience with the flowers visited during previous foraging. It has

been demonstrated, that *E. tenax* can be trained to show preferences toward another colours, however with remaining preference toward yellow colour (Lunau and Wacht, 1997). This limited ability to change the preferences under experience is in line with our results, as the preferences toward yellow colour decreased but not diminished, similarly to experiments with bumblebees (Gumbert, 2000). We also know, that individuals of *E. tenax* visit broad spectra of flowering plants at the study locality, which flower colour spectrum range from white and yellow to purple and blue (Fig. 1D). It is important to note that *E. tenax* predominantly visits blue-purple *Succisa pratensis* and several species from Apiaceae family with white flowers, while species with yellow-colored flowers represent only a small fraction of visited plant species (Fig. 1D). The effect of flower context and visitation history on pollinator visitation choice has been documented in several cases (Hill et al., 1997 ; Whitehead et al., 2019; Ye et al., 2020; Finnell and Koski, 2021), therefore, it could be expected that the experiment could possibly yield a different result in the community with different proportions of the visited plants species differing in flower colour. Hence it would be interesting to test the relative effect of community context on shaping the pollinator preference and to find the limitations of the pollinator learning abilities.

Eristalis tenax in our experiments in general showed lower flower constancy comparing to Honeybees (Hill et al., 1997; Grüter et al., 2011; Us Van Der Niet et al., 2020), bumblebees (Austin et al., 2019) or butterflies (Goulson and Cory, 1993), suggesting generalized foraging behaviour of *E. tenax*. The relatively low flower constancy of *E. tenax* can be explained by the lack of social behaviour which is possible explanation of strong constancy in social bees (Hayes and Grüter, 2023). Moreover, unlike bees, but similarly to butterflies, hoverflies are not interested only in the feeding, but also searching for mate, avoiding predators and searching for oviposition sites also play an important role in their choices during foraging. Flower constancy of *E. tenax* was also affected by the visual difference of the offered artificial flowers. *E. tenax* flower constancy was driven mainly by the difference in colour, while the combination of colour and size increased the constancy just slightly. This is surprising given to the previous experiments with bumblebees (Robert and Lavery, 2001; Gegear and Lavery, 2005), revealing that artificial flowers differing in more than one trait highly increased flower constancy. We argue, that in the case of *E. tenax*, colour does have superior importance in forming their preferences over other floral traits (Matoušková et al., 2023). Therefore, it is not surprising that the increase in constancy by adding one trait does not render stronger flower constancy. However, it is questionable if other traits, such as nectar guides or the depth of the flower would not play more significant role, similarly to another colour (e.g., blue or pink) or size variants of the flower forms. Combination of several traits in full-factorial experiment may then revealed structured network of trait interactions affecting flower constancy.

The remaining and compelling question rising from our results is why are experienced individuals of *E. tenax* less flower constant than the naive ones?

Similarly to our results, Gumbert (2000) detected higher degree of generalization of trained bumblebees while foraging between artificial flowers, suggesting that experience is affecting

flower constancy despite strong innate preferences toward particular flower form. This results highlight strong opportunistic behaviour of *E. tenax* during foraging, which may allow to adapt in different context of flowering plant community. The high level of opportunism of experienced hoverflies is supported by the difference between estimated constancy with minimal and maximal angular distance ratio (Fig. 3), i.e., hoverflies were less constant if they were close to the other form of flowers. The difference was more prone in experienced hoverflies, indicating that their constancy depends more on the local distribution of flowers, which may have implications for visitation pattern in patchily distributed plants in the vegetation. On top of that, there are two alternative explanation for experienced *E. tenax* showing lower flower constancy than the naive ones raising from potential methodological limitations of our study: First, experienced *E. tenax* were possibly not satisfied with the quality of offered reward in the artificial flowers, because artificial flowers carried reward with lower sugar concentration than can be found in the flowers (Comba et al., 1999; Pamminer et al., 2019). This may caused higher tendency to switch between flower forms as the individuals were searching for more satisfying reward (Chittka et al., 1999). Similarly, in experiments of Grüter et al. (2011) honeybees decreased their flower constancy in response to lower sucrose concentration in the in artificial flowers, but remained constant even in the treatment with the lowest level of the sucrose concentration. We used lower sucrose concentration to motivate *E. tenax* to forage among multiple flowers as the higher sucrose concentration in flowers seemed to prolong the time of the visit on flower (personal observation during the pilot experiment) and used concentration did not seemed to render unrealistic behaviour. Hence, study on the effect of the reward quality on the foraging behaviour and flower constancy of *E. tenax* is needed, particularly to investigate the response to different sucrose concentration. Second possible explanation is that *E. tenax* visits flowers with easily accessible reward (e.g., *Succisa pratensis*, all Apiaceae), similarly to the artificial flower, which does not stimulate flower constancy due to only modest difference in the handling time and reward intake. According to the learning investment hypothesis (Heinrich, 1976; Lavery, 1994), flower constancy may be induced only if the pollinator benefits from increased reward intake per time from the flower it learned to handle effectively. However, this benefit from experience is presumably limited to narrow set of plant species due to memory limitations of individual pollinators (Lavery, 1994; Gegear and Lavery, 1998; Gegear and Lavery, 2005). In the case of our study system, the benefits resulting from pollinator constancy may be limited by the minimal cost of switching between plant species occurring on the locality. Similarly, the artificial flowers does not restrict access to the reward, thus, the cost of switching between them was minimal (Lavery, 1994; Dukas, 1995). Thus, direct investigation of the the effect of flower restrictiveness on flower constancy would help us to explain which mechanisms are responsible for forming flower constancy of *E. tenax*.

Combined, our results suggest that *E. tenax* is able to adapt foraging behaviour based on the previous experience, despite strong innate preferences. We hypothesize that this ability allows *E. tenax* to adapt opportunistic foraging behaviour and generalized strategy when visiting flowers in species rich habitats. Hence, it may be advantageous for plants predominantly visited by *E.*

tenax to manipulate

8.3.2.8 Conclusions Our study shows the effect of foraging experience on preferences and flower constancy of *Eristalis tenax*. Experience with foraging within flower rich community modify pollinator preferences and decrease flower constancy, making *E. tenax* more opportunistic when compared to naive individuals. Thus, we conclude that *E. tenax* is able to behaviorally adapt to the changing “flower market” offer, which may be crucial in highly generalized plant-pollinator community. Temporal changes in *E. tenax* foraging behaviour may then play a role in inner structuring of the interactions with the pollinated plants.

8.3.2.9 Acknowledgments We thank the members of the Flower Ecology Group in Prague for discussions on the topic and valuable suggestions to earlier versions of the manuscript. We also thank the American Journal Editors for language editing of the manuscript. This work was supported by the Charles University Grant Agency [Grant No. 842120].

8.3.2.10 Authors contribution AH, ZJ and JŠ conceived the research idea. AH and ZJ designed the method for testing *E. tenax* preferences. EM and JŠ designed and produced artificial flowers. AH collected the data and ZJ analysed the data. JŠ, EM and ZJ provided funding. JŠ and AH? led writing of the manuscript with contribution of all coauthors to the final manuscript.

8.3.2.11 Data availability statement Data will be available at FigShare repository after publication of the article.

8.3.2.12 Supporting Information

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10 Appendix

10.1 Supplementary material for Article 1: Pollinator community and generalisation of pollinator spectra changes with plant niche width and local dominance

Zdeněk Janovský¹, Jakub Štenc^{2*}

¹Svatý Jan t. Krsovice 1, 285 04 Uhlířské Janovice, Czech Republic

²Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 41 Prague, Czech Republic

*Corresponding author, e-mail address: jakubstenc@gmail.com.

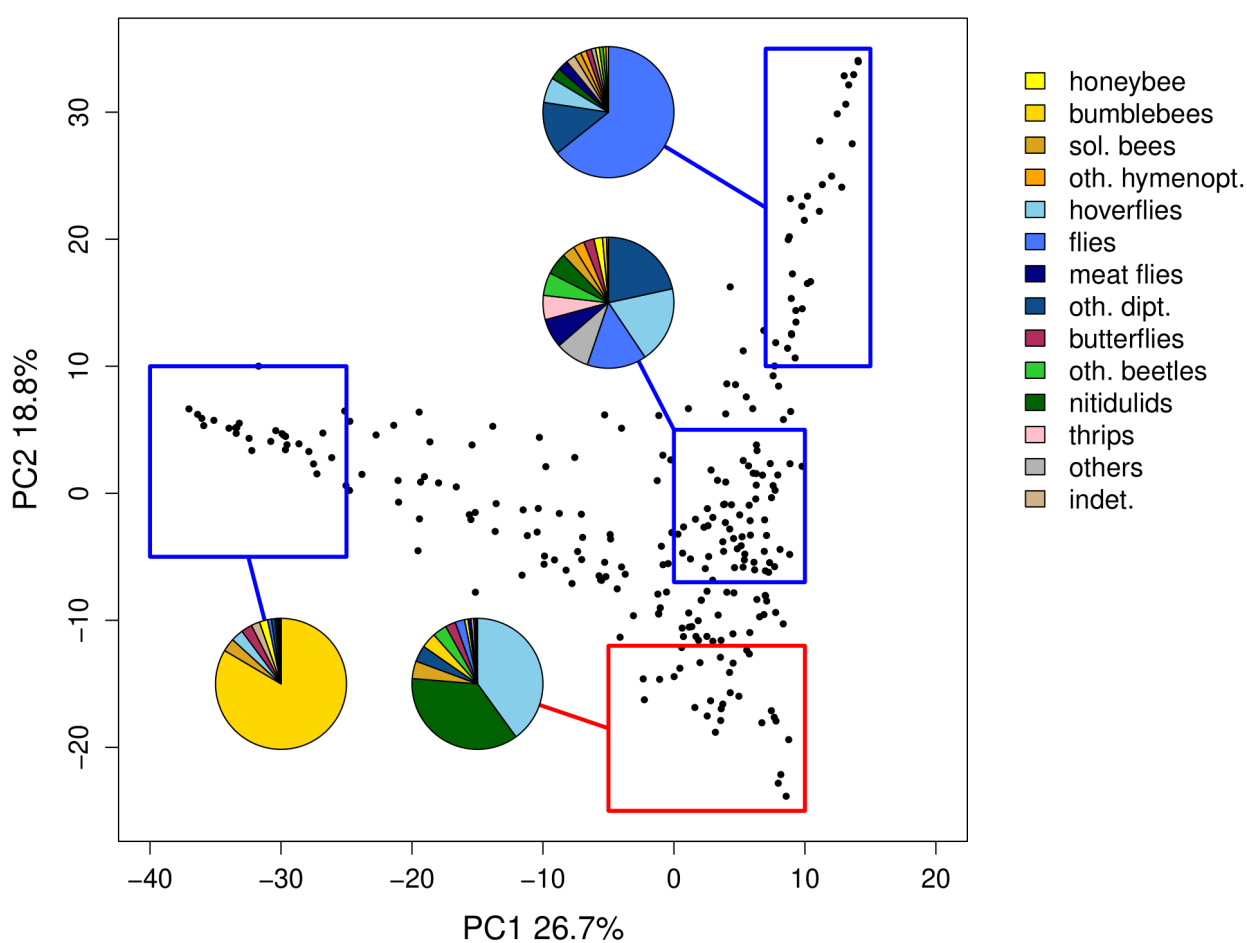


Fig. S1: Ordination diagrams of the phylogenetic PCA of plant pollinator spectra, 1st and 2nd principal components. Piecharts depict proportional composition of pollinator spectra for area in a rectangle.

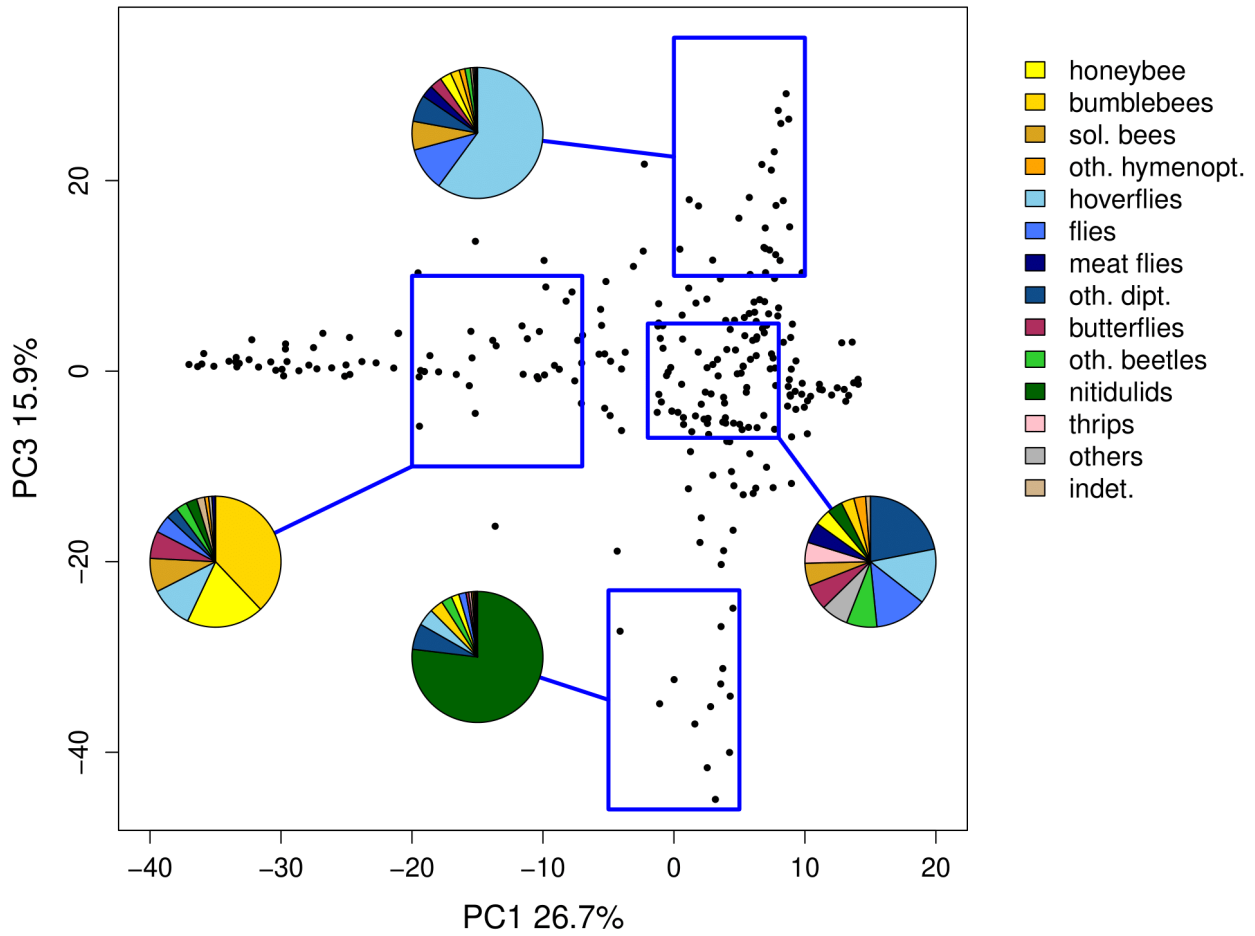


Fig. S2: Ordination diagrams of the phylogenetic PCA of plant pollinator spectra, 1st and 3rd principal components. Piecharts depict proportional composition of pollinator spectra for area in a rectangle.

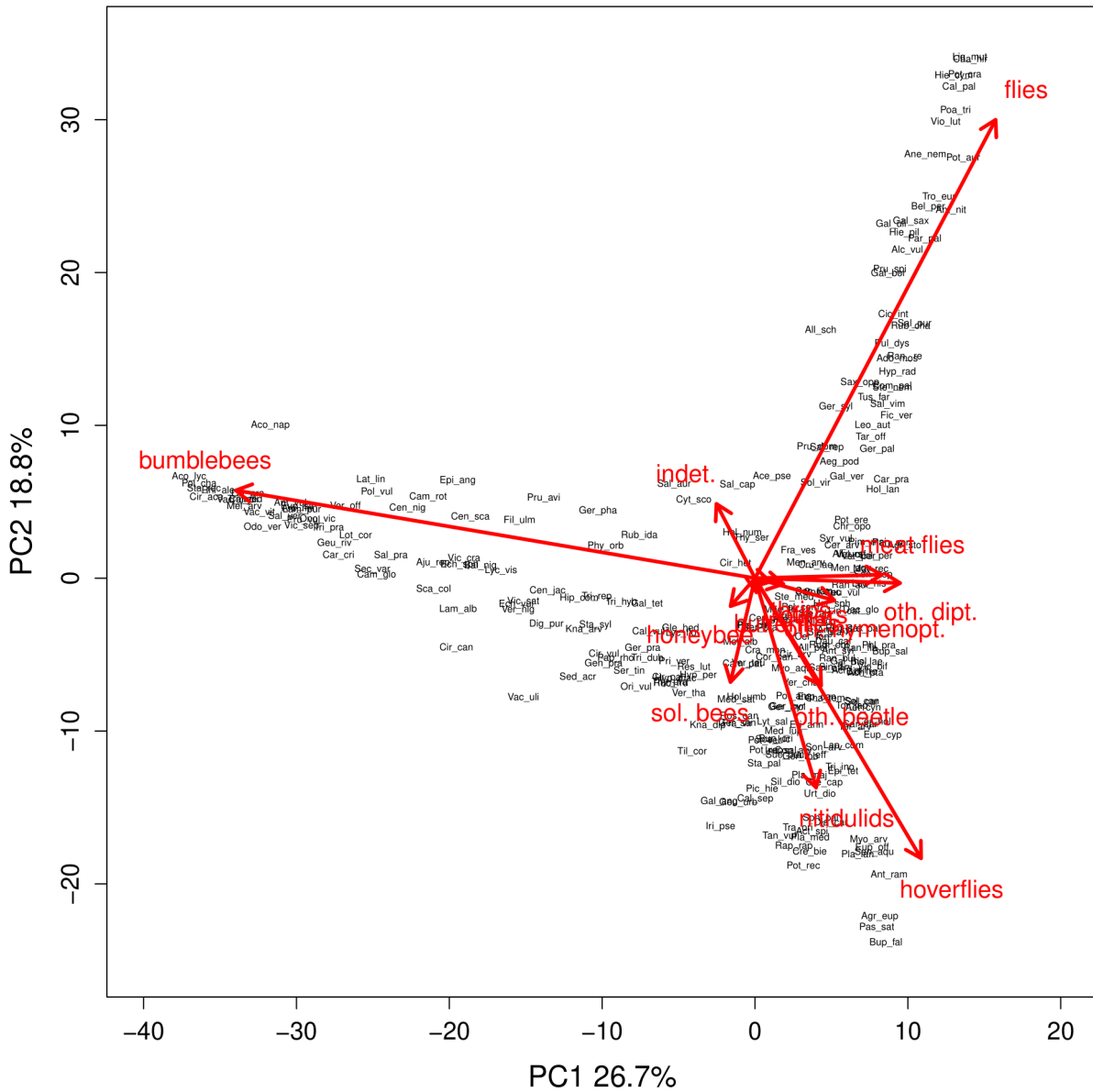


Fig. S3: Ordination diagrams of the phylogenetic PCA of plant pollinator spectra, 1st and 2nd principal components.

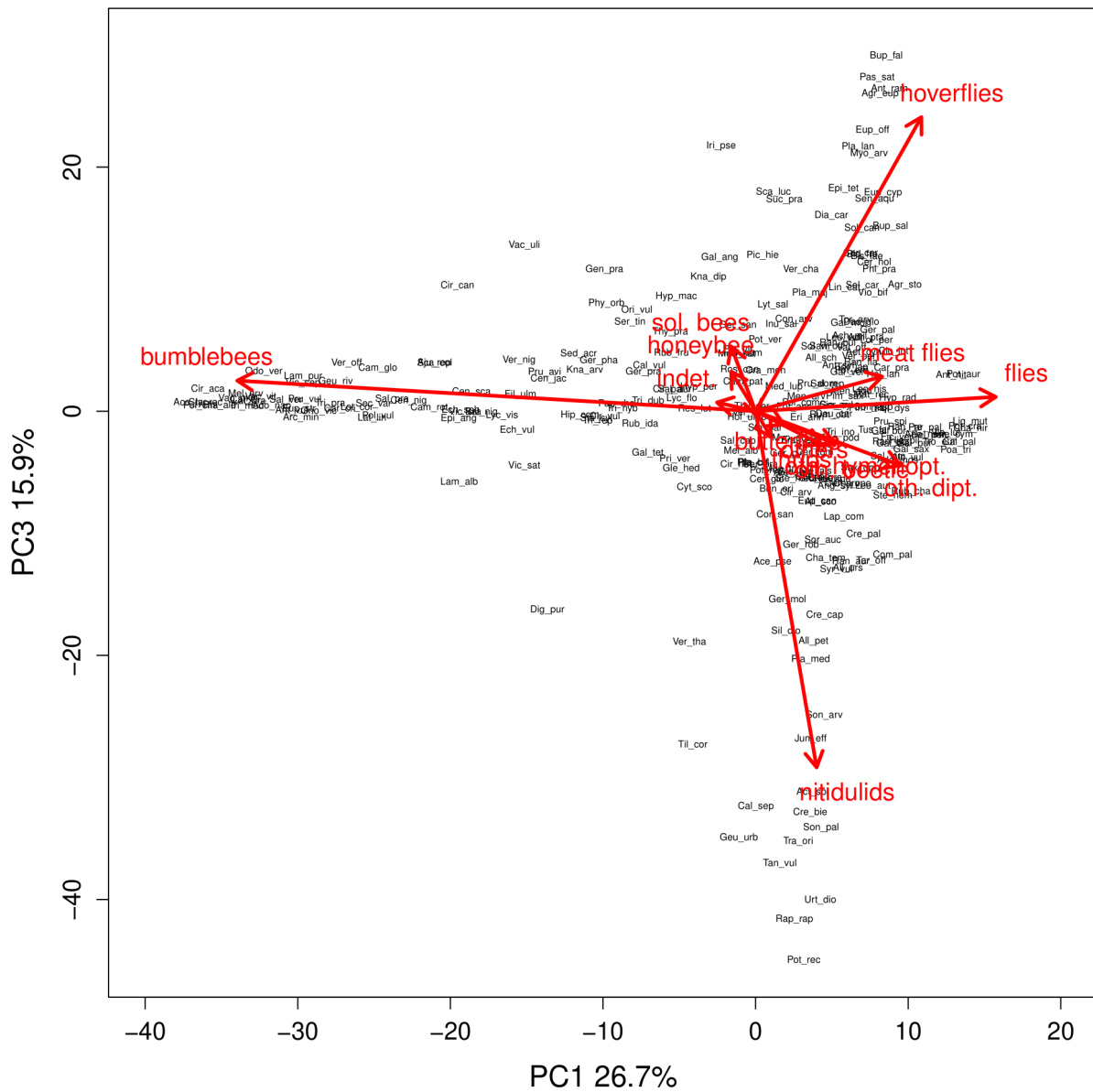


Fig. S4: Ordination diagrams of the phylogenetic PCA of plant pollinator spectra, 1st and 3rd principal components.

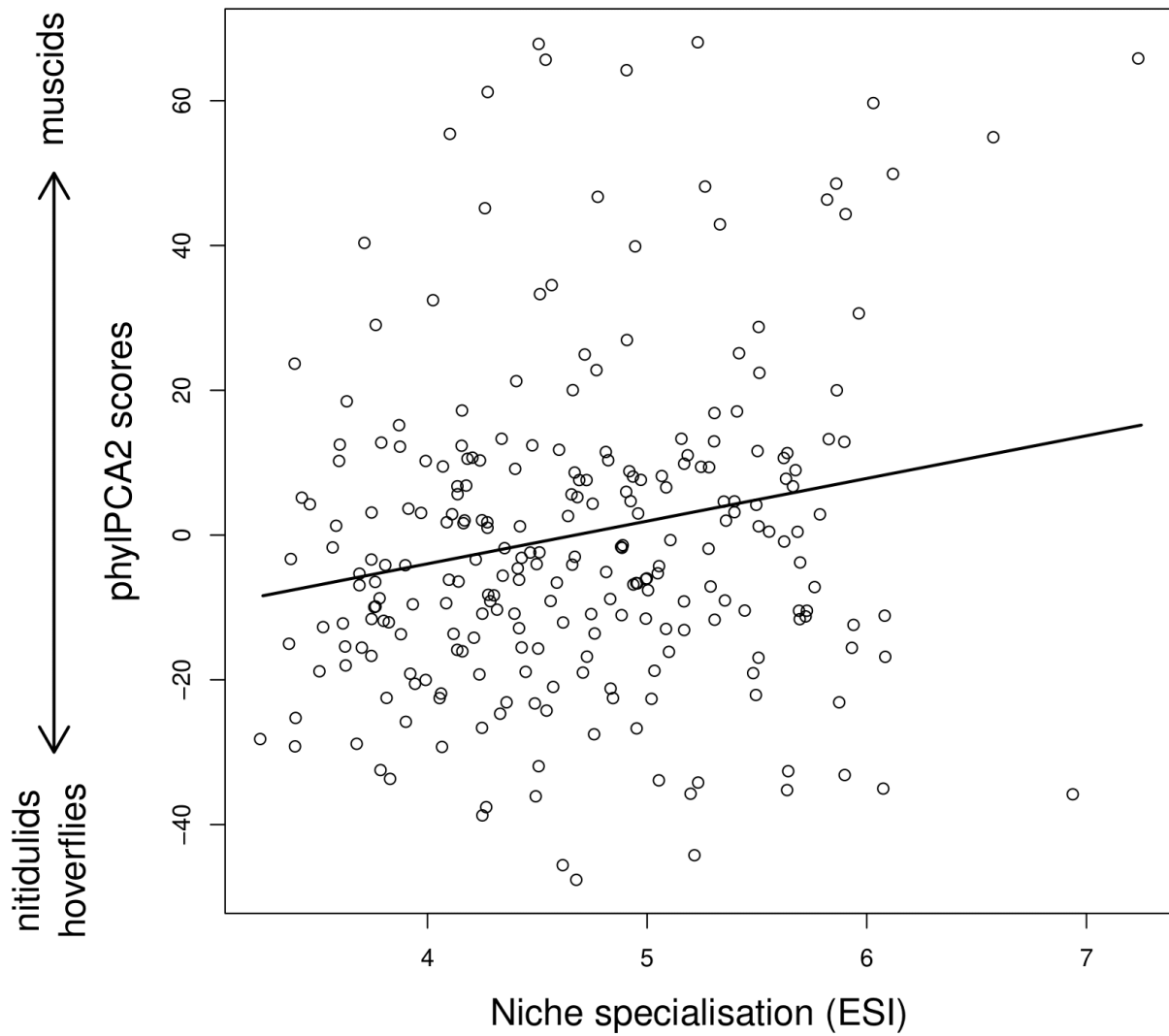


Fig. S5: The relationship between plant niche width (ESI) position in the phyPCA ordination space 1st principal component. The fitted relationships are predictions from pGLS regressions listed in Table 2.

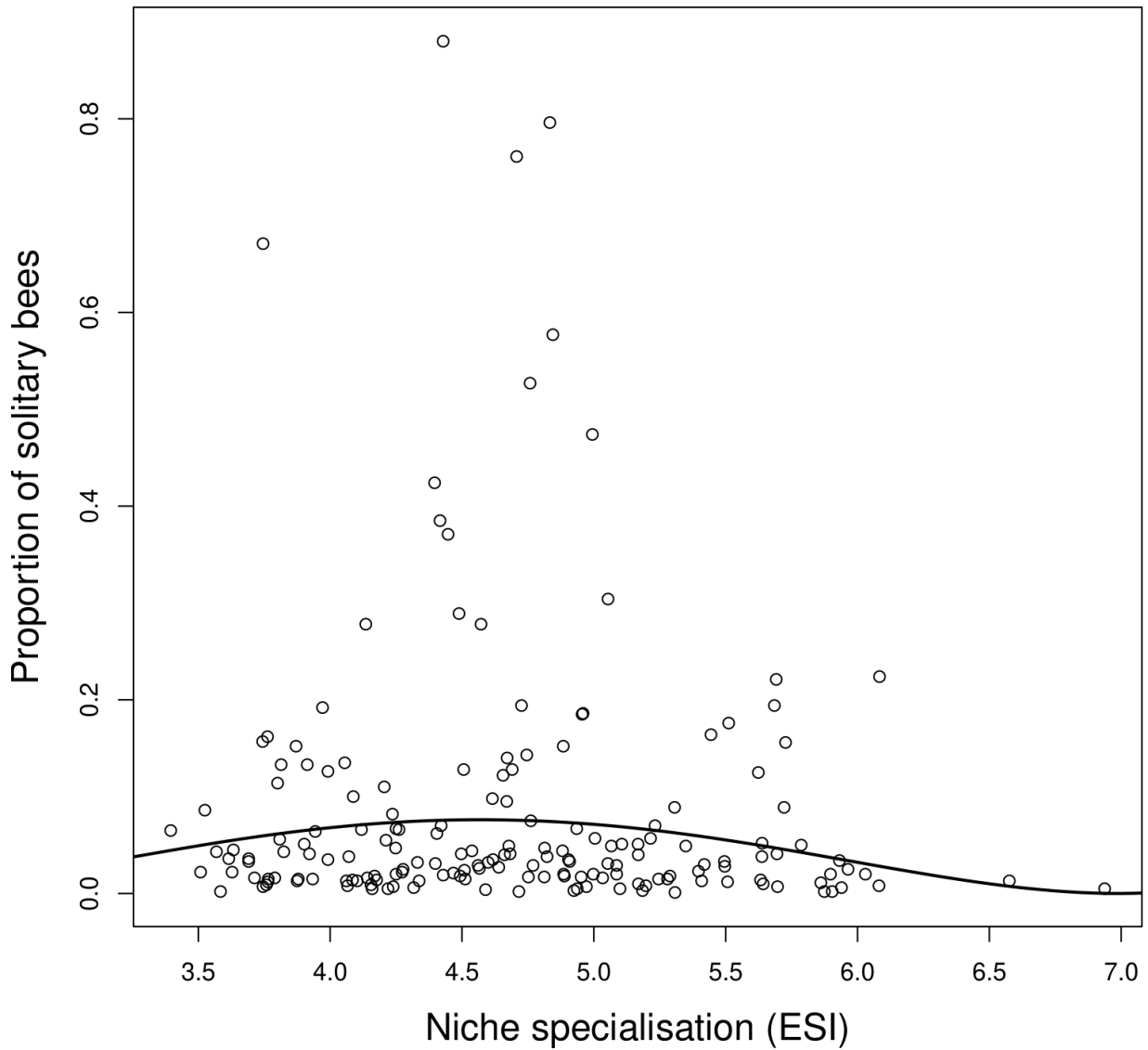


Fig. S6: The relationship between plant niche width (ESI) and proportion of other bees in plant pollinator spectra. The fitted relationships are predictions from pGLS regressions listed in Table 2

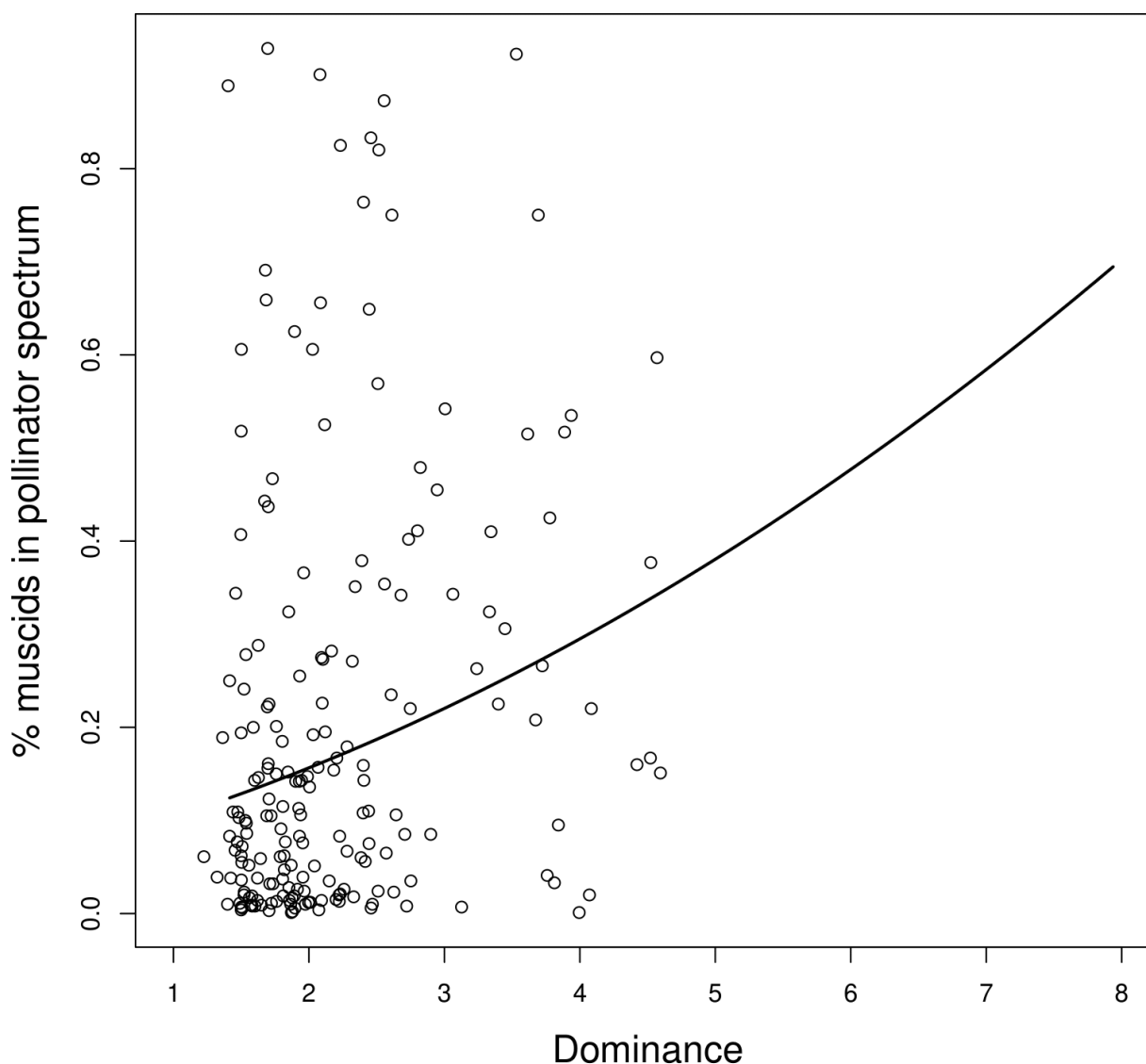


Fig. S7: The relationship between local dominance proportion of muscids in plant pollinator spectra. The fitted relationships are predictions from pGLS regressions listed in Table 2.

Species abbreviations:

Ace_pse - *Acer pseudoplatanus*, Aco_lyc - *Aconitum lycoctonum*, Aco_nap - *Aconitum napellus* agg., Act_spi - *Actaea spicata*, Ado_mos - *Adoxa moschatellina*, Aeg_pod - *Aegopodium podagraria*, Aet_cyn - *Aethusa cynapium*, Agr_eup - *Agrimonia eupatoria*, Agr_sto - *Agrostis stolonifera*, Ach_mil - *Achillea millefolium* agg., Ach_pta - *Achillea ptarmica*, Aju_rep - *Ajuga reptans*, Alc_vul - *Alchemilla vulgaris*, All_pet - *Alliaria petiolata*, All_sco - *Allium scorodoprasum*, All_sch - *Allium schoenoprasum*, All_urs - *Allium ursinum*, Ane_nem - *Anemone nemorosa*, Ang_syl - *Angelica sylvestris*, Ant_ram - *Anthericum ramosum*, Ant_nit - *Anthriscus nitidus*, Ant_syl - *Anthriscus sylvestris*, Ant_vul - *Anthyllis vulneraria*, Arc_min - *Arctium minus*, Are_ser - *Arenaria serpyllifolia* agg., Bal_nig - *Ballota nigra*, Bel_per

- *Bellis perennis*, Bid_fro - *Bidens frondosus*, Bis_lae - *Biscutella laevigata*, Bun_ori - *Bunias orientalis*, Bup_fal - *Bupleurum falcatum*, Cal_vul - *Calluna vulgaris*, Cal_pal - *Caltha palustris*, Cal_sep - *Calystegia sepium*, Cam_glo - *Campanula glomerata*, Cam_pat - *Campanula patula*, Cam_rot - *Campanula rotundifolia*, Car_pra - *Cardamine pratensis*, Car_cri - *Carduus crispus*, Car_car - *Carum carvi*, Cen_jac - *Centaurea jacea*, Cen_sca - *Centaurea scabiosa*, Cer_arv - *Cerastium arvense*, Cer_bra - *Cerastium brachypetalum*, Cer_fon - *Cerastium fontanum*, Cer_glo - *Cerastium glomeratum*, Cer_hol - *Cerastium holosteoides*, Cer_tom - *Cerastium tomentosum*, Cic_int - *Cichorium intybus*, Cir_aca - *Cirsium acaulon*, Cir_arv - *Cirsium arvense*, Cir_can - *Cirsium canum*, Cir_het - *Cirsium heterophyllum*, Cir_pal - *Cirsium palustre*, Cir_vul - *Cirsium vulgare*, Cal_cli - *Clinopodium vulgare*, Com_pal - *Comarum palustre*, Con_arv - *Convolvulus arvensis*, Cor_san - *Cornus sanguinea*, Cra_mon - *Crataegus monogyna*, Cre_bie - *Crepis biennis*, Cre_cap - *Crepis capillaris*, Cre_pal - *Crepis paludosa*, Cru_lae - *Cruciata laevipes*, Cyt_sco - *Cytisus scoparius*, Dac_glo - *Dactylis glomerata*, Dau_car - *Daucus carota*, Dia_car - *Dianthus carthusianorum*, Dig_pur - *Digitalis purpurea*, Ech_sph - *Echinops sphaerocephalus*, Ech_vul - *Echium vulgare*, Epi_ang - *Epilobium angustifolium*, Epi_tet - *Epilobium tetragonum*, Eri_ann - *Erigeron annuus*, Eup_can - *Eupatorium cannabinum*, Eup_cyp - *Euphorbia cyparissias*, Eup_off - *Euphrasia officinalis*, Eup_str - *Euphrasia stricta*, Fic_ver - *Ficaria verna*, Fil_ulm - *Filipendula ulmaria*, Fra_ves - *Fragaria vesca*, Fra_vir - *Fragaria viridis*, Gal_ang - *Galeopsis angustifolia*, Gal_tet - *Galeopsis tetrahit*, Gal_bor - *Galium boreale*, Gal_mol - *Galium mollugo* agg., Gal_sax - *Galium saxatile*, Gal_uli - *Galium uliginosum*, Gal_ver - *Galium verum*, Gen_pra - *Gentianella praecox*, Ger_mol - *Geranium molle*, Ger_pal - *Geranium palustre*, Ger pha - *Geranium phaeum*, Ger_pra - *Geranium pratense*, Ger_pyr - *Geranium pyrenaicum*, Ger_rob - *Geranium robertianum*, Ger_san - *Geranium sanguineum*, Ger_syl - *Geranium sylvaticum*, Geu_riv - *Geum rivale*, Geu_urb - *Geum urbanum*, Gle_hed - *Glechoma hederacea*, Hel_num - *Helianthemum nummularium*, Her_sph - *Heracleum sphondylium*, Hol_lan - *Holcus lanatus*, Hol_umb - *Holosteum umbellatum*, Hyp_mac - *Hypericum maculatum*, Hyp_per - *Hypericum perforatum*, Hyp_rad - *Hypochaeris radicata*, Cha_hir - *Chaerophyllum hirsutum*, Cha_tem - *Chaerophyllum temulum*, Chr_opo - *Chrysosplenium oppositifolium*, Inu_sal - *Inula salicina*, Iri_pse - *Iris pseudacorus*, Jun_eff - *Juncus effusus*, Kna_arv - *Knautia arvensis*, Kna_dip - *Knautia maxima*, Lam_alb - *Lamium album*, Lam_pur - *Lamium purpureum*, Lap_com - *Lapsana communis*, Lat_lin - *Lathyrus linifolius*, Lat_pra - *Lathyrus pratensis*, Leo_aut - *Scorzoneroideis autumnalis*, Leo_his - *Leontodon hispidus*, Leu_vul - *Leucanthemum vulgare* agg., Lig_mut - *Ligusticum mutellina*, Lin_cat - *Linum catharticum*, Lol_per - *Lolium perenne*, Lot_cor - *Lotus corniculatus*, Lyc_flo - *Lychnis flos-cuculi*, Lyt_sal - *Lythrum salicaria*, Lyc_vis - *Viscaria vulgaris*, Mat_rec - *Matricaria chamomilla*, Med_lup - *Medicago lupulina*, Med_sat - *Medicago sativa*, Mel_arv - *Melampyrum arvense*, Mel_alb - *Melilotus albus*, Men_aqu - *Mentha aquatica*, Men_arv - *Mentha arvensis*, Moe_tri - *Moehringia trinervia*, Myo_arv - *Myosotis arvensis*, Myo_aqu - *Myosoton aquaticum*, Odo_ver - *Odontites vernus*, Ono_vic - *Onobrychis vicifolia*, Ori_vul - *Origanum vulgare*, Pap_rho -

Papaver rhoeas, Par_pal - *Parnassia palustris*, Pas_sat - *Pastinaca sativa*, Phl_pra - *Phleum pratense*, Phy_orb - *Phyteuma orbiculare*, Pic_hie - *Picris hieracioides*, Hie_cym - *Pilosella cymosa*, Hie_pil - *Pilosella officinarum*, Pim_maj - *Pimpinella major*, Pim_sax - *Pimpinella saxifraga*, Pla_lan - *Plantago lanceolata*, Pla_maj - *Plantago major*, Pla_med - *Plantago media*, Pla_bif - *Platanthera bifolia*, Pla_chl - *Platanthera chlorantha*, Poa_tri - *Poa trivialis*, Pol_com - *Polygala comosa*, Pol_cha - *Polygala chamaebuxus*, Pol_vul - *Polygala vulgaris*, Pot_ans - *Potentilla anserina*, Pot_aur - *Potentilla aurea*, Pot_cra - *Potentilla crantzii*, Pot_ere - *Potentilla erecta*, Pot_rec - *Potentilla recta*, Pot_rep - *Potentilla reptans*, Pot_ver - *Potentilla verna*, Pri_ver - *Primula veris*, Pru_vul - *Prunella vulgaris*, Pru_avi - *Prunus avium*, Pru_dom - *Prunus domestica* agg., Pru_spi - *Prunus spinosa*, Pul_dys - *Pulicaria dysenterica*, Ran_acr - *Ranunculus acris*, Ran_aur - *Ranunculus auricomus*, Ran_bul - *Ranunculus bulbosus*, Ran_fla - *Ranunculus flammula*, Ran_re - *Ranunculus repens*, Rap_rap - *Raphanus raphanistrum*, Res_lut - *Reseda lutea*, Rhi_ale - *Rhinanthus alectorolophus*, Ros_can - *Rosa canina* agg., Rub_fru - *Rubus fruticosus* agg., Rub_ida - *Rubus idaeus*, Rum_obt - *Rumex obtusifolius*, Sal_aur - *Salix aurita*, Sal_cap - *Salix caprea*, Sal_pur - *Salix purpurea*, Sal_rep - *Salix repens*, Sal_vim - *Salix viminalis*, Sal_pra - *Salvia pratensis*, Sal_ver - *Salvia verticillata*, San_off - *Sanguisorba officinalis*, Sca_col - *Scabiosa columbaria*, Sec_var - *Securigera varia*, Sed_acr - *Sedum acre*, Sel_car - *Selinum carvifolia*, Sen_aqu - *Senecio aquaticus*, Sen_jac - *Senecio jacobaea*, Ser_tin - *Serratula tinctoria*, Sil_dio - *Silene dioica*, Sin_arv - *Sinapis arvensis*, Sol_can - *Solidago canadensis*, Sol_vir - *Solidago virgaurea*, Son_arv - *Sonchus arvensis*, Son_asp - *Sonchus asper*, Sor_auc - *Sorbus aucuparia*, Sta_pal - *Stachys palustris*, Sta_rec - *Stachys recta*, Sta_syl - *Stachys sylvatica*, Ste_als - *Stellaria alsine*, Ste_gra - *Stellaria graminea*, Ste_hol - *Stellaria holostea*, Ste_med - *Stellaria media*, Ste_nem - *Stellaria nemorum*, Suc_pra - *Succisa pratensis*, Syr_vul - *Syringa vulgaris*, Tan_vul - *Tanacetum vulgare*, Tar_off - *Taraxacum* sect. *Taraxacum*, Thy_pra - *Thymus praecox*, Thy_ser - *Thymus serpyllum*, Til_cor - *Tilia cordata*, Tor_jap - *Torilis japonica*, Tra_ori - *Tragopogon orientalis*, Tri_dub - *Trifolium dubium*, Tri_hyb - *Trifolium hybridum*, Tri_med - *Trifolium medium*, Tri_pra - *Trifolium pratense*, Tri_rep - *Trifolium repens*, Tri_ino - *Tripleurospermum inodorum*, Tro_eur - *Trollius europaeus*, Tus_far - *Tussilago farfara*, Urt_dio - *Urtica dioica*, Vac_myrt - *Vaccinium myrtillus*, Vac_uli - *Vaccinium uliginosum*, Vac_vit - *Vaccinium vitis-idaea*, Val_off - *Valeriana officinalis* agg., Ver_nig - *Verbascum nigrum*, Ver_tha - *Verbascum thapsus*, Ver_cha - *Veronica chamaedrys*, Ver_off - *Veronica officinalis*, Ver_per - *Veronica persica*, Ver_teu - *Veronica teucrium*, Vic_cra - *Vicia cracca*, Vic_sat - *Vicia sativa*, Vic_sep - *Vicia sepium*, Vio_bif - *Viola biflora*, Vio_lut - *Viola lutea*,

10.1.1 Supplementary material 1 – Dataset used in the analyses and references of data sources

The dataset used in the analyses is enclosed as a separate excel file. The data source reference numbers correspond to the column ‘References’ in the dataset table.

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10.2 Supplementary material: Appendix S1 Pollinator visitation closely tracks diurnal patterns in pollen release

Jakub Štenc¹, Lukáš Janošík¹, Eva Matoušková², Jiří Hadrava², Michael Mikát^{2,3,4}, Zdeněk Janovský¹

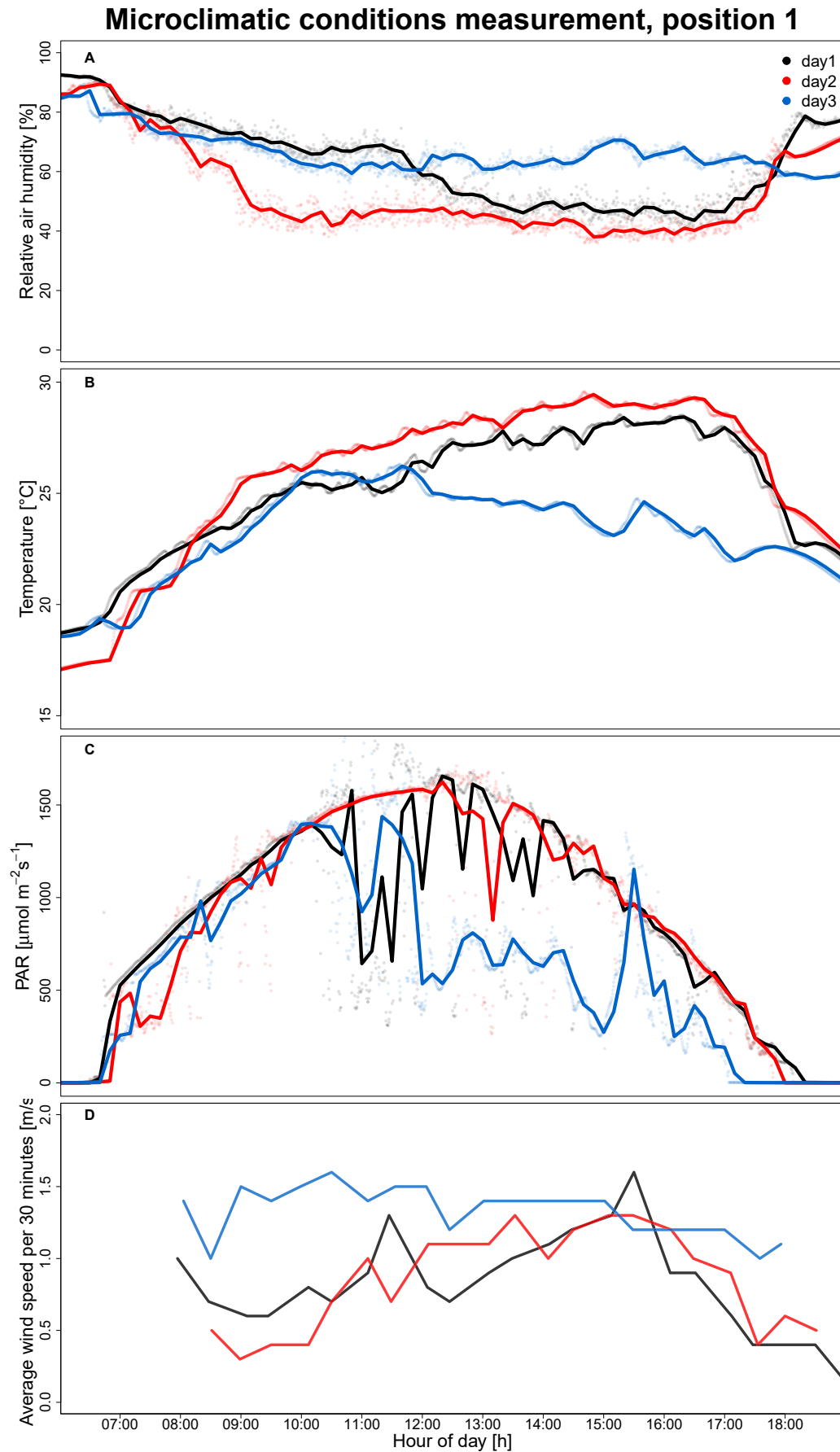
¹Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 41 Prague, Czech Republic

²Department of Zoology, Faculty of Science, Charles University, Viničná 7, 128 41 Prague, Czech Republic

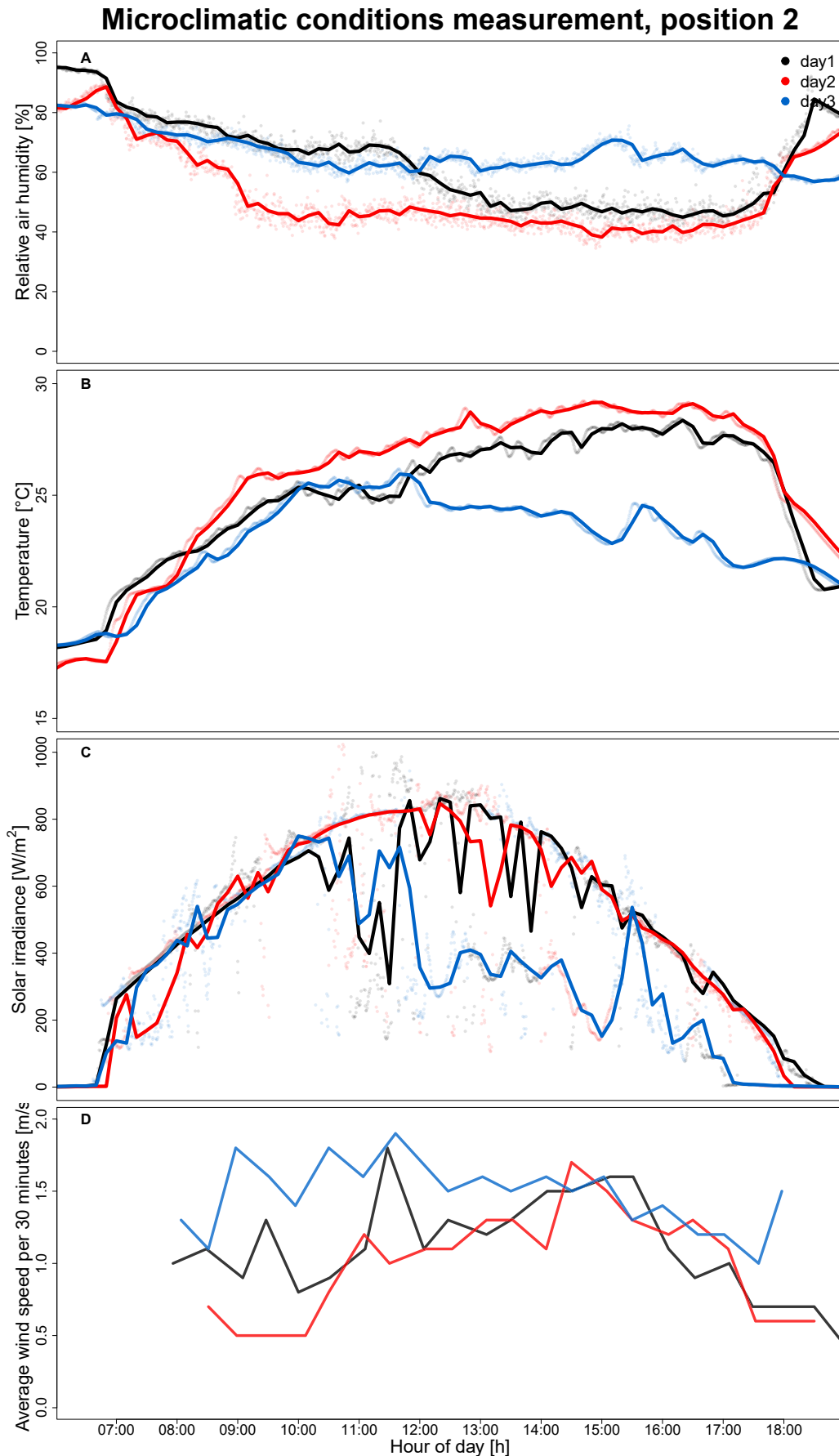
³Department of Biology, Faculty of Science, York University, 4700 Keele Street, 203D, M3J 1P3, Toronto, Canada

⁴Department of General Zoology, Faculty of Natural Sciences 1 - Biosciences, Martin Luther University, Hoher Weg 8 , 06100 Halle (Saale), Germany

*Corresponding author, e-mail address: jakubstenc@gmail.com.



Appendix 1, Fig. S1: Measured microclimatic variables on the 1 close to *Succisa pratensis* microsite. Details about measurement tools in Methods. Day 1 and 2 (14th and 15th August 2021, respectively) are days of pollen presentation sampling and Day 3 (16th August 2021) is the day of pollen presentation. The variables are: A) Relative air humidity, B) Temperature, C) PAR, and D) Average wind speed per 30 minutes.



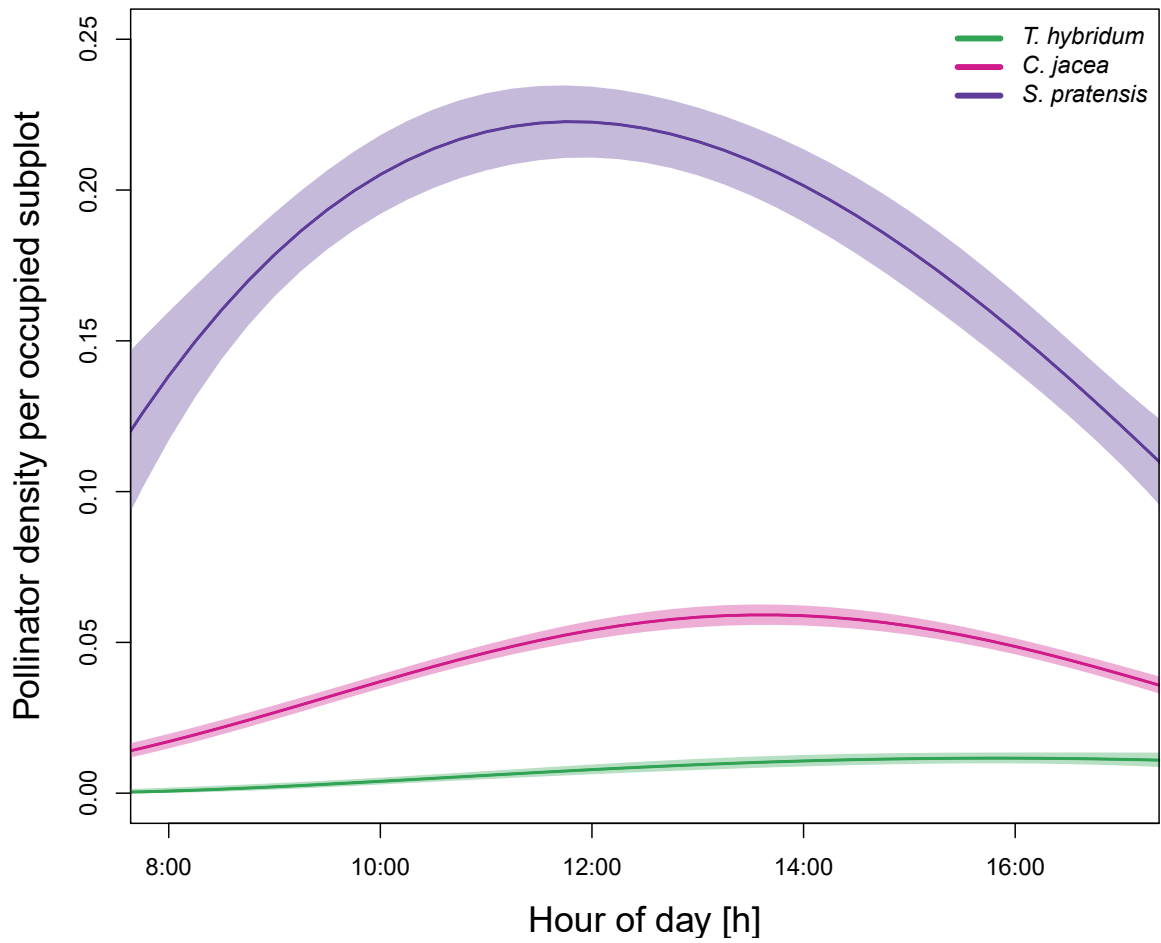
Appendix 1, Fig. S2: Measured microclimatic variables on the position 2 close to *Centaurea jacea* and *Trifolium hybridum* microsites. Details about measurement tools in Methods. Day 1 and 2 (14th and 15th August 2021, respectively) are days of pollen presentation sampling and Day 3 (16th August 2021) is the day of pollen presentation sampling. (For interpretation of the references to published material in this article, the reader is referred to the web version of this article.)

Appendix 1, Table. S1: Numbers of pollinators observed visiting studied plant species during seasons 2011-2021. Pollinators are in the table scored to functional groups.

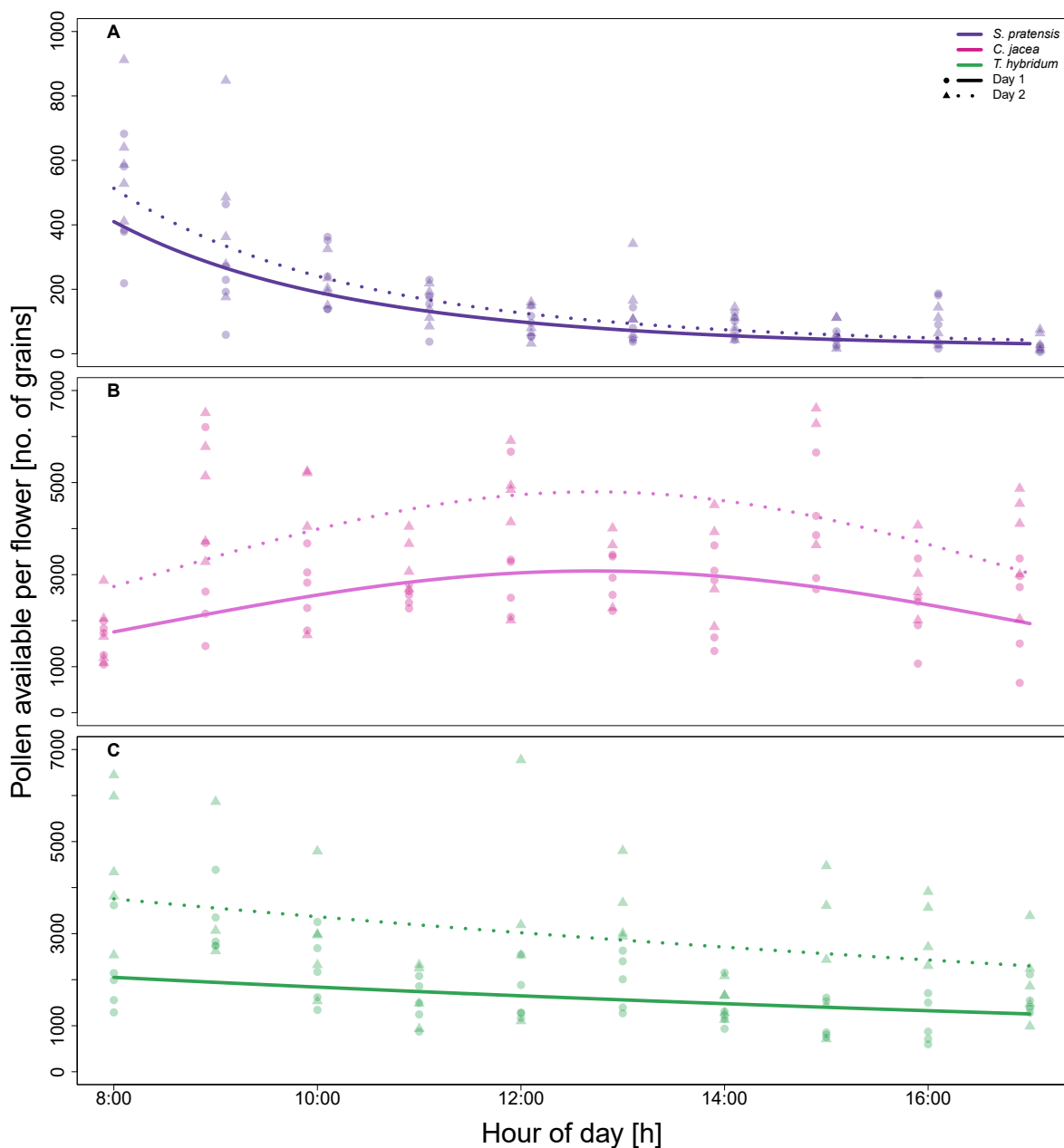
Pollinator	<i>T. hybridum</i>	<i>C. jacea</i>	<i>S. pratensis</i>
<i>Apis mellifera</i>	270	2,558	171
<i>Bombus spp.</i>	113	1,911	919
Coleoptera	0	17	9
Lepidoptera	7	315	288
Other bees	13	69	30
Other Diptera	18	123	356
Other Hymenoptera	2	10	8
Syrphidae	16	852	5,519

Appendix 1, Table. S2: Total pollen production per flower and number of flowers per inflorescence. Mean indicates arithmetic mean, SD stands for standard deviation.

Species	Total pollen production		Flowers per inflorescence	
	Mean	SD	Mean	SD
<i>T. hybridum</i>	5,162	349.96	50	8.13
<i>C. jacea</i>	6,008	785.37	43	14.12
<i>S. pratensis</i>	991	231.83	59	15.88



Appendix 1, Fig. S3: Absolute predicted pollinator densities. Lines represent predictions obtained from linear models; polygons represent confidence interval.



Appendix 1, Fig. S4: Pollen presentation schedule of studied species scaled by the estimated numbers of pollen grains. A) *Trifolium hybridum*; B) *Centaurea jacea*; C) *Succisa pratensis*. Lines represent predictions obtained from linear models (table 1); polygons represent confidence interval; points represent raw data. Solid lines and circles represent Day 1; dashed lines and triangles represent Day 2.

Appendix 1, Table. S3: Coefficients (Coef.) and Standard errors (SE) for linear models of amount of available pollen (log-transformed) depending on time of day and day of sampling.

Predictor	<i>T. hybridum</i>		<i>C. jacea</i>		<i>S. pratensis</i>	
	Coef.	SE	Coef.	SE	Coef.	SE
intercept	6.274	1.538	0.912	1.319	6.317	1.517
day	0.592	2.174	-0.432	1.866	1.894	2.145
time ²	-0.273	0.255	0.605	0.219	-0.470	0.252
day x time	0.007	0.010	-0.024	0.009	0.009	0.010
day x time ²	-0.008	0.361	0.113	0.309	-0.274	0.356
day × time ²	0.001	0.014	-0.003	0.012	0.011	0.014

Appendix 1, Table. S4: Coefficients (Coef.) and Standard errors (SE) for linear models of proportion of active flowers within an inflorescence (square-root transformed) depending on time of day.

Predictor	<i>T. hybridum</i>		<i>C. jacea</i>		<i>S. pratensis</i>	
	Coef.	SE.	Coef.	SE.	Coef.	SE.
intercept	1.738	0.445	-1.263	0.218	0.318	0.044
time	-0.454	0.112	0.244	0.036	-0.005	0.003
time ²	0.044	0.009	-0.009	0.001		
time ³	-0.001	0.000				

Appendix 1, Table. S5: Coefficients (Coef.) and Standard errors (SE) for linear models of pollinator density (square-root transformed) depending on the time of day.

Predictor	<i>T. hybridum</i>		<i>C. jacea</i>		<i>S. pratensis</i>	
	Coef.	SE	Coef.	SE	Coef.	SE
intercept	-0.225	0.056	-0.278	0.044	-0.843	0.274
time	0.042	0.009	0.113	0.007	0.212	0.067
time ²	-0.001	0	-0.004	0	-0.013	0.005
time ³					0.000	0.000

10.3 Supplementary material for Article 3: Strategies of pollen presentation mitigate competition for pollinators due to partial diurnal stratification of pollen transfer

Jakub Štenc^{1*}, Lukáš Janošík¹, Martin Freudenfeld¹, Eva Matoušková², Jiří Hadrava², Michael Mikát², Klára Daňková², Tereza Hadravová², Tadeáš Ryšan², Jasna Simonová², Klára Koupilová³, Alice Haveldová¹, Eliška Konečná¹, Jan Martínek⁴, Barbora Jelínková⁴, Stanislav Vosol sobě⁴, Karolína Dobešová⁴, Marie Smyčková^{1,5}, Jan Smyčka^{1,6}, Jitka Smyčková⁷, Lucie Studená⁷, Josef Nováček⁷, Štěpán Káně⁷, Kateřina Knotková⁸, Tomáš Figura^{4,9,10}, Jan Simon-Pražák^{2,11}, Kateřina Čermáková¹², Jiří Janoušek¹², Václav Bočan¹², Vojtěch Brož¹³, Anna Suchá¹⁴, Eva Horčíčková¹⁵, Tomáš Dvořák^{2,16}, Tomáš Jor^{2,16}, Doubravka Požárová¹⁷, Karel Plavec¹⁸, Zdeněk Janovský¹⁹

¹ Department of Botany, Faculty of Science, Charles University, Benátská 2, 128 41 Prague, Czech Republic

² Department of Zoology, Faculty of Science, Charles University, Viničná 7, 128 41 Prague, Czech Republic

³ Rovná 1333, Sulice, 25168, Czech Republic

⁴ Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 128 44 Prague, Czech Republic

⁵ Department of Forest Ecology, Faculty of Forestry and Wood Sciences, Czech University of Life Sciences Prague, Kamýcká 129, 165 00 Prague, Czech Republic

⁶ Center for Theoretical Study, Charles University and the Academy of Sciences of the Czech Republic, 110 00, Prague, Czech Republic

⁷ Faculty of Science, Charles University, Albertov 6, 128 00 Prague 2, Czech Republic

⁸ Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, Brno 611 37, Czech Republic

⁹ Department of Mycorrhizal Symbioses, Institute of Botany, Czech Academy of Sciences, Lesní 322, 25243 Průhonice, Czech Republic

¹⁰ Naturalis Biodiversity Center, Darwinweg 2, 2333 CR, Leiden, The Netherlands

¹¹ Museum of Eastern Bohemia in Hradec Králové, Eliščíno nábřeží 465, 500 03 Hradec Králové 3, Czech Republic

¹² Department of Cell Biology, Faculty of Science, Charles University, Viničná 7, 128 41 Prague, Czech Republic

¹³ Department of Ecology, Faculty of Science, Charles University, Viničná 7, 128 44 Prague 2, Czech Republic

¹⁴ Faculty of Education, Charles University, Magdalény Rettigové 4, Prague 1, 116 39, Czech Republic

¹⁵ Department of Environment, Faculty of Environment, Jan Evangelista Purkyně University in Ústí nad Labem, Pasteurova 3632/15, 400 96, Ústí nad Labem, Czech Republic

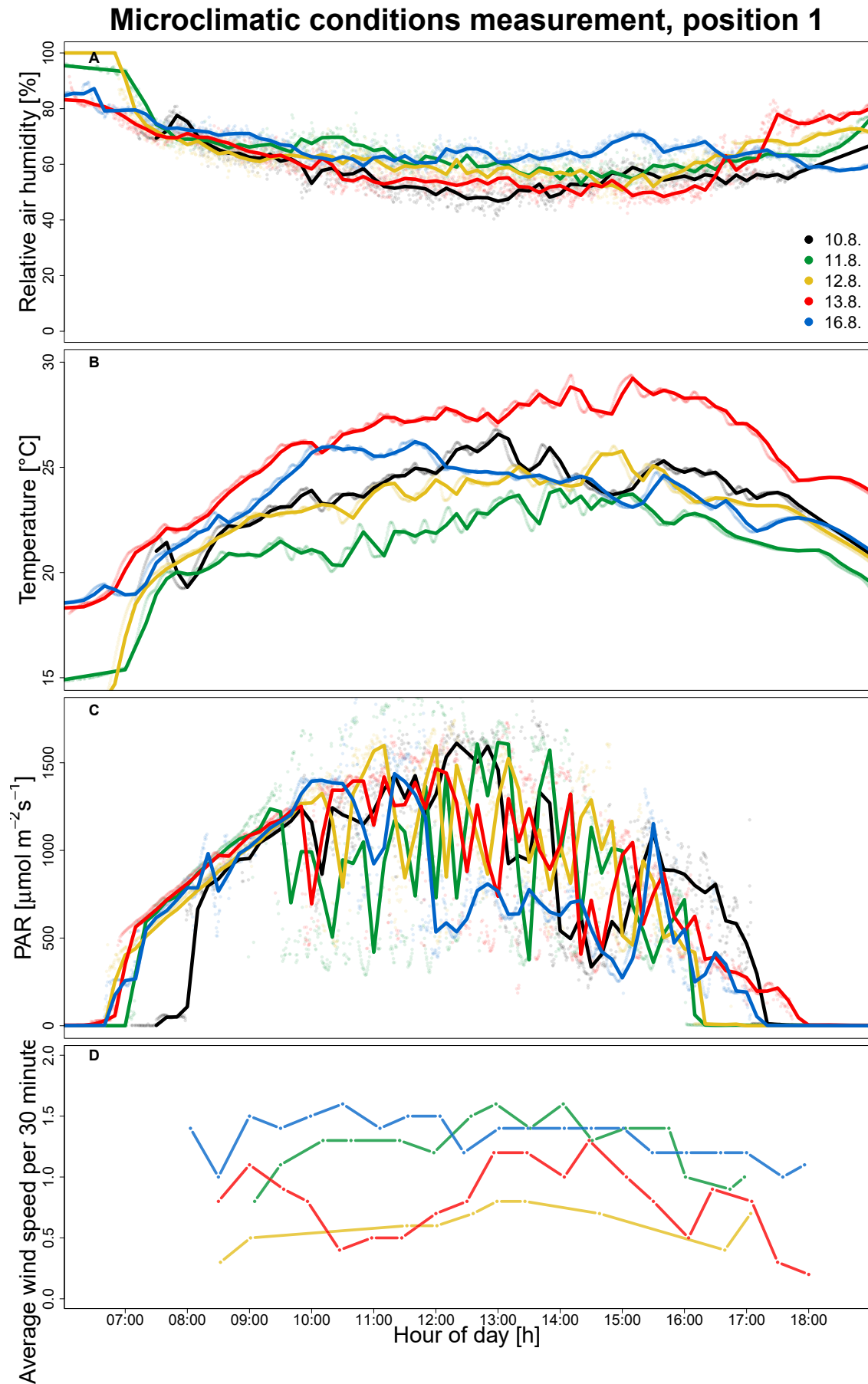
¹⁶ Department of Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences, Kamýcká 129, 165 00, Prague , Czech Republic

¹⁷ Lhota pod Džbánem 23, 27007 Mutějovice, Czech Republic

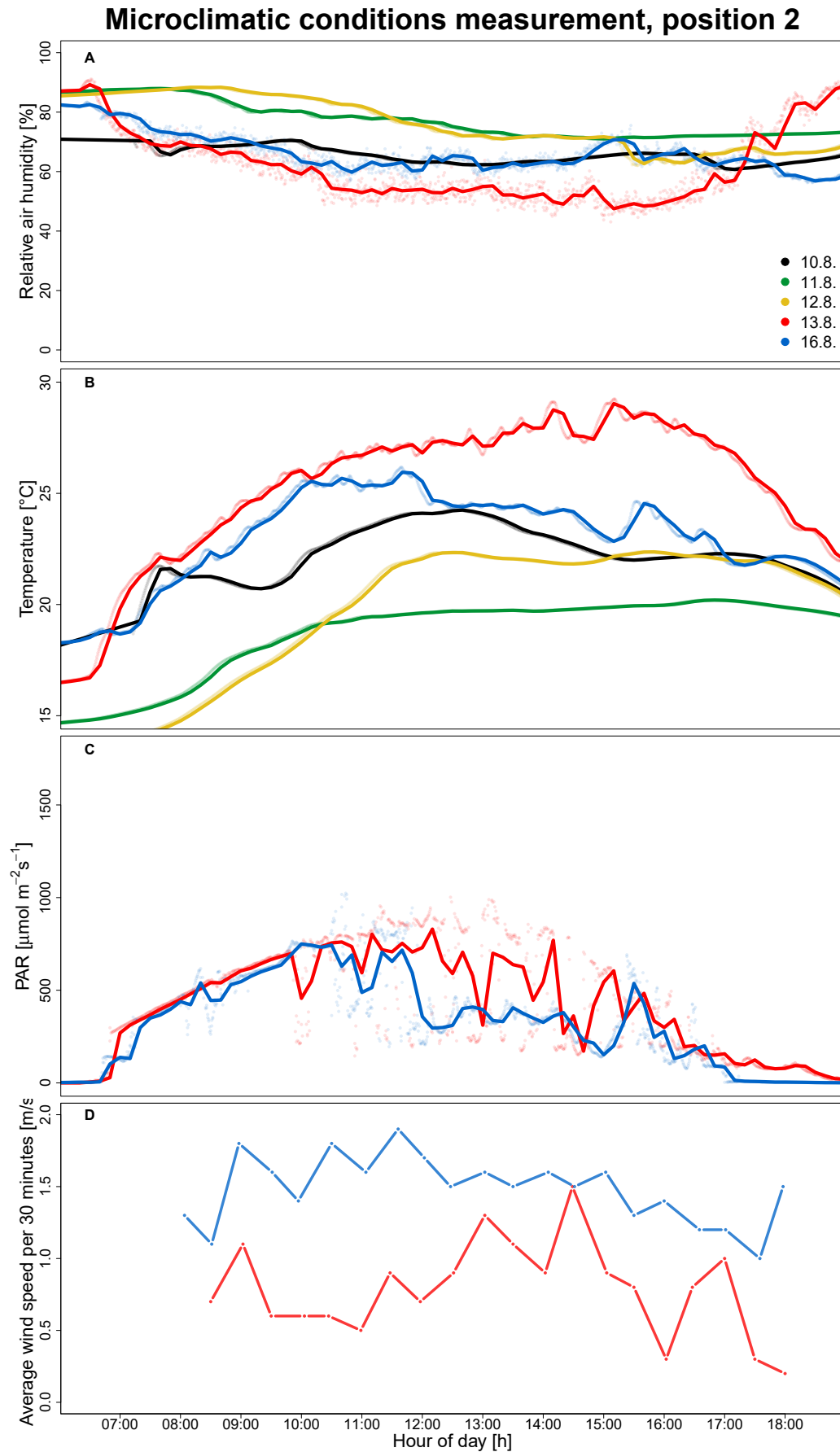
¹⁸ Gymnázium AD Fontes s.r.o, Jánská 14, 586 01, Jihlava, Czech Republic

¹⁹ Svatý Jan t. Krsovice 1, 285 04 Uhlířské Janovice, Czech Republic

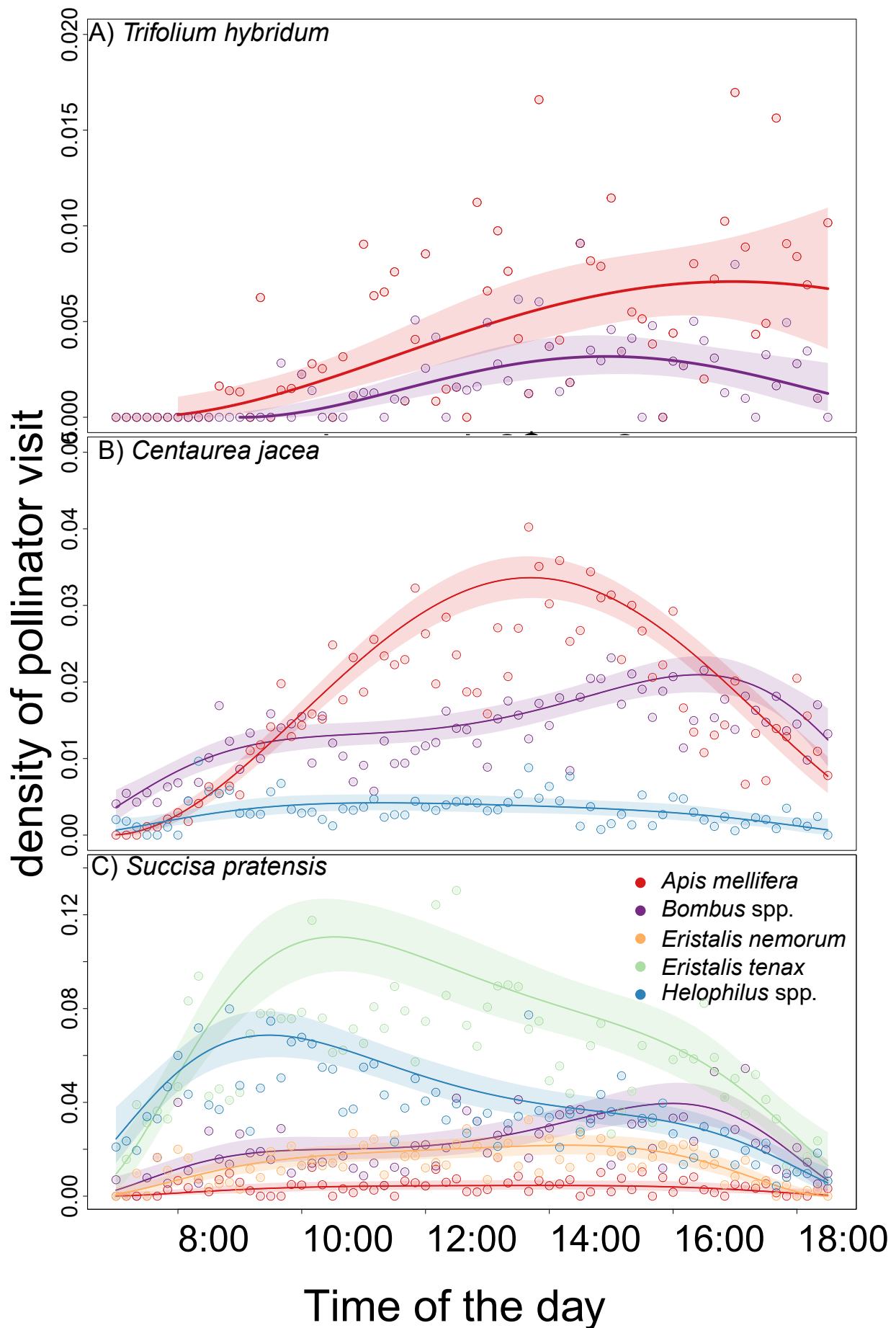
*Contact and corresponding author, e-mail address: jakubstenc@gmail.com.



Appendix 1, Fig. S1: Measured microclimatic variables on the 1 close to *Succisa pratensis* microsite. Details about measurement tools in Methods. Each line represents one day. In plots for A) Relative air humidity; B) Temperature and C) Photosynthetic active radiation show



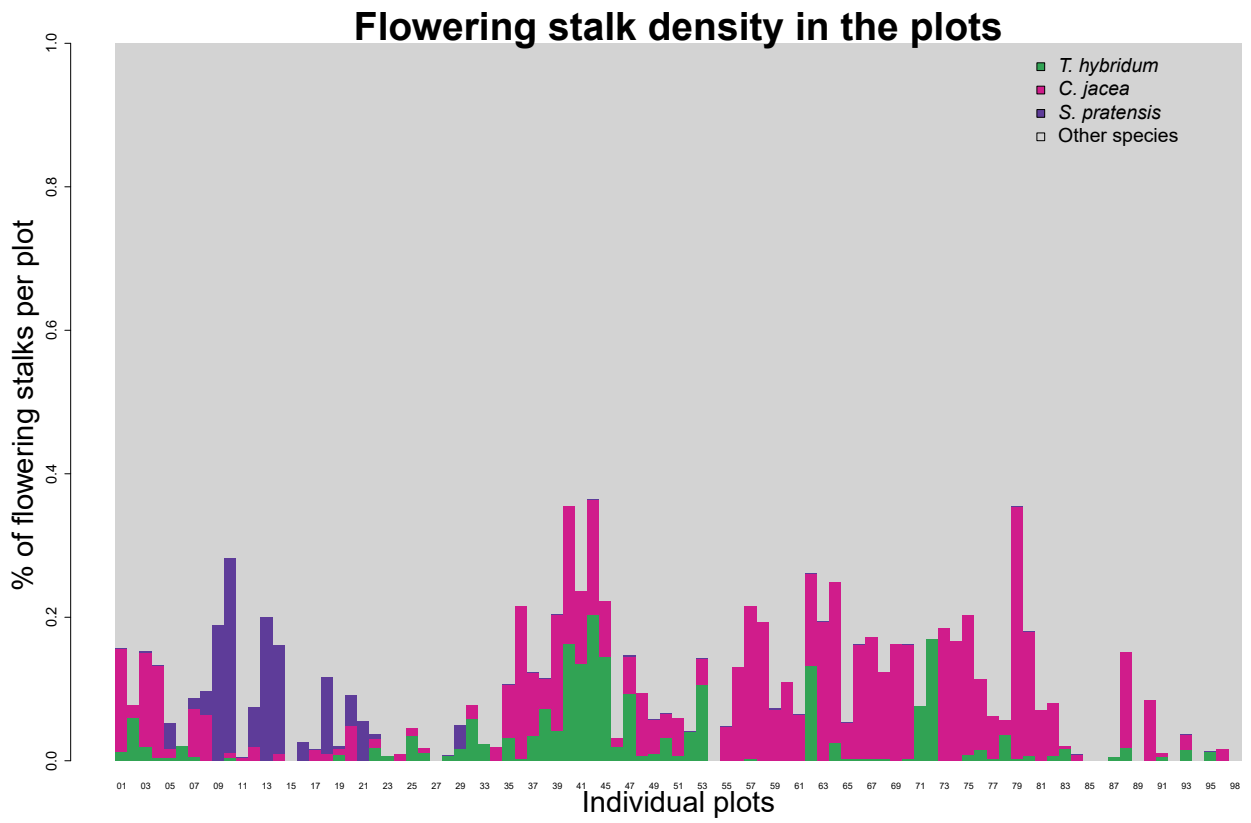
Appendix 1, Fig. S2: Measured microclimatic variables on the position 2 close to *Centaurea jacea* and *Trifolium hybridum* microsites. Details about measurement tools in Methods. Day 1 and 2 (14th and 15th August 2021, respectively) are days of pollen presentation sampling and Day 3 (16th August 2021) is the day of pollen presentation sampling. Inset plot



Appendix 1, Fig. S3: Pollinator density in time of the day. Every point represents averaged pollinator density for 10-minute time frame. Graphs A), B) and C) stands for *T. hybridum*, *C. jacea* and *S. pratensis*, respectively. Other groups of pollinators visiting plant species are not shown as they represent wide group sparsely interacting with the plant species.

Table S1: The overview of sampled individual pollinators per plant species.

Pollinator	<i>Trifolium hybridum</i>	<i>Centaurea jacea</i>	<i>Succisa pratensis</i>
<i>Apis mellifera</i>	80	85	59
<i>Bombus spp.</i>	0	45	0
<i>Eristalis nemorum</i>	0	0	77
<i>Eristalis tenax</i>	0	0	83
<i>Halictus spp.</i>	0	0	42



10.4 Supplementary material for Article 4: Appendix S1 Innate preferences of *Eristalis tenax* L. (Syrphidae) toward flower colour, size and symmetry

Running title: Innate preferences of *Eristalis tenax*

Eva Matoušková¹, Jakub Štenc², Zdeněk Janovský³

¹Department of Zoology, Faculty of Science, Charles University, Viničná 7, CZ-128 41 Prague, Czech Republic

²Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 41 Prague, Czech Republic

³Svatý Jan t. Krsovice 1, 285 04 Uhlířské Janovice, Czech Republic

*Corresponding author, e-mail address: jakubstenc@gmail.com.

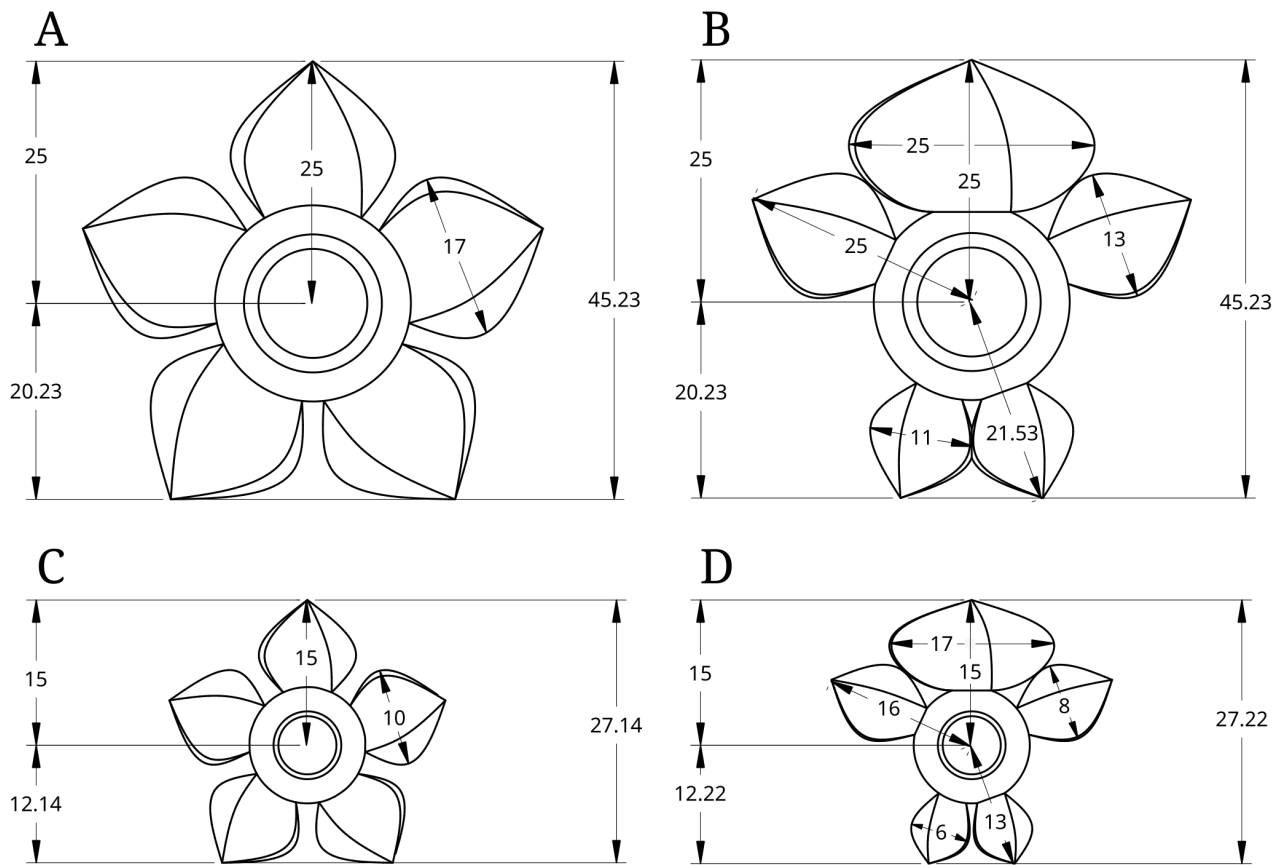


Fig. S1: Technical drawing of different forms of artificial flowers used in the experiment. Numbers indicates dimensions in millimeters. A) Large radial; B) large bilateral; C) small radial; D) small bilateral.

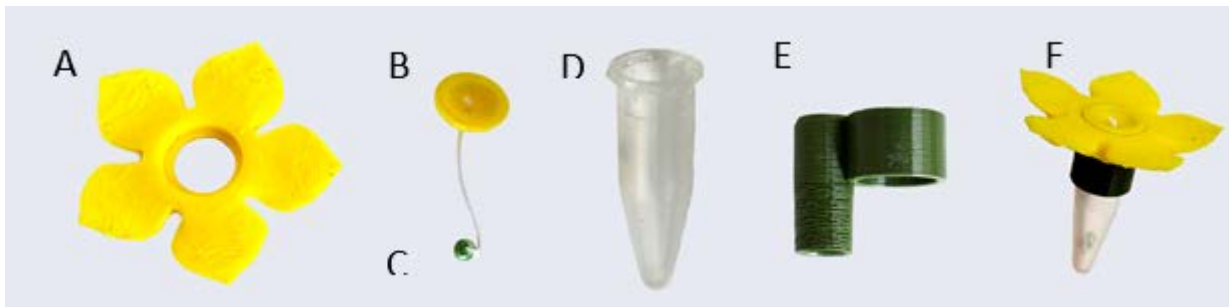


Fig. S2: Part of the artificial flowers. A) artificial corola; B) center of the flower with knot offering the sugar solution; C) wick allowing soaring of the sugar solution; D) Eppendorf tube used as sugar solution container; E) holder of the flower; F) the whole flower. Author: Alice Haveldová.

Sampling effort per experimental trials with the same combination of focal and anatagonist flower form. Numbers represent number of individuals recorded in total per combination of flower forms. Numbers in parenthesis represent number of experimental trials carried out.

	YLR	YLB	YSR	YSB	PLR	PLB	PSR	PSB
YLR	210 (6)							
YLB	334 (4)	210 (6)						
YSR	189 (4)	160 (6)	210 (6)					
YSB	270 (4)	311 (4)	³⁹⁰ (11)	210 (6)				
PLR	186 (6)	241 (6)	217 (4)	219 (8)	210 (6)			
PLB	334 (6)	²⁴⁴ (10)	197 (8)	168 (8)	178 (4)	210 (6)		
PSR	292 (4)	281 (8)	255 (7)	150 (6)	220 (6)	228 (8)	210 (6)	
PSB	247 (3)	133 (2)	220 (6)	168 (4)	239 (6)	154 (3)	239 (9)	210 (6)

10.5 Supplementary material for Article 5: Innate preferences of *Eristalis tenax* L. (Syrphidae) toward flower colour, size and symmetry

Running title: Innate preferences of *Eristalis tenax*

Eva Matoušková¹, Jakub Štenc², Zdeněk Janovský³

¹Department of Zoology, Faculty of Science, Charles University, Viničná 7, CZ-128 41 Prague, Czech Republic

²Department of Botany, Faculty of Science, Charles University, Benátská 2, CZ-128 41 Prague, Czech Republic

³Svatý Jan t. Krsovice 1, 285 04 Uhlířské Janovice, Czech Republic

*Corresponding author, e-mail address: jakubstenc@gmail.com.

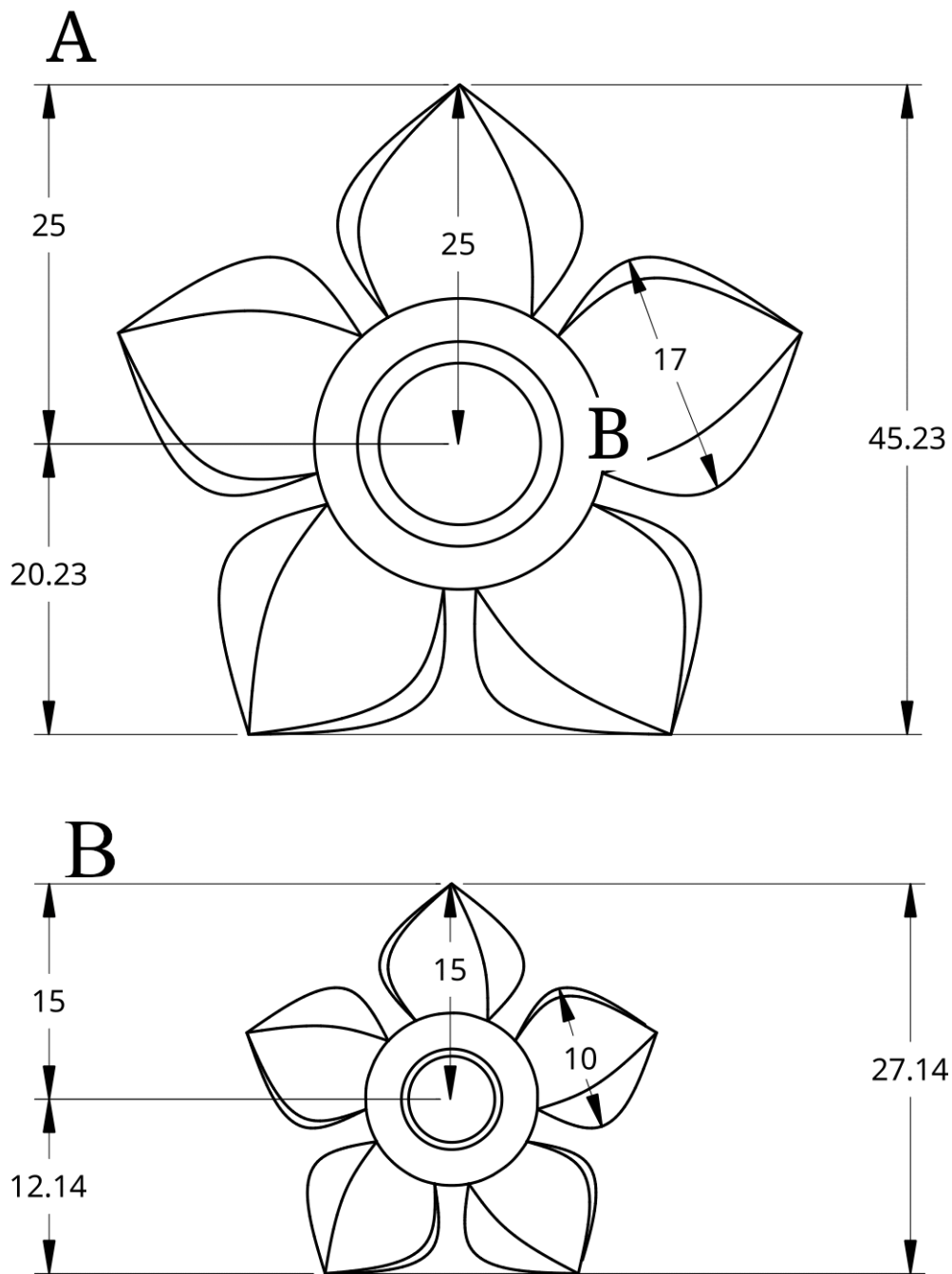


Fig. S1: Technical drawing of different forms of artificial flowers used in the experiment. Numbers indicates dimensions in millimeters. A) Large radial; B) small radial

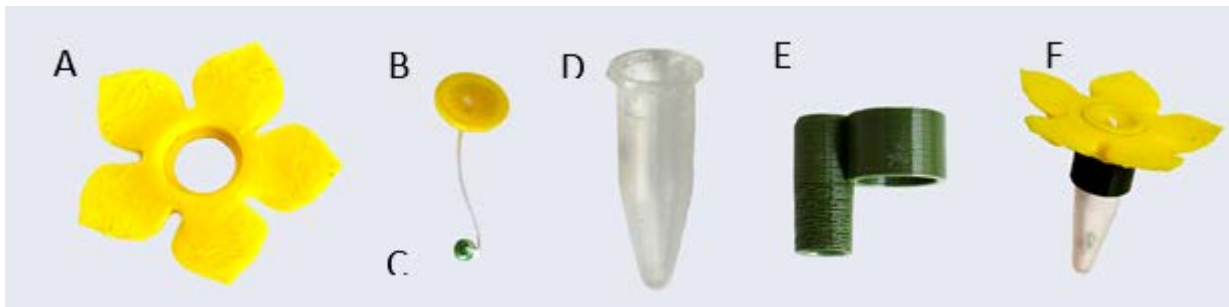


Fig. S2: Part of the artificial flowers. A) artificial corola; B) center of the flower with knot offering the sugar solution; C) wick allowing soaring of the sugar solution; D) Eppendorf tube used as sugar solution container; E) holder of the flower; F) the whole flower.