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Changes in pollinator visitation behaviour under different plant spatial aggregation

Změny chování opylovačů v rostlinných populací o různé míře shlukovitosti

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Diploma thesis

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Praha 2020

I declare that I have completed this master thesis by myself and that I have properly acknowledged and cited all used sources. The presented data was obtained in experiments carried out in collaboration with Klára Koupilová (she actively participated on all field experiments) under the supervision of Zdeněk Janovský. I declare that I played a major role in the preparation and execution of the experiments described in this thesis. I testify that neither this thesis nor any of its parts have been submitted to obtain any other academic degree. Part of the data presented in this thesis is part of manuscript that is currently submitted under the name "Pollinator-transmitted disease and plant spatial aggregation drive the pattern of pollen dispersal in plant populations"

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Data prezentovaná v práci pochází z experimentů na kterých se mnou spolupracovala Klára Koupilová. Prohlašuji, že jsem hrál podstatnou roli při přípravě a provedení experimentů. Tato práce ani její podstatná část nebyla předložena k získání dalšího akademického titulu. Část prezentovaných dat je součástí dosud nepublikovaného manuskriptu pod názvem "Pollinator-transmitted disease and plant spatial aggregation drive the pattern of pollen dispersal in plant populations".

Jakub Štenc
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Abstract

Plants often occur aggregated into clusters and this spatial pattern is supposed to affect pollinator behaviour and pollen dispersal. Such pollinator reaction may influence reproductive success of zoogamous plant species both in terms of number of available mates and their genetic diversity (nearby growing plant individuals are also often closely related, especially in clonal plants).

In the present thesis, I investigated the influence of plant spatial aggregation on pollinator behaviour and how this translates into pollen transfer. For that purpose, I carried three experiments. In the Experiment 1, I used potted plants placed into arrays and aggregated into four patches in order to track the pollen dispersal by means of a UV-dye pollen analogue. I manipulated distances between plants within clusters (dense × loose) and between clusters (near × far). I conducted this experiment for three plant species differing in their pollinator spectra. In the Experiment 2, I observed pollinator foraging sequences (sequences of visited plant individuals) under the same experimental design as for the first experiment, but I carried out this experiment for five plant species. In addition in one study species, *Dianthus carthusianorum*, I conducted the Experiment 3 to get better insight into pollination effectiveness of individual pollinator groups and estimate number of pollen grains carried on pollinator bodies.

While the Experiment 1 did not show any pattern in pollen dispersal except for an exponential decline from the source plant (possible due to low test power), the Experiment 2 indicate multiple important effects of spatial aggregation on pollinator foraging behaviour. Increasing between-cluster distance decreased mean relative flight distance and proportion of between-cluster flights undertaken by pollinators. Similarly, the probability of between-cluster flight also decreased in dense clusters. These effects differed in their magnitude for different pollinator functional groups (butterflies flew the furthest, while hoverflies, other diptera and solitary were least prone to fly further and between clusters). The Experiment 3 showed that solitary bees carried approximately a hundred times more pollen than butterflies and small hoverflies. However, this result cannot be taken as conclusive as the pollen from solitary bees originated from all over the insect body including the structures used for collecting to provide larvae with food.

I conclude that dominant Central European pollinator groups respond similarly to changes in plant spatial aggregation. However at the same time, pollinator functional groups greatly differ among themselves due to their other traits determinating their spatial activity. These between-functional group differences seem to be more important than the changes induced by varying plant spatial aggregation.

Key words: Pollination, plant spatial aggregation, pollen transfer, pollen carry-over, UV-dye

Abstrakt

Rostliny jsou v rámci populace často uspořádány do shluků. Toto prostorové uspořádání rostlin následně ovlivňuje chování opylovačů, kteří jsou zodpovědní za přenos pylu. To může hrát významnou roli při rozmnožování rostlin, jejichž pohlavní reprodukce přímo závisí na přenosu pylu. Nadto prostorové uspořádání rostlin ovlivňuje kvalitu přenášeného pylu z pohledu dostupnosti sexuálních partnerů a genetické diverzity (blízce rostoucí jedinci jsou si ve shluku příbuznější než jedinci mezi shluky, obzvlášť u klonálních rostlin).

Ve své práci se věnuji vlivu různé míry agregace a to jak na chování opylovačů, tak i na přenos pylu. Z toho důvodu jsem provedl tři experimenty: V pokuse 1, zaměřeném na přenos pylu jsem vytvořil arény z předpěstovaných rostlin, jejichž jedince jsem uspořádal do čtyř shluků a následně jsem manipuloval jak vzdálenost jedinci v rámci shluku (řídký × hustý) tak i mezi shluky (blízko × daleko). V experimentálních arénách jsem na jednu rostlinu aplikoval UV fluorescentní prášek sloužící jako analog pylu, přičemž tento pokus byl proveden se třemi druhy rostlin. V rámci arény jsem pak pozoroval jeho šíření ze zdrojové rostliny po jednom dni expozice. V pokusu 2, zaměřeném na chování opylovačů jsem ve stejně uspořádaných arénách pozoroval sekvence návštěv rostlin jednotlivými opylovači, přičemž tento pokus jsem provedl s pěti rostlinnými druhy. Následně jsem v doplňkovém pokusu 3 stanovil množství pylu neseného na těle opylovačů jednoho ze zkoumaných druhů, hvozdíku kartouzku (*Dianthus carthusianorum*).

Zatímco pokus 1 s UV analogem prokázal pouze slabý pokles přenášeného analogu (nejspíš kvůli malé síle testu), pokus 2 s chováním opylovačů zaznamenal více důležitých efektů prostorového uspořádání na chování opylovačů: Se zvýšením vzdálenosti mezi shluky vedlo ke snížení průměrné přeletové vzdálenosti mezi rostlinami a pravděpodobnosti přeletů mezi shluky. Podobně, pravděpodobnost přeletu mezi shluky se snížila v hustých shlucích. Tyto efekty se ve své velikosti významně lišili mezi funkčními skupinami opylovačů (motýli létali nejdále, zatímco pestřenky, mouchy a samotářské včely létaly na kratší vzdálensot). Dále jsem zaznamenal významné rozdíly mezi skupinami opylovačů. Největší množství pylu hvozdíku kartouzku na svém těle průkazně nosily samotářské včely, přičemž motýli a malé pestřenky přenášeli až stokrát méně pylových zrn. Pokus 3 ukázal, že samotářské včely přenášejí přibližně stokrát více pylových zrn než motýle a malé pestřenky. Nicméně, je nutné brát na zřetel, že tyto výsledky obsahují i pyl ze sběracích struktur včel, který většinou slouží jako potrava pro larvy.

Na základě těchto výsledků lze tvrdit, že dominantní skupiny opylovačů ve střední Evropě reagují podobně na prostorové uspořádání rostlin. Nicméně, zároveň funkční skupiny

opylovačů se velmi liší mezi sebou. Tyto rozdíly se zdají být důležitější než vliv prostorového uspořádání rostlin do shluků.

Klíčová slova: opylování, prostorové uspořádání rosltin do shluků, přenos pylu, kapacita přenosu opylovači

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1. Introduction

Pollen transfer by animal vectors is crucial for plant sexual reproduction: recent estimate states that about 80% species of vascular plants somehow rely on animal pollinators in their pollen transfer (Ollerton et al. 2011). Nonetheless, our knowledge about important processes and mechanisms underlying pollen transfer and gene-flow in plant populations is still insufficient. For instance, our insight into plant spatial aggregation on pollinators' behaviour and pollen transfer is still limited, while considerable influence on effective population size would be expected. Improving our understanding of the role of spatial aggregation in pollen transfer may lead to successful conservation of plant-pollinator diversity in time of increasing habitat fragmentation (Rathcke & Jules 1993) and its detrimental effect on pollinator communities (Nielsen et al. 2012).

Flowering plants in open habitats are typically patchily distributed (see Fig. 1. as an illustration) with clusters of flowering individuals separated by area without flowering conspecifics. Plant spatial patchiness could be caused by limited seed dispersal ability, clonal growth, disturbances or by spatial heterogeneity of sources and environmental conditions (Silvertown & Charlesworth 2001). Unsurprisingly, this pattern has considerable influence on pollinator behaviour (Cresswell 1997, 2000, Cresswell & Osborne 2004).

Insect pollinators usually fly from flower to flower during their foraging sequence and collect rewards. Due to the relative high cost of insect flight , pollinators tend to optimize their behaviour to maximize the energy intake gained from the reward and minimize the cost of flights. Given Marginal Value Theorem (Charnov 1976), forager decision to switch from cluster to cluster is based on the balance between their reward intake from the cluster and distance between clusters and foraging distance between sources in the cluster (Nonaka & Holme 2007). Thus, in array of patchily distributed conspecific plants, pollinators should visit more plants in cluster if A) clusters are farther apart or B) current cluster is densely clumped. Both should decrease the proportion of between cluster flights and decrease the mean flight distance (Cresswell 2000), due to pollinator preferences to visit densely spaced plant clusters or cluster (Kunin 1993).

Consequently, pollen transferred from individual plant within the array should decrease in the same manners as pollinator flights: with decrease of inter-plant distances within cluster and with increase of inter-cluster distances (Kwak et al. 1998) and thus decrease outcrossing rate (Duncan et al. 2004). The decline could be easily approximated by exponential model (Thomson 1986). This is caused by rapid decline of deposited pollen on stigma from pollinator body on the flowers visited during the foraging sequence (Rademaker et al. 1997, Parker et al. 2016, Minnaar & Anderson 2019), but pollen transfer on long distance is also possible (Schulke & Waser 2001) and it may extent the dispersal distance of the pollen grains.

Given this prediction, the pollen dispersal should decrease rapidly in more aggregated and isolated clusters in the the plant population. In that cases, majority of pollen deposited on plant stigma should theoretically originated from the very same cluster of co-flowering individuals. As consequence, in populations where are plants from the same cluster are closely related, it could resulted in higher risk of inbreeding depression or pollen limitation in self-incompatible plant species.

Pollinators considerably differ among species and higher taxa in their foraging behaviour: Social hymenopterans as bumblebees and honeybees are considered as effective foragers acting according to Optimal Foraging Theory (Pyke 1978). The optimization of their foraging decision could be showed on ability to decide to which plant species they visit (Gegear & Thomson 2004), when to switch from one reward source to another one (Hodges 1985), when to shorter their flight distance during foraging sequence (Hartling & Plowright 1979) and to avoid re-visitation of previously probed flowers (Hartling & Plowright 1979). Furthermore, they showed other optimization mechanisms as is trapline foraging (repeated visits of the same site) and systematic searching in the array during the foraging bout. While trapline foraging helps to find the same foraging route and increase pollinator efficiency (Ohashi & Thomson 2009), systematic searching increase foraging efficiency due to decrease re-visitation of the same plant in the array. This is caused by bees' ability to remember their direction of arrival to plant and tendency to continue in the same direction (Heinrich 1979, Waddington 1980, Pyke & Cartar 1992). The directionality increase in arrays, where reward level is lower (Heinrich 1979, Waddington 1980) and decrease when the probability of re-visiationt the previously visited flower is low (Zimmerman 1982). Directionality was reported also for butterflies (Levin et al. 1971), but in my knowledge, there is no evidence for directionality in dipterans,

possibly due to lack of relevant publications. Moreover, in comparison to hymenopterans we lack systematic investigation of foraging efficiency in other pollinator groups. Our knowledge is often restricted on length of flights, which indicate that butterflies fly in average on further distances (Schmitt 1983, Herrera 1987) and small dipterans togehter with small solitary bees are expected to fly on shorter distances (Herrera 1987).

Flight behaviour and visitation pattern was relatively well studied during past decades and many studies proved the validity of basic principles in pollination biology. Nonetheless, to investigate patterns in pollen transfer, these should be considered together with a direct measurement of pollinator contribution to pollen dispersal (King et al. 2013). For instance, the counting of pollen grains carried on pollinator body (pollen carry-over) and counting of deposited pollen grains on flower stigma (pollen deposition) could be used. However the possible high pollen loss from hymenopterans and dipterans bodies, due to pollen eating, body grooming and by feeding their larvae (Holloway 1976b, Gilbert 1981a, Thomson 1986, Haslett 1989), may caused overestimation of pollinator contribution to pollen transfer based on counting pollen grains on pollinator body. In addition, ratio between pollen load on pollinator body and on stigma also strongly depends on system-specific situation. For instance, specialistic bees could be considered as effective pollen vectors, but they were showed more likely as thieves of Claytonia virginica pollen, due their preferential visits of flowers in male phase, which reduce pollen deposition on stigma (Parker et al. 2016). Morphological fit of pollinator body and flower structure also plays an important role (Solís-Montero & Vallejo-Marín 2017). These examples are surely not the only one and it highlights needs of precisely conducted auto-ecological experiments and observations before the broad generalizations could be done.

Nevertheless, both type of estimating pollen transfer by pollinators shows strong correlation (Howlett et al. 2011) and they could be used to estimate pollinator contribution to pollen transfer. As a result of studies comparing different pollinators, it seems that butterflies, hairless dipterans and small hairless bees are more likely to carry and deposited fewer amount of pollen grains than hairy hymenopterans (Bloch et al. 2006, Stanghellini et al. 2015, Wagner et al. 2016).

As a consequence of above mentioned factors, pattern of pollen transfer within an array of conspecific, patchily distributed plants should respond to plant spatial distribution, pollinator behaviour and pollinator pollen carry-over. Therefore, in my thesis I focused on the effect of plant spatial aggregation on pollinator behaviour and pollen dispersion by major Central European pollinator groups and how these may translate into the pollen flow between plants. I conducted three experiments in order to answer my study questions.

2. Questions

The main aim of my thesis is to investigate the effect of changes in plant spatial aggregation on pollination. For further investigation, my thesis consists from three separate parts, each focused on separated questions and supported by experiment designed to test the influence of spatial aggregation on pollen transfer, pollinator foraging behaviour and how pollinator groups differ in the amount of transferred pollen. Specifically, parts should be answering on the following questions:

1. Does the pattern of pollen transfer change under different plant spatial aggregation?

The Experiment 1 aimed to answer proposed question by using the dispersal of UV-dye powder as a pollen proxy. Three plant species were used on two experimental sites where native populations of studied plant species and their pollinators were present. The plant spatial aggregation was manipulated by using potted plants.

UV-dye have been used in previous studies with relevant results (Campbell & Waser 1989, Rademaker et al. 1997, van Rossum 2010, van Rossum et al. 2011a). Benefits of this method are relative simplicity and low costs. Moreover, various colours of UV-dye can be used simultaneously and the method is applicable to various plant species without limitation by flower morphology. Although a tight correlation of pollen and UV-dye transfer has been observed, the UV-dye particles often overestimate real pollen transfer or gene flow (Adler & Irwin 2006, van Rossum et al. 2011b) and this disadvantage has to be considered. Nonetheless, I considered UV-dye to be the most suitable method to answer my study question due to its availability and simplicity to use on multiple plant species under the same experimental design.

2. Does the pollinator behaviour change under different plant spatial aggregation?
    2.1. Does the plant spatial aggregation affect pollinator mean flight distances?
    2.2. Does the spatial aggregation affect proportions of between-cluster flights?
    2.3. Do the main pollinator groups differ in recorded aspects of their foraging behaviour?

To answer the proposed questions Experiment 2 was designed. I recorded pollinator foraging sequences (i.e. coordinates of visited plants during a pollinator's visitation sequence) in the experimental arrays of five studied plant species on two study sites with native pollinators. The analyses focused on pollinator mean flight distances and proportion of between cluster flights during the foraging bout in the experimental array.

3. Do pollinators differ in their pollen carry-over capacities?
    3.1. How important is their change of foraging behaviour under different spatial treatments for plant's pollen flow?

The Experiment 3 focused on differences in pollinator pollen carry-over capacities and compared main pollinator groups visiting one of the studied plant species, *Dianthus carthusianorum*. The pollen was sampled from pollinators caught on flowers of the studied species by dabbing them with fuchsine jelly and subsequent preparation of microscope slides for counting the pollen grains.

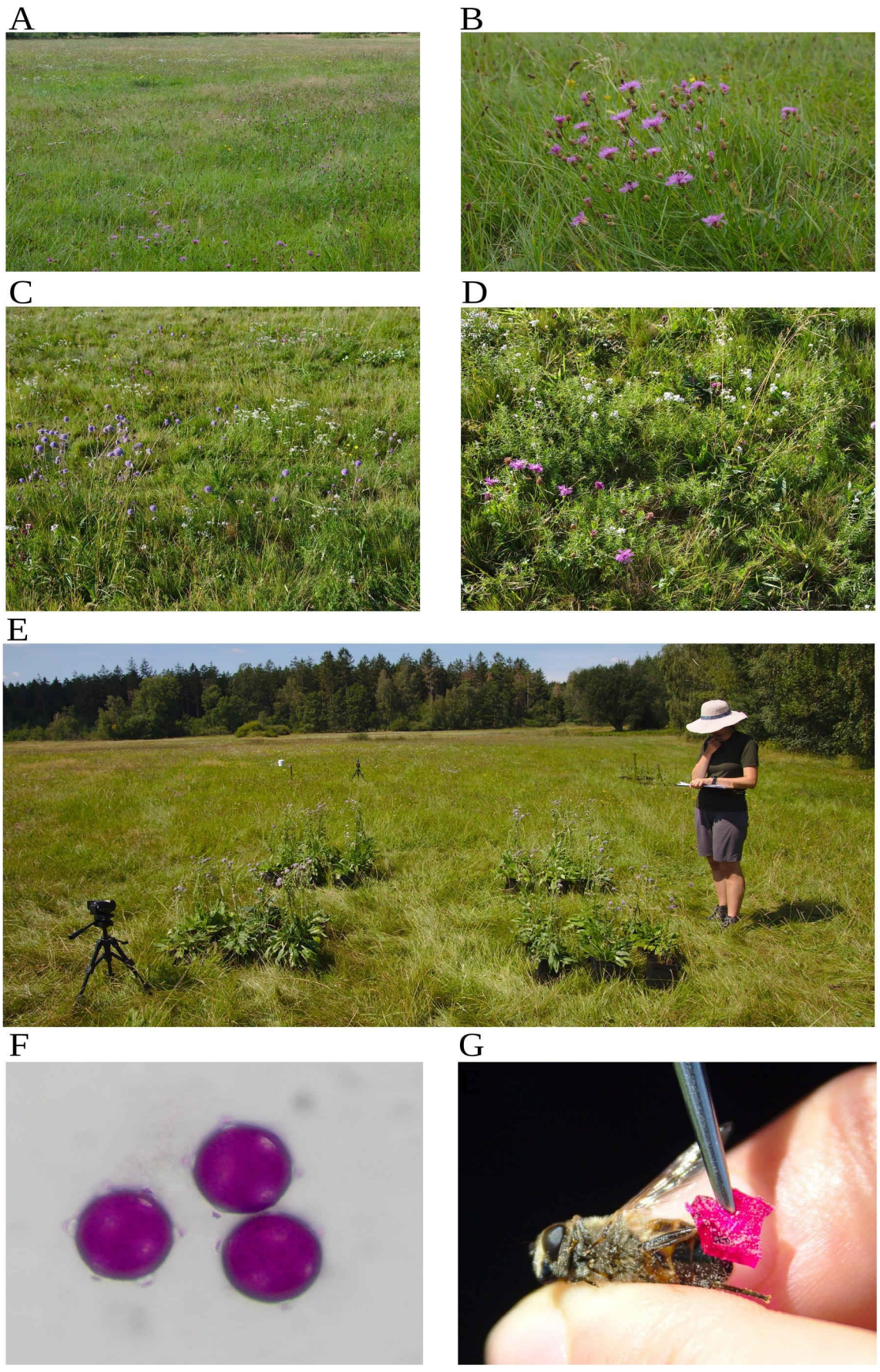

Fig. 1: A-D: plants in the meadow aggregated into clusters, illustration; E: an experimental array on Handrkov in dense-near treatment; F: D. carthusianorum *pollen grains; G: swabbing of pollen from pollinator body using fuchsine jelly.*

3. Methods

During summer of 2018 and 2019, I set up three experiments to answer the above mentioned questions. The Experiment 1 was conducted in order to observe pollinator behavioural response to changing plant spatial aggregation. The Experiment 2 was established to investigate patterns of pollen transfer and the experiment 3 was established to compare differences in pollinators' carry-over capacity.

To investigate the influence of spatial aggregation both on pollinator behaviour and pollen transfer, I established experimental arrays of potted plants placed in native populations of the study species. To simulate possible scenarios of plant spatial distribution in nature, I arranged plants in the array into spatially distinct clusters (see Fig. 3). I set up four treatments, which differed in within-cluster and among-cluster distances. In order to obtain data about main Central European pollinator groups, I used the same experimental design for 5 plant species which differ in their flower visitor spectra. Experimental arrays were used for experiment 1 and experiment 2:

Experiment 1: UV-dye tracking

In arrays with *Dianthus carthusianorum, Hypericum maculatum* and *Achillea ptarmica*, I used visually detectable UV-dye as pollen analogue to track pollen transfer. I applied UV-dye into flowers of one plant in the experimental array and counted transferred particles on flowers of other plants in respect to their spatial relation to the treated plant. Further description of the method follows in section 3.6. All experiments were carried during sunny days with no or light wind.

Experiment 2: Observation of pollinator behaviour

I observed pollinator behaviour in the experimental array during foraging flights from flower to flower. I recorded coordinates of plants whose flowers were visited by individual

pollinators. These observations I did for all 4 treatments and all 5 plant species used in the experiment. Further description of the method follows in section 3.5.

Experiment 3: Pollen carry-over capacity

In addition, I investigated pollen carry-over by collecting pollen samples from *D. carthusianorum* pollinators' body in order to point out possible difference between pollinator groups. I collected pollen samples from pollinators caught on flowers in native population of *D. carthusianorum* after their flower visit. Further description of the method follows in section 3.7.

3.1. Experimental array

Each experimental array consisted of 36 potted plant individuals of one species. The array was divided into 4 clusters with 9 plants (see Fig. 3). I excluded other attractive flowers both from the array and a 3 m wide buffer zone to avoid their influence on pollinator behaviour. Non-flowering or anemogamous plants were not removed in order to preserve site conditions as much as possible.

I manipulated within cluster distances (dense and loose) and among clusters distances (near and far) of each array. This resulted in total of 4 spatial treatments: dense near, dense far, loose near, loose far (see Table 1 and Fig. 2). Dense near treatment represents scenario of plants closely aggregated within the clusters and also clusters are close to each other. On the contrary, loose far represent scenario whereas clusters seem to be unconnected and individual plants within clusters are separated by further distance. All four treatments were replicated two times.

I arranged each experimental array in the morning hours before the start of pollinator activity (ca 8:00). I standardized height and number of flowers (or inflorescensces in case of *Serratula tinctoria*, *Succisa pratensis* and *A. ptarmica*) of the plants as much as possible in order to obtain plants with similar height and number of active flowers in the array. The resulting plant heights and flower/inflorescence numbers were recorded.

Table 1: Summary of spatial treatments used in the experiment.

		Among cluster distance	
		Near (190 cm)	Far (290 cm)
Within cluster distance	Dense (29 cm)	Dense near	Dense far
	Loose (44 cm)	Loose near	Loose far

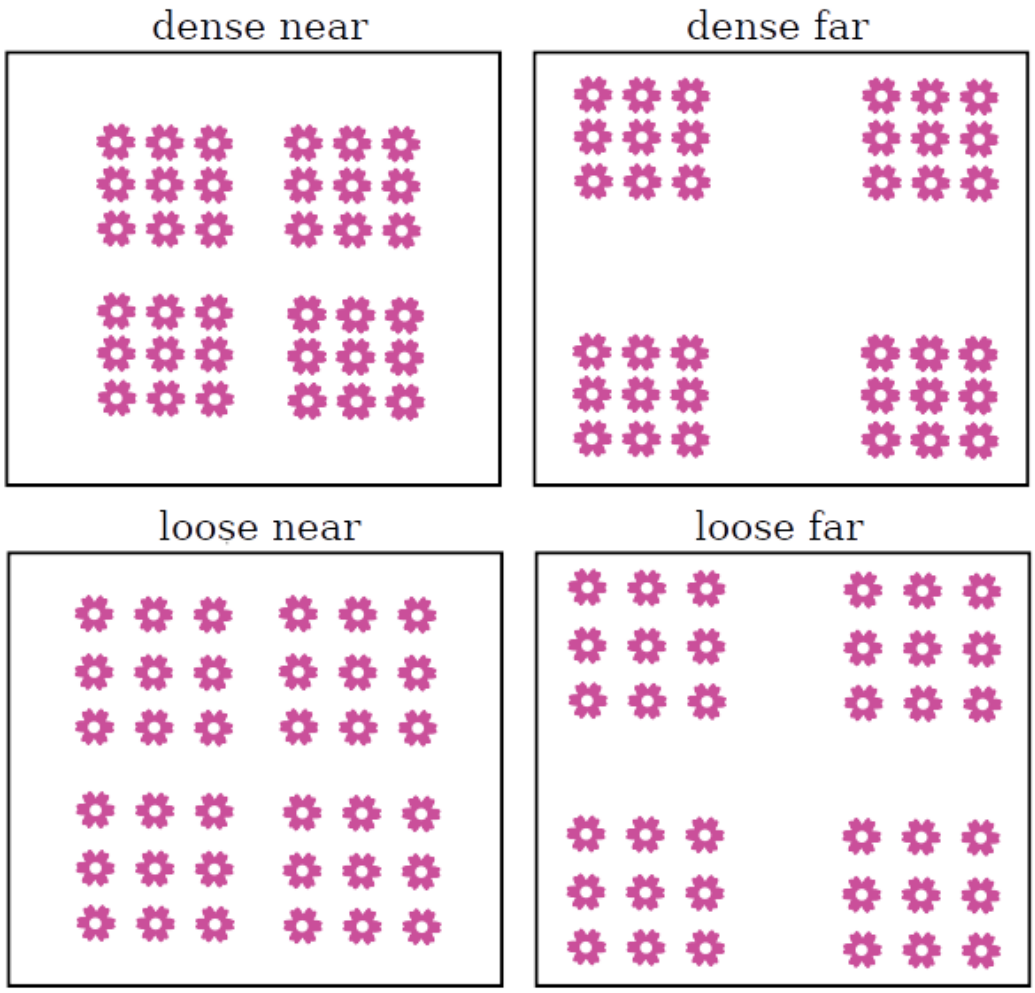

Fig. 2: Outline of spatial treatments used in the experiment. For distances see Table 1.

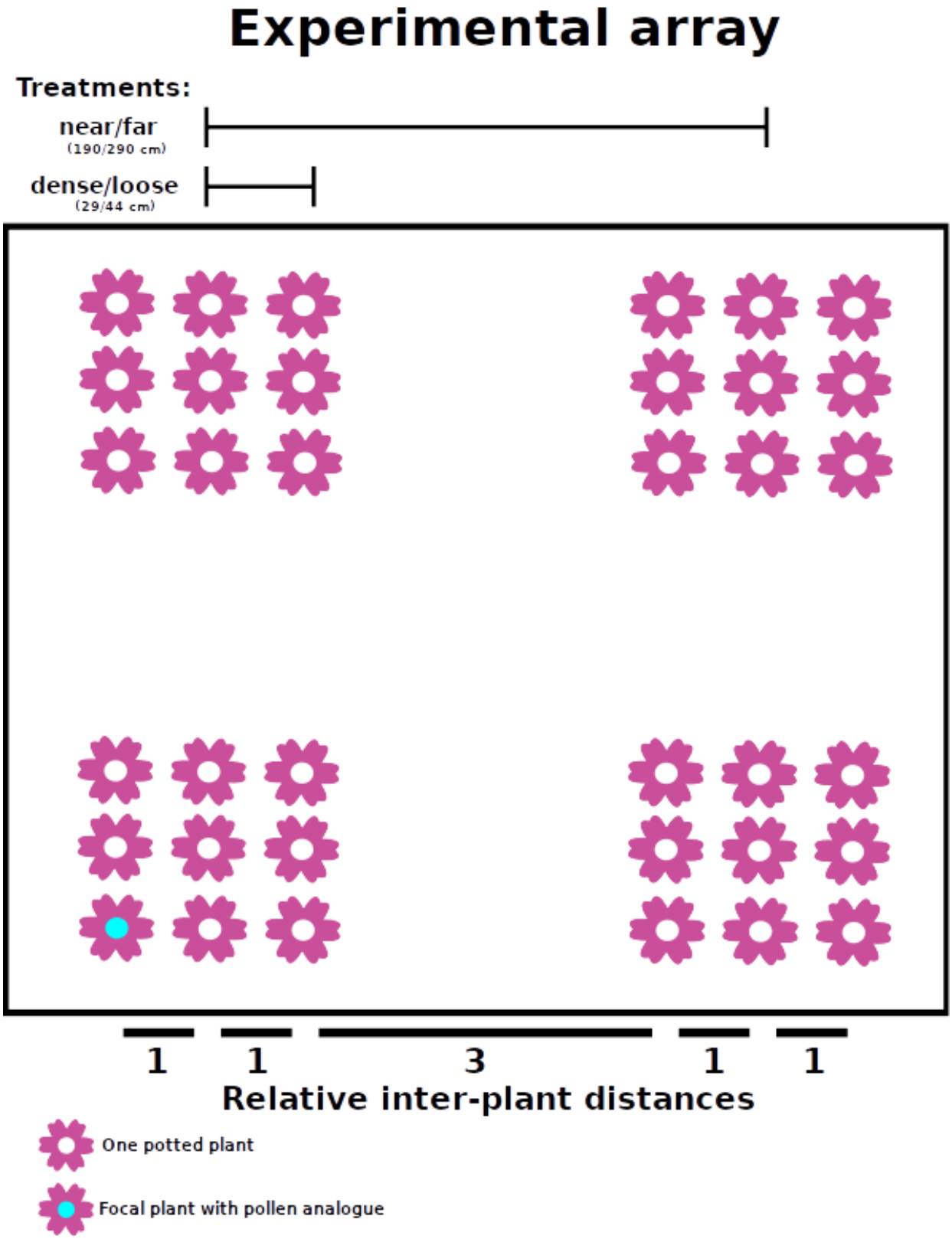

Fig. 3: Outline of an experimental array with 36 potted plants divided into four clusters of 9 plants. A flower represents a potted plant. The cyan dot in a flower represents the plant with applied UV-dye used to track pollen transfer.

3.2. Study species

Plants were cultivated from seeds and grown in common garden (Brožkova genetická zahrada, Prague) since 2017. Seeds of *H. maculatum* originated from Planta naturalis provider (Sobotka, Central Bohemia; www.plantanaturalis.com), seeds of *D. carthusianorum*, *S. tinctoria* and *S. pratensi*s came from were collected in natural population on study locality site. Adult plants of *A. ptarmica* were transferred to pots from an indigenous population at the study locality site. Seeds of planted species were sown in sand and after germination seedlings were transferred to flower pots (Ø 19 cm) with substrate of ¼ soil and ¾ sand and they were watered twice a day. I used granulated moluscicide (NEUDORFF Ferramol, active constituent $FePO_4$) to reduce herbivory during cultivation.

Short characteristic of studied plant species used in experiments follows:

3.2.1. *Dianthus carthusianorum L. (Caryophyllaceae)*

D. carthusianorum, known as Carthusian Pink is a perennial herb from Caryophyllaceae family. Occurring in European dry grasslands. Flowers are pink, hermaphrodite and protandrous, with pollen and nectar both relatively easily accessible to flower visitors and they are grouped in inflorescences of 2-10 flowers. *D. carthusianorum* flowers are mainly visited by diurnal butterflies (40.4 %; +- SE = 3.3 % ; n = 421), small hoverflies (33.7 %; +- SE = 3.5 %; n = 267), solitary bees (17.2 %; +- SE = 2.3 % ; n = 148) and they are irregularly visited by bombylids, bumblebees, solitary bees and beetles (Koupilová et al., unpublished data, manuscript under revision, data collected by using video camera recorded focal plant in experimental array with UV-dye).

3.2.2. *Achillea ptarmica L. (Asteraceae)*

Achillea ptarmica, the sneezewort**,** is self-incompatible, clonal perennial herb from *Asteraceae* family, occurring in wet grasslands of West and Central Europe. It produces compact

composite inflorescences of 4-12 white-coloured capitula. The whole inflorescence looks like a single flower from pollinator's point of view and they usually pass from one capitulum to another (personal observation). Inflorescences, producing both nectar (Käpylä 1978) and pollen, are mainly visited by small hoverflies (Syrphidae), house flies (Muscidae), tachinids (Tachinidae) and meat flies (Sarcophagidae).

3.2.3. *Hypericum maculatum* Crantz (*Hypericaceae*)

Hypericum maculatum is a clonal perennial herb from Hypericaceae family native to European grasslands (except of Southern Europe). *H. maculatum* produces flowers in a long period with about 4-8 yellow flowers open at a time producing pollen, but without nectaries (Stevens 2007). Flowers are visited mainly by European honeybee (*Apis melifera*) and hoverflies (Syrphidae).

3.2.4. *Serratula tinctoria* L. (*Asteraceae*)

S. tinctoria, the dyer's plumeless saw-wort, is a clonal perennial herb from Asteraceae family. It is native to Czech Republic and large parts of Europe, growing in transiently wet grasslands and oak forests.Flowering in mid-summer with red-violet inflorescence and attracts mainly honeybee (*Apis melifera*), bumblebees (*Bombus* spp.) and large hoveflies (mainly *Eristalis* spp.).

3.2.5. *Succisa pratensis* Moench (*Caprifoliaceae*)

S. pratensis , also known as devil's-bit scabious, is a clonal perennial herb from Caprifoliaceae family. It grows in transiently wet grasslands of Europe and Western part of Siberia. Inflorescences are blue, ball-like and occur in August and September. Flowers produce both nectar and pollen, and they are attractive mainly for large hoveflies (mainly *Eristalis* spp.), honeybee and bumblebees.

3.3. Study sites

For experiments I chose two sites according to the occurrence of native populations of experimental plant species (see Table 2). Both sites are located in the Central Bohemia region in agricultural landscape. The sites are about 70 kilometers apart.

The study site called Kamýk is located near the village Kamýk nad Vltavou (N 49°38'10.9", E 14°13'35.7") in a semi-natural dry grassland on rocky outcrops. It hosts a native population of *D. carthusianorum*. The field experiments took place from 3rd to 9th July in 2018 and 8th to 17th July in 2019.

Study site called Handrkov is located near the village Vernýřov (N 49.84°66'32.2", E 15.14°89'44.2") in a meadow mown twice a year (ca end of May, beginning of September). It hosts native populations of *A. ptarmica, H. maculatum, S. tinctoria and S. pratensis* and other flowering plant species. This meadow is used as study site in long term experiment by Zdeněk Janovský's group thus local pollinator spectrum is well-known. The field experiments took place from 8rd to 17th August in 2018 and 9th to 19th August in 2019.

Table 2: Summary of plant species, study sites and conducted experiments.

Plant species	Study site	UV-dye observation	Pollinator observation	Pollen carry-over sampling
Dianthus carthusianorum	Kamýk VII/2018	x	x	
	Kamýk VII/2019		x	x
Achillea ptarmica	Handrkov VIII/2018	x	x	
Hypericum maculatum	Handrkov VIII/2018	x	x	
Serratula tinctoria	Handrkov VIII/2019		x	
Succisa pratensis	Handrkov VIII/2019		x	

3.4. Experiment 1: UV-dye transfer

I used the fluorescent powdered dye (Radiant Colour NV, Houthalen, Belgium), which emits light in visible colour spectrum after UV light illumination, as pollen analogue (hereafter UV-dye) for tracking pattern of pollen transfer. I applied the UV-dye thoroughly to each flower of a randomly chosen plant in the corner position in each array (see Fig. 3) in the morning before pollinator observation started. After first experiments with *D. carthusianorum*, the dose of applied UV-dye was standardized to 1 mg. I used different colours of the UV-dye to distinguish possible between-array transfer.

The UV-dye was exposed on flowers from early morning (ca 8:00) to sunset (ca 21:00). , I counted the amount of UV-dye particles carried to flowers with UV lamp Brinyte 502B (Brinyte Technology Co., Ltdm Shenzhen, China) after sunset. I recorded the number of UV-dye particles on a semi-quantitive scale: 1, 2, 3, 4, 5-9, 10-14, 15-24 and 25+ particles visible. After recording, I removed the flowers with detected UV-dye and reused plants in another run of experiment with different pollen analogue colour to avoid contamination.

3.5. Experiment 2: Pollinator foraging behaviour

I observed pollinator flight behaviour as sequences of all visited flowers in experimental array for the time of the pollinator presence. I recorded 1) time of arrival into the array; 2) the coordinates of visited plants; 3) the number of visited flowers per plant; 4) time of departure from the array and 5) pollinator identity. The coordinates of visited plants were recorded sequentially.

All records were taken during the time of main pollinator activity (9:00 to 17:00). Observation periods lasted 15 minutes and were replicated at least ten times per array. The observation periods were distributed evenly during the day considering the pollinator activity, i.e. more observations were done in the peak of pollinator visitation activity.

I was able to observe and record only a single pollinator at a time. Thus, my observations cannot be used to describe plant visitation frequency or pollinator density. Furthermore, I did not observe nocturnal visitors due to logistic reasons and problematic observation.

I consulted pollinator identification with Jiří Hadrava and Michael Mikát from Dept. of Zoology, CUNI. Pollinators could be mostly identified to species level, but animals from subsection Calyptratae (Diptera) and superfamily Apoidea (Hymenoptera) except honeybees and bumblebees were distinguished only to family or genera level.

3.6. Experiment 3: *D. carthusianorum* pollen carry-over

I measured the amount of pollen grains carried on pollinator bodies. Every examined pollinator was caught by a net on the flower of *D. carthusianorum*. It was killed immediately and determined to species or functional group. A half of its body was dabbed as soon as possible by a ca 5x5x3 mm fuchsine-jelly block (I followed the protocol of Dafni, Kevan, & Husband (2005). Afterwards, the fuchsine-jelly block was placed on a microscope slide, melted and covered by a cover slip on the same day. The persistence of the sample was achieved by sealing the cover slip with transparent nail polish. To avoid contamination, I cleaned all tools used as often as possible. After the dabbing procedure, all pollinator specimens were stored in ethanol (all hymenopterans and dipterans) or dried or stored in paper sack (butterflies) for further determination.

I counted pollen grains in the sample under the light microscope at 100× magnification. I used a square grid of 1×1 mm squares mounted on the cover slip in order to facilitate the counting. I determined the pollen grains to species, whenever it was possible and I counted the abundance of each species in the sample. For further description of pollen counting see the protocol in Supplement 2. I used pollen collected from anthers of plants on the study site by the same method for reference. Pollen grains, which I could not determine, were scored as “other”.

3.7. Data analysis

Linear Mixed Effect model (hereafter LME) and Generalized Linear Mixed Effect Model (GLMM) were used for data analyses. Significance in LME was assessed using the F-tests with the Satterthwaite approximation of the denominator degrees of freedom (Satterthwaite, 1946). Significance in GLMMs was tested by the likelihood-ratio tests comparing subsetted models differing always in a single fixed effect term in model formula (see Bolker et al. (2009)) for more details).

All computations and data analyses were conducted in R statistical environment, v. 3.5.2 (R Core Team, 2012) using the base installation and packages *lme4* v. 1.1-14 (Ba*tes et* al., 2015) and *lmerTest* v. 3.0-1 (Kuznets*ova et* al., 2017).

Boxplots illustrate corresponding data: The boxes range from the first to the third quartile, the whiskers extend to the highest value 1.5 x inter-quantile range, outliners are represented as dots and median is represented by horizontal line inside the box.

3.7.1 Experiment 1: UV-dye transfer

I tested the effect of distance from the focal plant and spatial treatments on the number of UV-dye particles detected on plants in the array. I applied an LME model with the following model formula:

sqrt (standardized number of UV-dye particles) ~ (log(relative distance from focal plant) + within cluster distance + among cluster distance) ^ 3 + (1|plant species/array)

The dependent variable is number of UV-dye particles per plant standardized to sum of UV-dye particles in array to avoid the differences in applied UV-dye doze for different arrays a different numbers of pollinators that arrived to the focal plant. Number of UV-dye particles on focal plant was excluded from all computations. I used the log-transformed relative distance from focal plant, within- and among-cluster distances and all interactions of the above

mentioned predictors as fixed effects and the identity of array nested within plant species as random effect.

I used the relative distance instead of the absolute one in order to avoid the bias introduced by manipulation of absolute distances in different spatial treatments (see Fig. 2 for illustration). I transformed the number of UV-dye particles by square root transformation to satisfy model assumptions, specifically the homogeneity of residual variance.

3.7.2 Experiment 2: Pollinator foraging behaviour

To examine pollinator behaviour, I used two characteristics of their foraging sequences: 1) mean relative flight distance between the two successively visited plants per pollinator individual; 2) the proportion of between-cluster flights per pollinator foraging sequence. For such analyses, I therefore included only foraging sequences including at least two individual plants visited in the array.

In the first analysis, I applied LME model to test the difference in mean relative flight distances per pollinator foraging sequence in the array. To meet the assumptions of the model, I used the logarithmic transformation of the dependent variable. I treated pollinator group, within- and among-cluster distances and all their interactions as fixed effects and array identity and pollinator species identity as random effect. See model formula:

log (mean relative flight distance) ~ (pollinator group + among cluster distance + within cluster distance) ^ 3 + (1|array) + (1|pollinator species)

I used model without plant species as fixed effect to maintain model convergence. The model did not significantly differ from the model with similar model where was plant species treated as random effect (see Table 3).

Table 3: Summary of LME models with and without plant species as random effect comparison. X^2 - likelyhood-ratio; DF – difference of model 1 and model 2 in degrees of freedom.

Model	deviance	χ^2	DF	P-value
Model 1 with plant species as random effect	395.9			
Model 2 without plant species as random effect	387.4	8.49	5	0.131

Then I applied an LME model to each pollinator group separately, since this allowed me to include plant species as a fixed effect (each pollinator group was present only on a subset of studied plant species). Due to model failure in convergence, I excluded models for large hoverflies and other dipterans. Summary of all used models is illustrated in Table 4.
Model equations differ in used fixed and random effects:

- Butterflies were observed on only one plant species; thus, the effect of plant species were excluded from the model.
- Interactions are excluded from small hoverflies' and bumblebees' formulas in order to stabilize the model.
- Random effect of pollinator species was excluded in honeybee and solitary bee models (solitary bees were not determined into species).

To test the influence of spatial treatments and pollinator group on proportion of between-cluster flights per pollinator sequence, I applied a binomial GLMM model with following equation:

proportion of between cluster flights ~ (pollinator group + among cluster distance + within cluster distance) ^ 3 + (1|array) + (1|pollinator species)

The dependent variable was the proportion of between-cluster flights to all flights recorded in a foraging sequence in the array. As fixed effects, I used pollinator group, within- and among-cluster distances and all interactions of the above mentioned predictors. I used identity of array and pollinator species as random effects of the model.

For this model, I used only a subset of pollinator foraging sequences excluding solitary bees, other dipterans and large hoverflies in order to obtain a sufficiently converging model.

Table 4: Summary of models for tested pollinator groups. Model equations correspond to model results in Table 10.

	Model equation	Plant species
Butterflies model	*log (mean relative flight distance) ~ (within cluster distance+ among cluster distance) ^ 2 + (1|array) + (1|pollinator species)*	*Dianthus carthusianorum*
Honey bee model	*log (mean relative flight distance) ~ (plant species + within cluster distance+ among cluster distance) ^ 3 + (1|array)*	*Hypericum maculatum; Serratula tinctoria; Succisa pratensis*
Bumble bees model	*log (mean relative flight distance) ~ (plant species + within cluster distance+ among cluster distance) + (1|array) + (1|pollinator species)*	*Serratula tinctoria; Succisa pratensis*
Solitary bees model	*log (mean relative flight distance) ~ (plant species + within cluster distance+ among cluster distance) ^ 3 + (1|array)*	*Achillea ptarmica; Dianthus carthusianorum*
Small hoverflies model	*log (mean relative flight distance) ~ (plant species + within cluster distance+ among cluster distance) + (1|array) + (1|pollinator species)*	*Achillea ptarmica; Dianthus carthusianorum; Hypericum maculatum*

3.7.3 Experiment 3: Pollen carry-over sampling

For analysis of differences in pollinator carry over capacities, I applied an LME model. I used number of all pollen grains per sample as a dependent variable and I considered it as predictors with fixed effect time of the day when the pollinator was caught, and pollinator functional group. I distinguished three functional groups: butterflies (Lepidoptera), hoverflies (Diptera, Syrphidae) and solitary bees (Hymenoptera, Apoidea). Pollinator species and date of sampling were considered as factors with random effect. See model formula:

number of pollen grains ~ (scale(time)+ pollinator group) ^ 2 + (1|date) + (1|pollinator species)

To satisfy the assumption of normality, I transformed the dependent variable by the logarithmic transformation as log(x+1).

In the analysis, the number of counted pollen grains in the sample was used, but it represent just ¼ of the pollen carried on pollinator body, because only ½ of the body was swabbed and only ½ of the slide was counted and determined. The relevant graph shows number of pollen grains after multiplication by four in order to obtain relevant estimate of pollen grains carried on the whole body of the pollinator.

4. Results

Pattern of UV-dye dispersion was observed in experimental arrays in 2018 for three plant species; *D. carthusianorum*, *A. ptarmica* and *H. maculatum*, with two replicates per species and treatment combination (Table 1). In total, data on dye dispersal were collected from 840 plants in 24 arrays.

The LME model showed a significant decrease of UV-dye load on plants with increasing relative distance from focal plant. None spatial treatment had a significant effect on the pattern of dispersion of UV-dye (see Table 5). The interaction between log-transformed distance from focal plant and within cluster distance was marginally significant and indicated a steeper decrease in dense treatments (Fig. 4)

Variation between arrays nested within a plant species was more important (SD = 0.049) than variation between plant species (SD = 0.023), but most of the variation was observed between individual plants within the array (SD = 0.085).

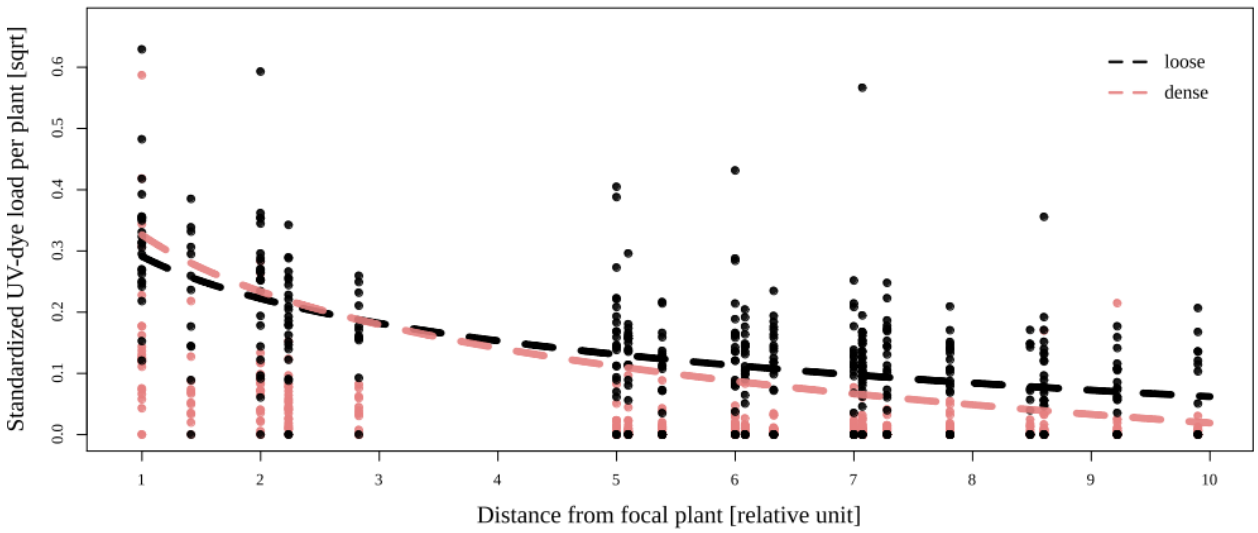

Fig. 4: Standardized UV-dye load per plant plotted against relative distance from focal plant. The decrease is steeper in the dense treatment (pink colour) than in the loose treatment (black), however the term was only marginally significant. Lines are predicted from minimal adequate model (for results of the full model see Table 5). Dots represent individual plants. Pink dots are partly covered by black dots.

Table 5: Summary of LME of UV-dye load in arrays and effect of distance from focal plant and spatial treatment.

	Standardized number of UV-dye particles [sqrt]					
Fixed effects	DF	Den DF	F-value	P-value	Coefficient	Estimate
					Intercept	0.3264
Log. of relative distance from focal plant	**1**	**2**	**67.08**	**0.015**	**Log. distance**	**-0.1335**
Within cluster distance	1	18	1.90		n.s.	
Among cluster distance	1	18	2.26		n.s.	
Log. of distance from focal plant x within cluster distance	1	18	4.04	0. 060	Log. Distance x loose	0.0340
Log. of distance from focal plant x among cluster distance	1	18	2.83		n.s.	
Within cluster distance x among cluster distance	1	18	0.73	0.404		
Log. of distance from focal plant x within cluster distance x among cluster distance	1	18	1.4		n.s.	

Random effects	SD	Factor levels
Array x plant species	0.0485	24
Plant species	0.0227	3
Residual	0.0849	

Note: The upper part shows summary of the fixed effects on UV-dye load in arrays. DF – numerator degrees of freedom; Den DF - denominator degrees of freedom estimated by the Satterthwaite approximation. Significant predictors are in bold. Estimates of coefficients are shown just for significant or marginally significant results and they are derived from the minimal adequate model. Intercept represents dense treatment.

4.1. Experiment 2: Pollinator foraging behaviour

In 2018 and 2019, 1798 individual pollinator sequences of at least two visited individual plants in array were observed and pollinators were scored to functional groups (= pollinator groups, see overview in Table 6).

*Table 6: Summary of pollinator sequences longer than two visited plants observed in the arrays (grouped by spatial treatments). Plant species marked with * correspond to species used in Experiment 1 to track UV-dye dispersal.*

*Achillea ptarmica**

Treatment	Solitary bee	Small hoverflies	Other diptera
Dense Near	20	57	34
Dense Far	16	51	25
Loose near	13	55	27
Loose far	9	45	15

*Dianthus carthusianorum**

Treatment	Butterflies	Solitary bee	Small hoverflies
Dense Near	61	11	34
Dense Far	47	8	46
Loose near	47	7	28
Loose far	83	8	25

*Hypericum maculatum**

Treatment	Honeybee	Small hoverflies
Dense Near	64	15
Dense Far	83	16
Loose near	69	6
Loose far	73	20

Serratula tinctoria

Treatment	Honeybee	Bumblebees
Dense Near	38	43
Dense Far	51	32
Loose near	47	36
Loose far	44	34

Succisa pratensis

Treatment	Honeybee	Bumblebees	Large hoverflies
Dense Near	15	27	57
Dense Far	18	33	30
Loose near	21	34	36
Loose far	18	27	39

Mean relative flight distance of pollinator foraging sequence significantly differed between pollinator groups and between among-cluster distance treatments (Table 7). Mean relative flight distance was generally higher in near-spaced treatments than in far-spaced ones (see Table 7 and Fig. 6).

Butterflies showed the highest mean relative flight distance (2.2±0.062, mean±SE, 238 sequences), followed by bumblebees (mean = 1.67±0.030, mean±SE, 266 sequences), honeybees (mean = 1.64±0.018, mean±SE, 544 sequences), solitary bees (mean = 1.43±0.044, mean±SE, 92 sequences), Small hoverflies (mean = 1.38±0.018, mean±SE, 388 sequences), large hoverflies (mean = 1.37±0.026, mean±SE, 162 sequences) and other dipterans (mean = 1.36±0.051, mean±SE, 101 sequences) (see Fig. 3).

Table 7: Results of LME of the influence of pollinator group and spatial treatment on the mean relative flight distance.

	log(mean relative flight distance per pollinator foraging sequence)					
Fixed effect	DF	Den DF	F-value	P-value	Coefficient	Estimate
Pollinator group	**6**	**13.9**	**9.08**	**<0.001**	Intercept	0.439
					Bumblebee	**-0.023**
					Large hoverflies	**-0.187**
					Butteflies	**+0.225**
					Other diptera	**-0.221**
					Small hoverflies	**-0.228**
					Solitary bee	**-0.144**
Within cluster distance	1	38.7	1.07		n.s.	
Among cluster distance	**1**	**39.2**	**5.11**	**0.029**	**near**	**+0.055**
Pollinator group x within cluster distance	6	190.3	0.45		n.s.	
Pollinator group x among cluster distance	6	191.4	1.08		n.s.	
Within cluster distance x among cluster distance	1	39.8	0.10		n.s.	
Pollinator group x within cluster distance x among cluster distance	6	193.5	1.28		n.s.	

Random effect	SD	Factor levels
Array	0.019	42
Pollinator species	0.107	25
Residual	0.268	

Note: The upper part shows summary of the fixed effects on the mean relative flight distance per pollinator foraging sequence. DF – numerator degrees of freedom; Den DF - denominator degrees of freedom estimated by the Satterthwaite approximation. Significant predictors are in bold. Estimates of coefficients are shown just for significant or marginally significant results and they are derived from the minimal adequate model. Reference levels of the intercept represent far treatment and honeybee.

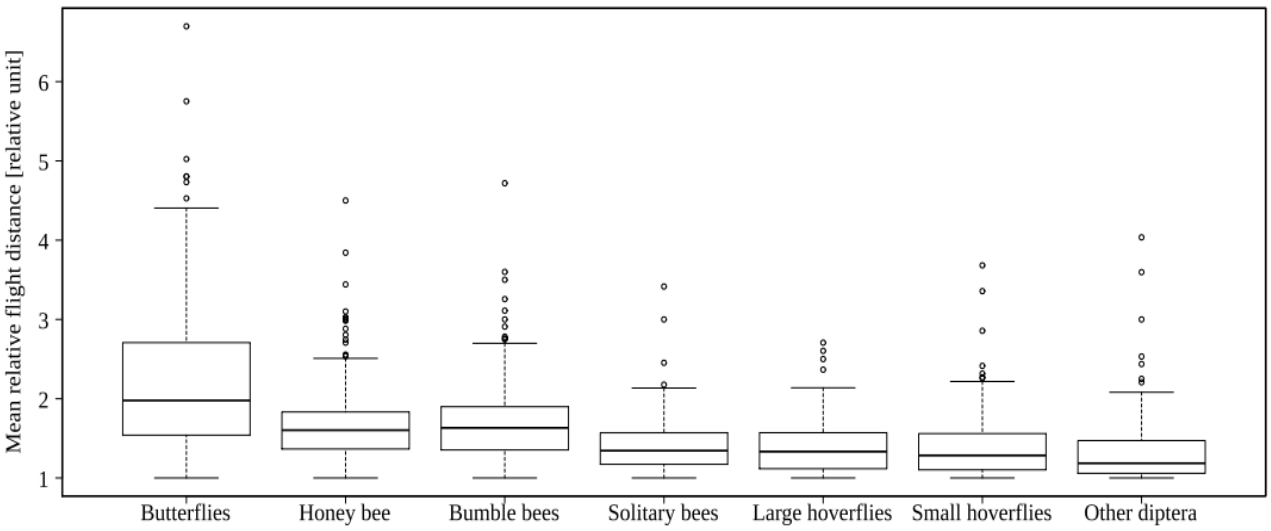

Fig. 5: Mean relative flight distance by pollinator group. Explanations to boxplot notation – see data analysis.

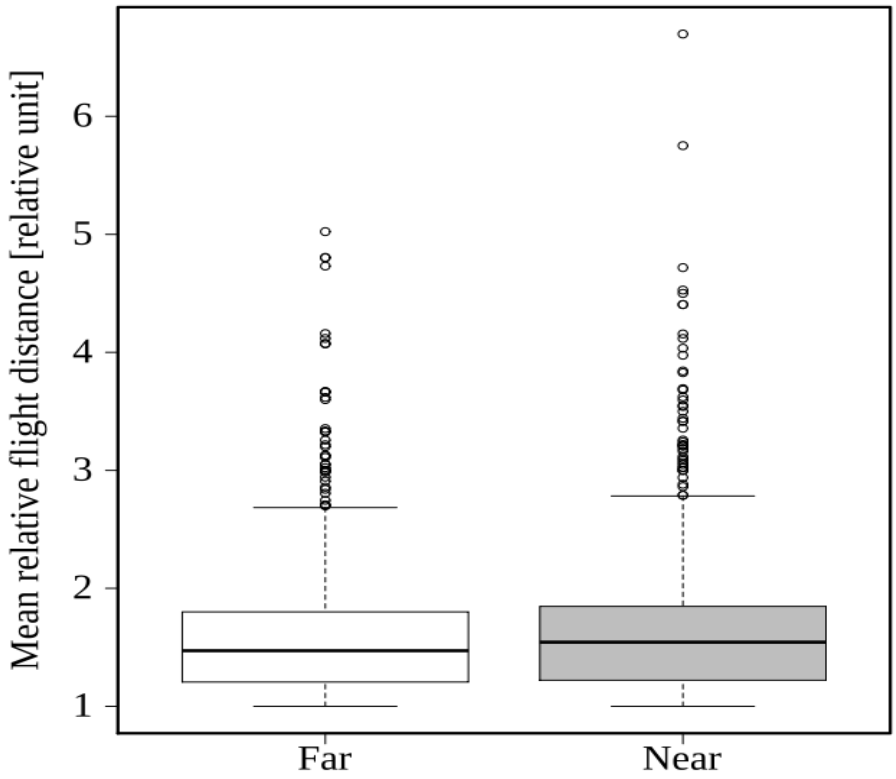

Fig. 6: Mean relative flight distance by among cluster distance. Arrays with near-spaced clusters tend to increase slightly pollinator mean relative flight distance. Explanations to boxplot notation – see data analysis.

Separate models for pollinator groups are shown in Table 4. Only models for honeybee and bumblebees – showed significant difference in among cluster distance (see Table 8). Interestingly, all four models with plant species (honeybee, bumblebees, solitary bees and small hoverflies) as predictor did not show differences of plant species in pollinator mean relative flight distance (see discussion).

Proportion of between cluster flights per pollinator sequence

The GLMM showed significant effects of pollinator group, within-cluster distance and among-cluster distance to proportion of between-cluster flights per foraging sequence while showing no significant interaction terms (Table 9). A significantly higher proportion of between-cluster flights was in loose and near treatments (Fig. 7). The highest proportion of between-cluster flights was exhibited by butterflies, followed by honeybees and bumblebees. Small hoverflies showed only little tendency to fly between clusters (see Fig. 7).

Table 8: Separate models for individual pollinator groups. For models formulas see data analysis. Den DF - denominator degrees of freedom estimated by the Satterthwaite approximation; horizontal lines represent predictors, which were not used in the model.

Fixed effect	Butterflies			Honey bee			Bumble bees			Solitary bees			Small hoverflies		
	Den DF	F-value	P-value	Den DF	F-value	P-value	Den DF	F-value	P-value	Den DF	F-value	P-value	Den DF	F-value	P-value
Plant species		-		17.964	0.4710	ns	25.3331	0.4998	ns	5.9847	1.8194	ns	14.235	0.6720	ns
Within patch distance	6.8537	0.3548	ns	13.388	0.0712	ns	3.6063	1.5331	ns	5.9847	0.1753	ns	15.676	0.1392	ns
Among patch distance	7.3087	0.9394	ns	13.388	5.6797	0.033	3.5144	6.8598	0.067	5.9847	1.0302	ns	15.967	1.6196	ns
Plant species x within patch distance		-		17.964	0.1241	ns		-		5.9847	1.0232	ns		-	
Plant species x among patch distance		-		13.388	0.0218	ns		-		5.9847	0.0243	ns		-	
Within patch distance x among patch distance	7.5453	2.4948	ns	13.388	0.0218	ns		-		5.9847	0.0004	ns		-	
Plant species x within patch distance x among patch distance		-		17.964	0.7250	ns		-		5.9847	0.7821	ns		-	

Table 9: Results from the LME model with random effects (individual array and pollinator species).

	Proportion of between cluster flights per pollinator sequence				
Factor	DF	Deviance	P-value	Coefficient	Estimate
				Intercept	2.072
				Bumblebee	**+0.036**
Pollinator group	**3**	**3231.5**	**<0.001**	**Butteflies**	**+0.277**
				Small hoverflies	**-1.373**
Within cluster distance	**1**	**3226.5**	**0.025**	**loose**	**+0.170**
Among cluster distance	**1**	**3199.1**	**<0.001**	**near**	**+0.332**

Random effect	SD	Factor levels
Array	0.058	42
Pollinator species	0.242	25

Null deviance	Residual deviance	Residual DF
3265.1	3199.1	1435

Note: The upper part shows summary of the fixed effects on the proportion of between-cluster flights per pollinator sequence. The lower table shows standard deviation (SD) and no. of factor levels for random effects of the model. DF – degrees of freedom. Significant predictors are in bold and non-significant interactions are not shown in the table. Estimates of coefficients are shown just for significant results and they are taken from minimal adequate model (for equation see methods). Reference levels of the intercept represent far and dense treatment and honeybee..

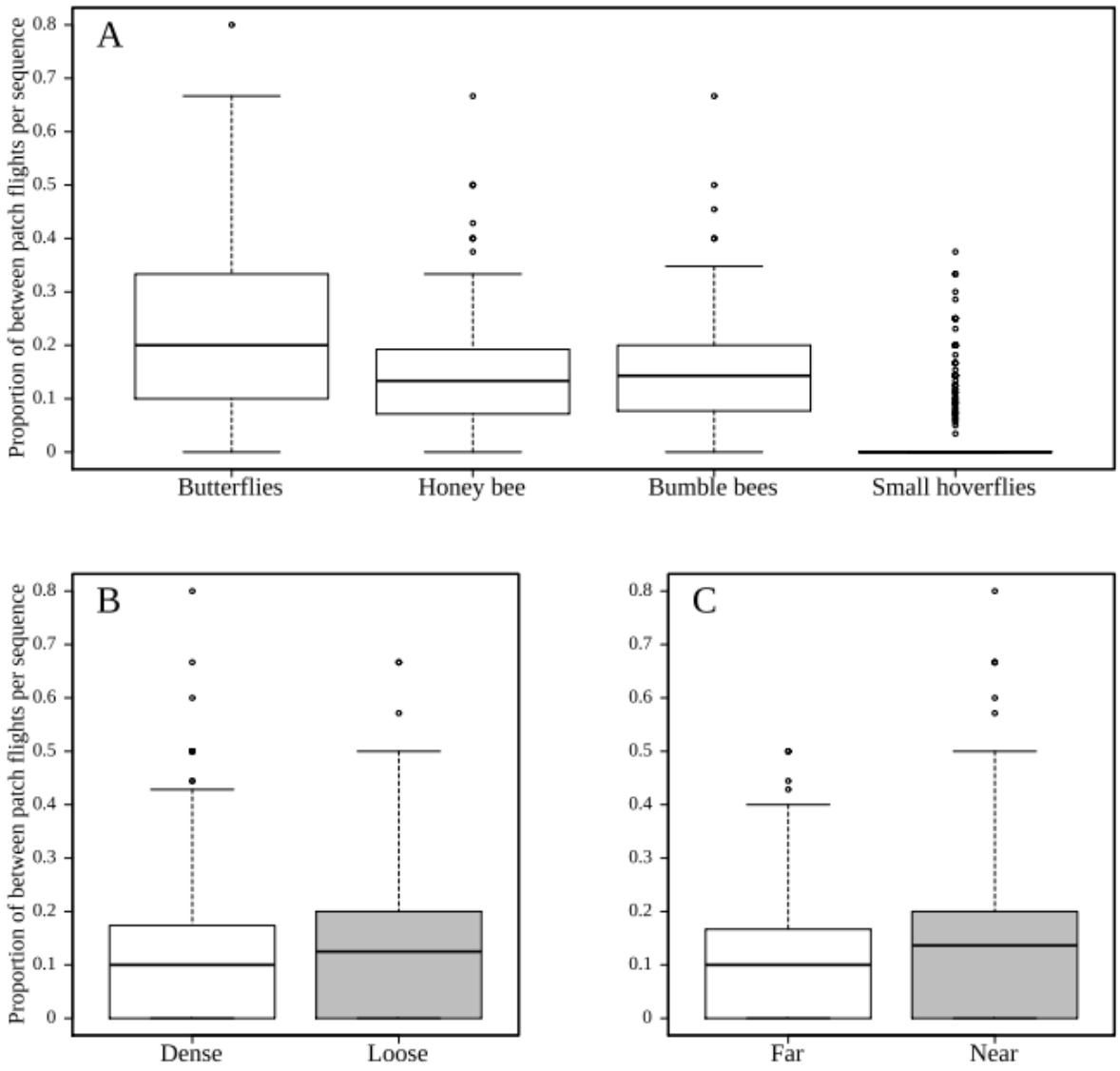

Fig. 7: Proportions of between cluster flights in a foraging sequence: A) by pollinator groups; B) by among cluster distance; C) by within cluster distance. Explanations to boxplot notation – see data analysis.

4.1. Experiment 3: *D. carthusianorum* pollen carry-over

In total, 124 samples of pollen from pollinator bodies were collected (see Table 11.). The highest number of *D. carthusianorum* pollen grains was carried by solitary bees (1024.7±620.2, mean±SE), followed by butterflies (17.2±3.9, mean±SE) and hoverflies (12.3±3.5, mean±SE). Although relatively small sample size (butterflies = 74, Small hoverflies = 43 and solitary bees = 7) the pattern was firmly significant due to large difference between the groups (see Fig. 8). Effect of time of the day and interaction of time of the day and pollinator group on the number of carried pollen grains were not significant (table 10).

Table 10: Summary of linear mixed-effect model of effects of time and pollinator group on number of pollen grains on pollinator body.

	Number of pollen grains					
Fixed effects	DF	DenDF	F-value	P-value	Coefficient	Estimate
					Intercept	1.063
Time	1	108.1	0.05		n.s.	
Pollinator group	**2**	**19.3**	**19.23**	**< 0.0001**	**Small hoverflies**	-0.193
					Solitary bees	+3.919
Time x pollinator group	2	111.0	0.92		n.s.	

Random effects	SD	Factor levels
Pollinator species	0.43	21
Date	0.20	7
Residual	0.83	

Note: The upper part shows summary of the fixed effects on number of pollen grains. The lower table shows standard deviation (SD) and no. of factor levels for random effects of the model. Df – numerator degrees of freedom; DenDf - denominator degrees of freedom estimated by the Satterthwaite approximation. Significant predictors are in bold. Estimates are taken from minimal adequate model. Reference levels of the intercept represent butterflies.

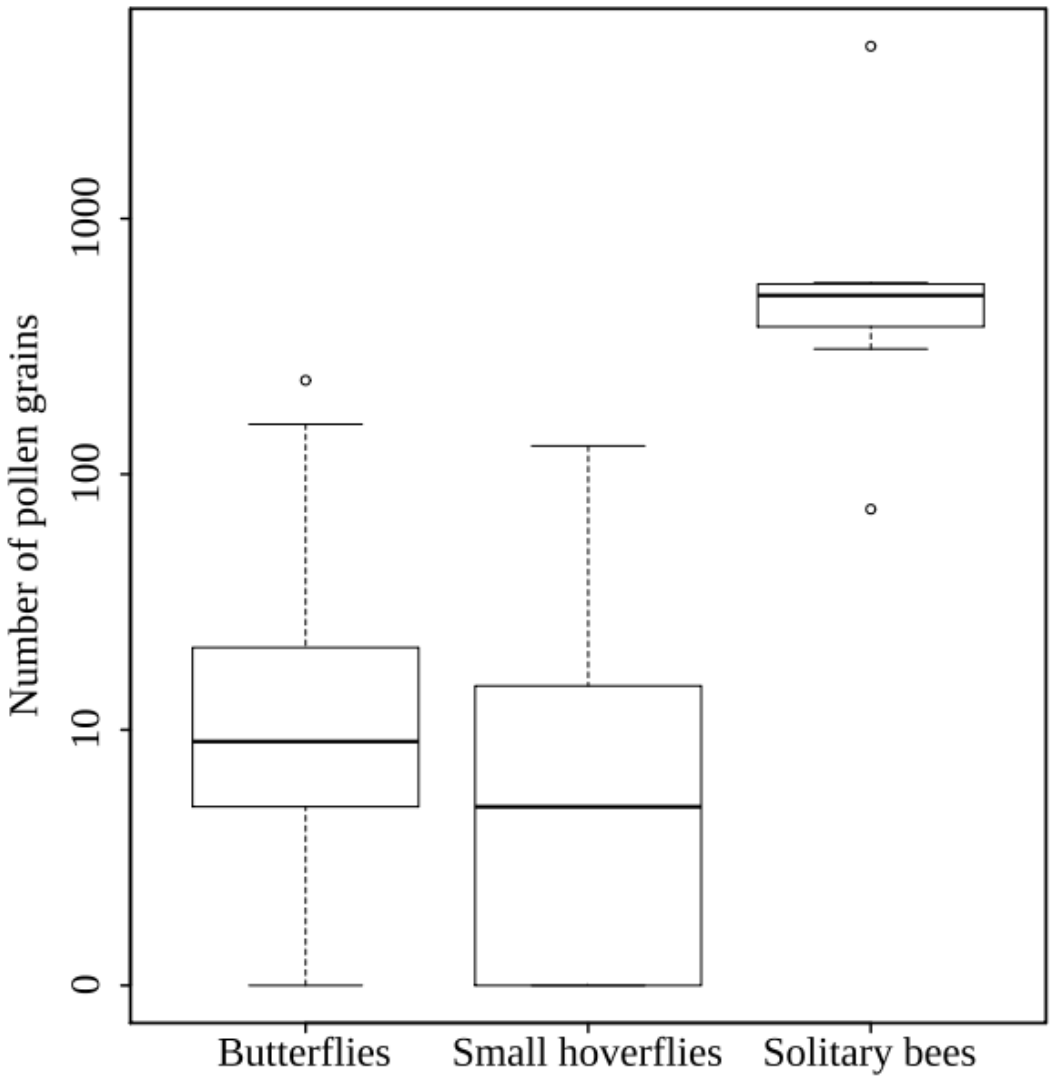

Fig. 8: Number of D. carthusianorum pollen grains carried on bodies of different pollinators. Graph shows number of counted pollen grains multiplied by 4 (½ of the body was swabbed and ½ of the slide was counted). Explanations to boxplot notation – see data analysis

Table 11: Summary of sampled pollinators by species and pollinator group

Pollinator group	Pollinator species (or higher taxa)	Number of samples
Butterfly	*Colias hyale*	2
	Goneopteryx rhamni	2
	Issoria_lathonia	3
	Lycanea phleas	1
	Lycanea sp.	3
	Maniola jurtina	1
	Melanargia galathea	22
	Pieris brassicae	9
	Pieris rapae	1
	Pontia edusa	1
	Thymelicus sp.	18
	Vanessa cardui	2
	Zygaena sp.	9
Small hoverfly	*Sphaneophoria scripta*	12
	Episyrphus balteatus	8
	Eupeodes sp.	1
	Scaeva pyrastri	15
	Syrphus sp.	1
	Other syrphid	6
Solitary bee	Solitary bee (Halictidae)	7

5. Discussion

5.1 Experiment 1: UV-dye transfer

The analysis showed a general decrease of the UV-dye load with increasing distance from the focal plant, but almost no effects of different plant aggregation level; only the interaction between within cluster distance and distance from the focal plant was marginally significant. That means that if the plants were aggregated in dense clusters, lower UV- dye load was found on flowers located furthest from the focal plant. Other tested variables did not show any effect on UV-dye load. This pattern corresponded with previous studies using UV-dye pollen analogue (Cresswell et al. 1995, Adler & Irwin 2006, van Rossum et al. 2011b), as well as with studies tracking gene flow (Lavigne et al. 1998) or orchid and milkweed pollinia removal (Pleasants 1991, Nilsson et al. 1992). This supports the expectation, that plants receive majority of pollen from the closest neighbourhood and from the nearest possible mates.

The rapid decline in UV-dye load on flowers could be explained by two possible mechanisms: 1) rapid loss of pollen from pollinator's body with increasing number of successive flower visits; 2) a pollinator tendency to disproportionately visit the nearest neighbours. The first mechanism was documented on hymenopterans (Rademaker et al. 1997, Adler & Irwin 2006) and it could be expected for other pollinators as well (but see Courtney et al. (1982)). The second mechanism corresponds with the results of Experiment 2 and it is discussed in the following part of the discussion.

On the other hand, the lack of substantial difference between spatial treatments does not correspond with the predictions that pollen dispersal should be lower should be lower in spatially aggregated populations due at lower proportion of flights between isolated clusters (Cresswell & Osborne 2004). The high variability of the total amount of deposited UV-dye between individual experimental arrays (documented by the magnitude of the random effect of experimental array identity) could be caused by pollinator densities varying between the experimental arrays or by the effects of weather condition on pollinator behaviour. Both factors should be considered in further analyses.

5.2 Experiment 2: Pollinator foraging behaviour

On average, foraging pollinators made shorter flights when the plant clusters were far from each other. This result corresponds to the Optimal Foraging Theory derived from the Marginal Value Theorem (Charnov 1976, Pyke 1978). According to these theories, large distances between clusters should stimulate pollinators to a more thorough inspection of the local cluster due to higher costs of between cluster flights (Hartling & Plowright 1979). Consequently, this optimization should maximize pollinator's reward uptake and save energy and should lead to a decrease in proportion of between cluster flights in densely spaced arrays (Osborne & Williams 2001).

Despite the high variability within pollinator groups, there were also detected considerable differences in foraging behaviour among the pollinator groups studied: Butterflies tended to fly further and more frequnently between clusters than any other studied group. This result is consistent with other studies comparing butterflies with other groups of insect pollinators (Schmitt 1983, Herrera 1987) and highlights the expected potential of butterflies to highly contribute to between cluster pollen transfer. The mean flight distance of other pollinator groups decreased in order: honeybee, bumblebees, solitary bees, hoverflies and flies. The proportion of between-cluster flights decreased in similar way: honeybee, bumblebees and small hoverflies (other pollinators groups was not tested due to model failure). This pattern indicates relatively high tendency of dipterans and solitary bees to fly to the nearest neighbour within the cluster in comparison to other groups.

Differences in pollinator behaviour could have several explanations: 1) difference in the costs of flight per distance unit among pollinator groups; 2) differences in foraging strategy between central place foragers and free roaming foragers; 3) whether pollinators focus only on reward collection or whether they simultaneously follow other aims (such as seeking to find mates, breeding sites etc.) 4) differences in the anti-predation behaviour; and 5) differences in sensory and memory limitations among pollinator groups.

1) difference in the costs of flight per unit distance among pollinator groups

According to the ¾ power law, the absolute cost of flight is higher for large insect, but the relative cost is higher for smaller insect (Kaufmann et al. 2013). Thus, flight efficiency tends to increase disproportionately with the pollinator body size (Harrison & Roberts 2000). Therefore, large pollinators should be foraging on larger area, due to their need of greater amount of rewards to satisfy their demands. This is supported by studies of the relation between body or wing size and pollinator flight distance in solitary bees (Araújo et al. 2004, Greenleaf et al. 2007). Moreover, the relation between body size and foraging rate is also known for individual workers of the same bumble bee colony (Spaethe & Weidenmüller 2002), which leads to the division of labour and spatial organization of bumblebees within the colony, where the bigger individuals collect rewards in farther distances.

2) difference between central place foragers and free roaming foragers

Pollinator groups could be divided into central place foragers (in our case honeybee, bumblebees and solitary bees) possessing nests around which they have their home range, and free roaming pollinators without a nest (butterflies and dipterans in this thesis). This division assumes that central place foragers are limited by their home range around the nest and by the need to travel back to the nest after collecting sufficient amount of reward (Cresswell et al. 2000). Thus, their foraging sequences are restricted within a roughly circular area, whose diameter differ among species (Greenleaf et al. 2007, Redhead et al. 2016). Moreover, their foraging sequence is driven by the need to return back to the nest with collected reward, while free roaming pollinators can continue in their foraging sequence or fly to different sites. To increase their efficiency, central place foragers usually tend to visit the same feeding location in consecutive foraging sequences (Ohashi et al.). This behaviour, called trapline foraging, may increase the pollen dispersion and number of mates in array due to increased fraction of trapliners visiting the cluster (Ohashi & Thomson 2009).

3) whether pollinators focus only on reward collection or whether they also follow simultaneously other aims

While workers of social bees, which are usually exclusively foraging for pollen and nectar to feed larvae and which are not interested in mating with males, optimise on reward gain, butterflies and dipterans tend to seek for rewards as well as for mates (Rodríguez-Gasol et al. 2019) or suitable condition for laying eggs during the foraging sequence (Stanton 1984). Thus, higher level of optimization to maximize reward intake should be expected in larva feeding hymenopterans than in other pollinator groups, which should be more likely distracted by other aims in order to increase their fitness. This could explain the difference in mean foraging distance among butterflies and bumblebees observed in previous studies (Waser 1982, Schmitt 1983, Herrera 1987).

4) differences in anti-predation behaviour

Pollinators, which are more vulnerable to be captured by predators (as hoverflies or flies, but other groups as well), optimize their behaviour in order to lower the predation risk (Jones 2010, Romero et al. 2011). As a consequence, plant fitness could be reduced by higher density of pollinator predators, which reduced pollinator density (Dukas 2005).

5) *difference in sensory limitations.*

Pollinators are limited in their sensoric and processing abilities. For instance, the resolution of a bee's eye is 100 times worse than the resolution of a human eye (Chittka & Raine 2006). The difference in compound eyes characteristic (but also in other sensory organ) may lead into variable sensory limitation in different pollinator groups: butterflies (Stavenga & Arikawa 2006), hymenopterans (Chittka & Raine 2006) and hoverflies (Lunau 2015). The magnitude of sensory limitation is considerable, since e.g. bees are able to recognize a flower with 1 cm in diameter only from ca 11 cm. Thus, densely spaced flowers in cluster may be easily recognizable
for pollinators from large distance than sparsely placed individual plants (Dafni et al. 1997).

In conclusion, above mentioned differences may result in discrepancy from optimal foraging strategy due to influence of complex set of aspects on pollinator behaviour. The higher level of optimization could be expected in workers of social hymenopterans, given their lack of interest in mating, trapline foraging strategy and relatively low level of predation. In contrast, dipterans and butterflies are expected to show lower level of optimization. However, honeybee and bumblebees need to gather up greater amount of rewards to feed larvae and to satisfy energy costs of flights by visiting more flowers per array. Therefore, honeybee and bumblebees mean flight distance and proportion of flight between clusters should be higher than in other groups except butterflies, which fly further possibly due their better flying ability and aim into seeking in larger area for mates and egg-laying sites.

Interestingly, plant species identity does not change pollinator foraging behaviour of individual pollinator groups (see Table 8). Thus, my results indicate that at least for unspecialised plant species the conclusions of laboratory studies on pollinator foraging behaviour (e.g. Cartar & Real (1997), Gegear & Laverty (2005)) could be relatively transferable to pollinator behaviour in monospecific stands under (nearly) field conditions conducted with different plant species.

Beyond the scope of this thesis remains the analysis of pollinator directionality of flight between visited plants. The directionality of flight should decrease the risk of revisiting previously visited plant again and optimize pollinator gain from visited flowers (Hodges & Miller 1981). Directionality tends to decrease with increasing degree of plant aggregation (Cresswell 1997, 2000, Cartar & Real 1997) and in clusters with possibility to obtain higher rewards (Heinrich 1979).

5.3 Experiment 3: *D. carthusianorum* pollen carry-over

Pollinator groups differed in the number of *D. carthusianorum* pollen carried on their body: Butterflies and hoverflies carried lower number of pollen grains than solitary bees. The low amount of pollen transferred by butterflies is corresponds with other studies (Larsson 2005, Bloch et al. 2006, Sahli & Conner 2007, Orford et al. 2015). Comparable lower amount of pollen was carried by small hoverflies, probably due to their small body size and low hair density (Phillips et al. 2018, Roquer-Beni et al. 2020). Solitary bees carried expectable higher

number of pollen grains on their bodies. This number could be increased and biased by swabbing pollen collecting structures, where is stored pollen used to feed larvae and which never reach flower stigma. On the contrary, previous studies suggested that pollen stigma deposition by solitary bees is still proportionally higher than deposition by butterflies and non-furry dipterans (Larsson 2005, Sahli & Conner 2007, Phillips et al. 2018). However, in further studies should be considered if swabbing of all pollinator body or selecting parts, which can possibly reach stigma, is in accordance to study question.

5.4 Synthesis of the combined results of Experiment 1, 2 and 3

Pattern observed in the Experiment 1 is not completely consistents with results from the Experiment 2. The Experiment 1 did not show strong difference between spatial treatments in UV-dye transfer whereas Experiment 2 rendered strong effect of spatial treatments (mainly between cluster distance) on pollinator behaviour. This inconsistency could be possibly explained by disparity between pollen and UV-dye transfer leading into weak ability of the experiment to detect significant difference between spatial treatments. Discrepancy between pollen transfer and UV-dye was previously reported (Thomson et al. 1986) usually with mentioning of possible overestimation of pollen dispersion and gene-flow by UV-dye (Adler & Irwin 2006, van Rossum et al. 2011a). This could be possibly caused by different physical properties of UV-dye, such a size (UV-dye particle diameter ca 20 μm compared to pollen grains of *D. carthusianorum* ca 40 μm (Halbritter 2016) adhesion and weight. A new approach proposed and developed by Minnaar and Anderson (2019), promising more accurate method to detect pollen transfer by using quantum dots to labelling pollen grains.

Unlikely, the pattern of UV-dye dispersion could be also shifted by activity of nocturnal pollinators visiting plants outside the observation period. While the presence of nocturnal pollinators on the study site could not be rejected, their influence on UV-dye dispersion seems to be unlikely due to relatively short period of array exposure to nocturnal pollinators (UV-dye pattern was examined immediately after sunset) and due to rare observation on flowers in experimental arrays (up to ten times on flowers of *D. carthusianorum* and *S. pratensis* combined).

However, the overall rapid decline of UV-dye load with increasing distance from the focal plant is congruent with the distribution of pollinator flight distances: Given the majority of short flights, most of the UV-dye particles is dispersed to the nearest neighbours of focal plant. This pattern seems to be possible generalized to pollen (Adler & Irwin 2006) with taking account to the discrepancy in absolute load of UV-dye and pollen grains. Moreover, studies using different methods to track pollen transfer and gene-flow confirmed similar pattern (Pleasants 1991, Cresswell et al. 1995, Lavigne et al. 1998).

Pollinator tendency to fly on shorter distances and less frequently between clusters increases in densely aggregated plant clusters. An expected result for plant sexual reproduction may be the decrease of the genetic variability of progeny in dense clusters of closely related plants. The restriction of pollen flow to the nearest neighbours may even reduce influx of the outcross pollen and intensify problems with sexual reproduction faced by clonal plants (Vallejo-Marín et al. 2010). However, these implications should be tested by a more precisely aimed approach incorporating a study of the genetic structure of plant the population and a more sensitive method to track pollen transfer.

Experiment 2 and 3 combined highlights the differences in contribution to pollen transfer by different pollinator groups. In study system of *D. carthusianorum* butterflies made up 40.2% of pollinator visits followed by small hoverflies (26.4%) and solitary bees (17.2%; Koupilová et al. under review), which combined with mean loads of *D. carthusianorum* pollen found on their bodies (1024.7, 17.2 and 12.3 respectively), renders that ca 94% of pollen transferred to pollinators is carried by solitary bees as compared to ca 4% by butterflies and ca 2% by hoverflies. This render that majority of *D. carthusianorum* pollen is transferred to close neighbours (mean relative flight distances are 1.34 and 1.23 (inter-plant distance) for solitary bees and small hoverflies respectively), and smaller proportion of pollen is carried by butterflies and transported to further distances (mean relative flight distance is 2.2 respectively).

However, neither Experiment 2 and 3 do not inspected several other parameters affecting pollen transfer, thus suggested conclusion could not be considered as firm estimate of pollen transfer. First, I did not conducted experiment comparing pollen deposition on stigma after visit between pollinator groups. Although strong correlation between the pollen carry-over and

pollen stigma deposition was reported by Rader et al. (2009), in their study pollen collecting structures (e.g. scopae) were excluded, which may shifted relationship between number of carried and deposited pollen grains by solitary bees and decrease their contribution to pollination. The pollen-ovule ratio of *D. carthusianorum* (P/O = 400, ca 30 000 pollen grains and ca 75 ovules per flower respective) (Jürgens et al. 2002) suggested relatively high loss of pollen. Since fruit development is induced if ca 50 pollen grains reach stigma, thus, considered mean pollinator pollen carry-over, plants could face to pollen limitation (Bloch et al. 2006). Second, I did not considered the number of flower visitors (visitation rate) per flower and also visitation of different flower stages, which also play an important role in pollinator contribution to pollen transfer (Parker et al. 2016).

In conclusion, results of Experiment 2 and 3 together highlight that butterflies may likely serve as mediators of pollen transfer between clusters more than other pollinator groups. However, major amount of pollen is transferred to nearest neighbourhood by solitary bees despite their relatively lower abundance. Given general decline in insect abundance (Hallmann et al. 2017) and high temporal variation in butterfly (Harrison et al. 2015) and solitary bees abundance (Franzén & Nilsson 2013), the plants may face to the pollen limitation and potential pollination crisis (Bloch et al. 2006).

6. Conclusion

In my thesis I focused on the effect of different plant spatial aggregation on changes in pollen transfer and visitation behaviour of the main Central European pollinator groups. As a side project, I also compared pollinator pollen carry-over capacity among three pollinator groups visiting one of the studied plant species (*D. carthusianorum*) to highlight difference between pollinator groups and their contribution to pollen transfer.

Experiment 1 and 2 combined render the effect of plant spatial aggregation. While the pattern is not strongly supported by UV-dye transfer, pollinator behaviour showed firm effect of plant spatial aggregation on pollinator behaviour. Pollinators shorter both flight distances and decrease proportion of between cluster flights in arrays with clusters separated by larger distance (between cluster distance – far; according to experimental design) and also decrease proportion of between cluster flights in densely spaced clusters (within cluster distance – dense; according to experimental design). Thus, if is plant population separated into clusters about high density and separated by large distances, it may decrease pollen flow even within population.

Moreover, pollinator groups differed in their foraging behaviour. Butterflies flown to further distances and more often between clusters followed by honeybee and bumblebees, whereas large hoverflies and small pollinators such a dipterans and solitary bees flown to shorter distances. Possible explanations for these differences were disused. Additionally, the effect of plant species on behaviour of individual pollinator groups was not detected, therefore pollinator behaviour seems to be constant among conspecific arrays of different plant species. It may supported possibility of generalization between experiments conducted under similar design with different plant species or with artificial flowers.

In addition, Experiment 3 underlines different contribution of individual pollinator groups to pollen transfer. Linked with Experiment 2, results showed that pollen dispersion between plants is influenced by combination of pollinator behaviour, relative proportion of visits and pollen carry-over. Despite the fact that butterflies were major visitors of *D. carthusianorum* flowers, majority of pollen grains was transferred on short distances by solitary bees. These

findings highlight importance of evaluation pollinators carry-over in studies focused on effect of pollinator foraging behaviour to pollen transfer.

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Haiku about pollen fate and finding a suitable mate through manifold ways:

The pollen fate,
driven in manifold ways
to find a mate.

7. References

Adler L. S. & Irwin R. E. (2006): Comparison of pollen transfer dynamics by multiple floral visitors: Experiments with pollen and fluorescent dye. – Ann. Bot. 97: 141–150.

Araújo E. D., Costa M., Chaud-Netto J., & Fowler H. G. (2004): Body size and flight distance in stingless bees (Hymenoptera: Meliponini): inference of flight range and possible ecological implications. – Brazilian J. Biol. 64: 563–568.

Barrett S. C. H. (2010): Darwin's legacy: The forms, function and sexual diversity of flowers. – Philos. Trans. R. Soc. B Biol. Sci. 365: 351–368.

Bloch D., Werdenberg N., & Erhardt A. (2006): Pollination crisis in the butterfly-pollinated wild carnation Dianthus carthusianorum? – New Phytol. 169: 699–706.

Bolker B. M., Brooks M. E., Clark C. J., Geange S. W., Poulsen J. R., Stevens M. H. H., & White J. S. S. (2009): Generalized linear mixed models: a practical guide for ecology and evolution. – Trends Ecol. Evol. 24: 127–135.

Campbell D. R. & Waser N. M. (1989): Variation in pollen flow within and among populations of Ipomopsis aggregata. *Evolution*. 43: 1444–1455.

Cartar R. V. & Real L. A. (1997): Habitat structure and animal movement: the behaviour of bumble bees in uniform and random spatial resource distributions. – Oecologia 112: 430–434.

Charnov E. L. (1976): Optimal foraging, the marginal value theorem. – Theor. Popul. Biol. 9: 129–136.

Chittka L. & Raine N. E. (2006): Recognition of flowers by pollinators. *Current Opinion in Plant Biology*. 9: 428–435.

Courtney S. P., Hill C. J., & Westerman A. (1982): Pollen Carried for Long Periods by Butterflies. – Oikos 38: 260.

Cresswell J. E. (1997): Spatial Heterogeneity, Pollinator Behaviour and Pollinator-Mediated Gene Flow: Bumblebee Movements in Variously Aggregated Rows of Oil-Seed Rape. – Oikos 78: 546.

Cresswell J. E. (2000): A comparison of bumblebees' movements in uniform and aggregated distributions of their forage plant. – Ecol. Entomol. 25: 19–25.

Cresswell J. E., Bassom A. P., Bell S. A., Collins S. J., & Kelly T. B. (1995): Predicted pollen dispersal by honey-bees and three species of bumble-bees foraging on oil-seed rape: A comparison of three models. – Funct. Ecol. 9: 829–841.

Cresswell J. E. & Osborne J. L. (2004): The effect of patch size and separation on bumblebee foraging in oilseed rape: Implications for gene flow. – J. Appl. Ecol. 41: 539–546.

Cresswell J. E., Osborne J. L., & Goulson D. (2000): An economic model of the limits to foraging range in central place foragers with numerical solutions for bumblebees. – Ecol. Entomol. 25: 249–255.

Dafni A., Kevan P. G., & Husband B. C. (2005): Practical pollination biology. – Pract. Pollinat. Biol.

Dafni A., Lehrer M., & Kevan P. G. (1997): Spatial flower parameters and insect spatial vision. – Biol. Rev. Camb. Philos. Soc. 72: 239–282.

Dauber J., Biesmeijer J. C., Gabriel D., Kunin W. E., Lamborn E., Meyer B., Nielsen A., Potts S. G., Roberts S. P. M., SÃµber V., Settele J., Steffan-Dewenter I., Stout J. C., Teder T., Tscheulin T., Vivarelli D., & Petanidou T. (2010): Effects of patch size and density on flower visitation and seed set of wild plants: a pan-European approach. – J. Ecol. 98: 188–196.

Dukas R. (2005): Bumble bee predators reduce pollinator density and plant fitness. – Ecology 86: 1401–1406.

Duncan D. H., Nicotra A. B., Wood J. T., & Cunningham S. A. (2004): Plant isolation reduces outcross pollen receipt in a partially self-compatible herb. – J. Ecol. 92: 977–985.

Franzén M. & Nilsson S. G. (2013): High population variability and source–sink dynamics in a solitary bee species. – Ecology 94: 1400–1408.

Funk H. (2013): Adam Zaluz?anský ’s “de sexu plantarum” (1592): An early pioneering chapter on plant sexuality. – Arch. Nat. Hist. 40: 244–256.

Gegear R. J. & Laverty T. M. (2005): Flower constancy in bumblebees: A test of the trait variability hypothesis. – Anim. Behav. 69: 939–949.

Gegear R. J. & Thomson J. D. (2004): Does the Flower Constancy of Bumble Bees Reflect Foraging Economics? – Ethology 110: 793–805.

Geslin B., Baude M., Mallard F., & Dajoz I. (2014): Effect of local spatial plant distribution and conspecific density on bumble bee foraging behaviour. – Ecol. Entomol. 39: 334–342.

Gilbert F. S. (1981): Foraging ecology of hoverflies: morphology of the mouthparts in relation to feeding on nectar and pollen in some common urban species. – Ecol. Entomol. 6: 245–262.

Greenleaf S. S., Williams N. M., Winfree R., & Kremen C. (2007): Bee foraging ranges and their relationship to body size. – Oecologia 153: 589–596.

Hallmann C. A., Sorg M., Jongejans E., Siepel H., Hofland N., Schwan H., Stenmans W., Müller A., Sumser H., Hörren T., Goulson D., & Kroon H. De. (2017): More than 75 percent decline over 27 years in total flying insect biomass in protected areas (E. G. Lamb, Ed.). – PLoS One 12: 1–21.

Harrison J. F. & Roberts S. P. (2000): Flight Respiration and Energetics. – Annu. Rev. Physiol. 62: 179–205.

Harrison J. G., Shapiro A. M., Espeset A. E., Nice C. C., Jahner J. P., & Forister M. L. (2015): Species with more volatile population dynamics are differentially impacted by weather. – Biol. Lett. 11:.

Hartling L. K. & Plowright R. C. (1979): Foraging by bumble bees on patches of artificial flowers: a laboratory study. – Can. J. Zool. 57: 1866–1870.

Haslett J. R. (1989): Adult feeding by holometabolous insects: pollen and nectar as complementary nutrient sources for Rhingia campestris (Diptera: Syrphidae). – Oecologia 81: 361–363.

Heinrich B. (1979): Resource heterogeneity and patterns of movement in foraging bumblebees. – Oecologia 40: 235–245.

Herrera C. M. (1987): Components of Pollinator “Quality”: Comparative Analysis of a Diverse Insect Assemblage. – Oikos 50: 79.

Hodges C. M. (1985): Bumble Bee Foraging: The Threshold Departure Rule. – Ecology 66: 179–187.

Hodges C. M. & Miller R. B. (1981): Pollinator flight directionality and the assessment of pollen returns. – Oecologia 50: 376–379.

Holloway B. A. (1976): Pollen feeding in hover flies (Diptera: Syrphidae). – New Zeal. J. Zool. 3: 339–350.

Hort A. & Theophrastus; (1916): Enquiry into plants and minor works on odours and weather signs, with an English translation by Sir Arthur Hort, bart. – London W. Heinemann, London.

Howlett B. G., Walker M. K., Rader R., Butler R. C., Newstrom-Lloyd L. E., & Teulon D. A. J. (2011): Can insect body pollen counts be used to estimate pollen deposition on pak choi stigmas? – New Zeal. Plant Prot.

Jones E. I. (2010): Optimal foraging when predation risk increases with patch resources: An analysis of pollinators and ambush predators. – Oikos 119: 835–840.

Jürgens A., Witt T., & Gottsberger G. (2002): Pollen grain numbers, ovule numbers and pollen-ovule ratios in Caryophylloideae: Correlation with breeding system, pollination, life form, style number, and sexual system. – Sex. Plant Reprod. 14: 279–289.

Käpylä M. (1978): Amount and type of nectar sugar in some wild flowers in Finland. – Ann. Bot. Fenn.

Kaufmann C., Reim C., & Blanckenhorn W. U. (2013): Size-dependent insect flight energetics at different sugar supplies. – Biol. J. Linn. Soc. 108: 565–578.

King C., Ballantyne G., & Willmer P. G. (2013): Why flower visitation is a poor proxy for pollination: Measuring single-visit pollen deposition, with implications for pollination networks and conservation. – Methods Ecol. Evol. 4: 811–818.

Kunin W. E. (1993): Sex and the Single Mustard : Population Density and Pollinator Behavior Effects on Seed-set. 74: 2145–2160.

Kwak M. M., Velterop O., & Andel J. van. (1998): Pollen and gene flow in fragmented habitats. – Appl. Veg. Sci. 1: 37–54.

Larsson M. (2005): Higher pollinator effectiveness by specialist than generalist flower-visitors of unspecialized Knautia arvensis (Dipsacaceae). – Oecologia 146: 394–403.

Lavigne C., Klein E. K., Vallée P., Pierre J., Godelle B., & Renard M. (1998): A pollen-dispersal experiment with transgenic oilseed rape. Estimation of the average pollen dispersal of an individual plant within a fieldA pollen-dispersal experiment with transgenic oilseed rape. Estimation of the average pollen dispersal of an indivi. – Theor. Appl. Genet. 96: 886–896.

Levin D. A., Kerster H. W., & Niedzlek M. (1971): Pollinator Flight Directionality and its Effect on Pollen Flow. – Evolution (N. Y). 25: 113.

Minnaar C. & Anderson B. (2019): Using quantum dots as pollen labels to track the fates of individual pollen grains (E. Shepard, Ed.). – Methods Ecol. Evol.

Minnaar C., Anderson B., Jager M. L. de, & Karron J. D. (2018): Plant–pollinator interactions along the pathway to paternity. – Ann. Bot.

Nielsen A., Dauber J., Kunin W. E., Lamborn E., Jauker B., Moora M., Potts S. G., Reitan T., Roberts S., Sõber V., Settele J., Steffan-Dewenter I., Stout J. C., Tscheulin T., Vaitis M., Vivarelli D., Biesmeijer J. C., & Petanidou T. (2012): Pollinator community responses to the spatial population structure of wild plants: A pan-European approach. – Basic Appl. Ecol. 13: 489–499.

Nilsson L. A., Rabakonandrianina E., & Pettersson B. (1992): Exact tracking of pollen transfer and mating in plants. – Nature 360: 666–668.

Nonaka E. & Holme P. (2007): Agent-based model approach to optimal foraging in heterogeneous landscapes: effects of patch clumpiness. – Ecography (Cop.). 30: 777–788.

Ohashi K. & Thomson J. D. (2009): Trapline foraging by pollinators: Its ontogeny, economics and possible consequences for plants. – Ann. Bot. 103: 1365–1378.

Ohashi K., Thomson J. D., & Souza D. D'. Trapline foraging by bumble bees: IV. Optimization of route geometry in the absence of competition.

Ollerton J., Winfree R., & Tarrant S. (2011): How many flowering plants are pollinated by animals? – Oikos 120: 321–326.

Orford K. A., Vaughan I. P., & Memmott J. (2015): The forgotten flies: the importance of non-syrphid Diptera as pollinators. – Proceedings. Biol. Sci. 282: 20142934.

Osborne J. L. & Williams I. H. (2001): Site constancy of bumble bees in an experimentally patchy habitat. – Agric. Ecosyst. Environ. 83: 129–141.

Parker A. J., Williams N. M., & Thomson J. D. (2016): Specialist pollinators deplete pollen in the spring ephemeral wildflower Claytonia virginica. – Ecol. Evol. 6: 5169–5177.

Phillips B. B., Williams A., Osborne J. L., & Shaw R. F. (2018): Shared traits make flies and bees effective pollinators of oilseed rape (Brassica napus L.). – Basic Appl. Ecol.

Pleasants J. M. (1991): Evidence for Short-Distance Dispersal of Pollinia in Asclepias syriaca L. – Funct. Ecol. 5: 75.

Pyke G. H. (1978): Optimal foraging in bumblebees and coevolution with their plants. – Oecologia 36: 281–293.

Pyke G. H. & Cartar R. V. (1992): The flight directionality of bumblebees: do they remember where they came from? – OIKOS.

Rademaker M. C. J., Jong T. J. de, & Klinkhamer P. G. L. (1997): Pollen dynamics of bumble-bee visitation on Echium vulgare. – Funct. Ecol. 11: 554–563.

Rader R., Howlett B. G., Cunningham S. A., Westcott D. A., Newstrom-Lloyd L. E., Walker M. K., Teulon D. A. J., & Edwards W. (2009): Alternative pollinator taxa are equally efficient but not as effective as the honeybee in a mass flowering crop. – J. Appl. Ecol. 46: 1080–1087.

Rathcke B. J. & Jules E. S. (1993): Habitat fragmentation and plant-pollinator interactions. – Curr. Sci.

Redhead J. W., Dreier S., Bourke A. F. G., Heard M. S., Jordan W. C., Sumner S., Wang J., & Carvell C. (2016): Effects of habitat composition and landscape structure on worker foraging distances of five bumble bee species. – Ecol. Appl. 26: 726–739.

Rodríguez-Gasol N., Avilla J., Alegre S., & Alins G. (2019): Sphaerophoria rueppelli adults change their foraging behavior after mating but maintain the same preferences to flower traits. – BioControl 64: 149–158.

Romero G. Q., Antiqueira P. A. P., & Koricheva J. (2011): A meta-analysis of predation risk effects on pollinator behaviour. *PLoS ONE*. 6:.

Roquer-Beni L., Rodrigo A., Arnan X., Klein A. M., Fornoff F., Boreux V., & Bosch J. (2020): A novel method to measure hairiness in bees and other insect pollinators. – Ecol. Evol. 10: 2979–2990.

Rossum F. van. (2010): Reproductive success and pollen dispersal in urban populations of an insect-pollinated hay-meadow herb. – Perspect. Plant Ecol. Evol. Syst. 12: 21–29.

Rossum F. van, Stiers I., Geert A. van, Triest L., & Hardy O. J. (2011a): Fluorescent dye particles as pollen analogues for measuring pollen dispersal in an insect-pollinated forest herb. – Oecologia 165: 663–674.

Rossum F. van, Stiers I., Geert A. van, Triest L., & Hardy O. J. (2011b): Fluorescent dye particles as pollen analogues for measuring pollen dispersal in an insect-pollinated forest herb. – Oecologia 165: 663–674.

Sahli H. F. & Conner J. K. (2007): Visitation, effectiveness, and efficiency of 15 genera of visitors to wild radish, Raphanus raphanistrum (Brassicaceae). – Am. J. Bot. 94: 203–209.

Schmitt J. (1983): Flowering plant density and pollinator visitation in Senecio. – Oecologia 60: 97–102.

Schulke B. & Waser N. M. (2001): Long-distance pollinator flights and pollen dispersal between populations of delphinium nuttallianum. – Oecologia 127: 239–245.

Silvertown J. W. & Charlesworth D. (2001): Introduction to Plant Population Biology. – Blackwell Science.

Solís-Montero L. & Vallejo-Marín M. (2017): Does the morphological fit between flowers and pollinators affect pollen deposition? An experimental test in a buzz-pollinated species with anther dimorphism. – Ecol. Evol. 7: 2706–2715.

Spaethe J. & Weidenmüller A. (2002): Size variation and foraging rate in bumblebees (Bombus terrestris). – Insectes Soc. 49: 142–146.

Stanghellini M. S., Ambrose J. T., & Schultheis J. R. (2015): Diurnal activity, floral visitation and pollen deposition by honey bees and bumble bees on field-grown cucumber and watermelon.

Stanton M. L. (1984): Short-term learning and the searching accuracy of egg-laying butterflies. – Anim. Behav. 32: 33–40.

Stevens P. F. (2007): Hypericaceae. In Kubitzki K. (ed.), Flowering Plants · Eudicots, pp. 194–201. – Springer Berlin Heidelberg, Hamburg.

Thomson J. D. (1986): Pollen Transport and Deposition by Bumble Bees in Erythronium: Influences of Floral Nectar and Bee Grooming Published by : British Ecological Society Stable URL : http://www.jstor.org/stable/2260258 REFERENCES Linked references are available on JST. – J. Ecol. 74: 329–341.

Thomson J. D., Price M. V., Waser N. M., & Stratton D. A. (1986): Comparative studies of pollen and fluorescent dye transport by bumble bees visiting Erythronium grandiflorum. – Oecologia 69: 561–566.

Vallejo-Marín M., Dorken M. E., & Barrett S. C. H. (2010): The Ecological and Evolutionary Consequences of Clonality for Plant Mating. – Annu. Rev. Ecol. Evol. Syst. 41: 193–213.

Waddington K. D. (1980): Flight patterns of foraging bees relative to density of artificial flowers and distribution of nectar. – Oecologia 44: 199–204.

Wagner J., Lechleitner M., & Hosp D. (2016): Pollen limitation is not the rule in nival plants: A study from the European Central Alps. – Am. J. Bot. 103: 375–387.

Waser N. M. (1982): A comparison of distances flown by different visitors to flowers of the same species. – Oecologia 55: 251–257.

Zimmerman M. (1982): Optimal foraging: Random movement by pollen collecting bumblebees. – Oecologia 53: 394–398.

8. Supplements

8.1 Supplement 1: Summary of individual sequences by pollinator groups and species.

Pollinator group	Pollinator species (or higher taxa)	Sequences
Butterflies	Pieris_brassicae	135
	Melanargia_galathea	33
	Gonepteryx_rhamni	10
	Macroglossum_stellatarum	20
	Thymelicus sp.	16
	other_Lepidoptera	24
Honeybee	Honeybee (Apis melifera)	541
Bumblebees	Bombus lapidarius	168
	Bombus pascuorum	21
	Bombus terrestris	77
Small hoverflies	Eupeodes sp	10
	Episyrphus balteatus	46
	other_Syrphids	3
	Scaeva_pyrastri	51
	Syrphus	24
	Syritta_pipiens	248
	Sphaerophoria_sp	16
Large hoverflies	Eristalis tenax	121
	Helophilus	32
	other_Eristalis	9
Other dipterans	Gymnosoma sp	34
	Meatfly	25
	Muscidae	25
	Other_diptera	17
Solitary bees	Solitary_bee	92

8.2 Supplement 2: Pollen carry-over sampling protocol

Phases:

1) Pollinator swabbing

2) Sample preparation

3) Sample counting

Materials:

- Insect net
- Tweezers
- Fuchsine jelly
- Microscope slides
- Cover slides
- Pernament fix
- Lighter
- Microscope

1) Pollinator swabbing

Catch pollinator on flower of study species using insect net. Immobilize it immediately and kill it. Avoid necessary shaking.

Swab the ½ of pollinator body with 5x5x3 mm cube of fuchsine jelly. Hold the cube in tweezers and avoid any contamination. After swabbing, put the cube into the clean jar with label. Write all information with ID into the sheet. Put pollinator body into the labelled jar with 70% ethanol for later identification (butterflies to the paper sack).

Write down at least these information:

- ID
- Date
- Time
- Pollinator species
- Information about the plant on which was the pollinator caught
- Note
- ID of the person who caught the pollinator

2) Sample preparation

Samples should be transferred on microscope slide immediately. Put the jelly cube on microscope slide and warm it with lighter (or candle). Be aware of boiling it! Mix the jelly wit clean tweezers and put the cover slide up. Label the sample with ID. After cooling, seal the sample with nail polish. Keep samples in a fridge.

3) Sample counting

For counting is used grid with cells about 1*1 mm. Every second cell is counted. Cells are scored into category by number of pollen grains of all species inside.

0 – 0 pollen grains
1 – 1 pollen grains
2 – 2-10 pollen grains
11 – 11-25 pollen grains
26 – 26 and more pollen grains

Pollen grains reaching left and bottom border of the cell are counted, but grains reaching right and top borders are not counted (see illustration).

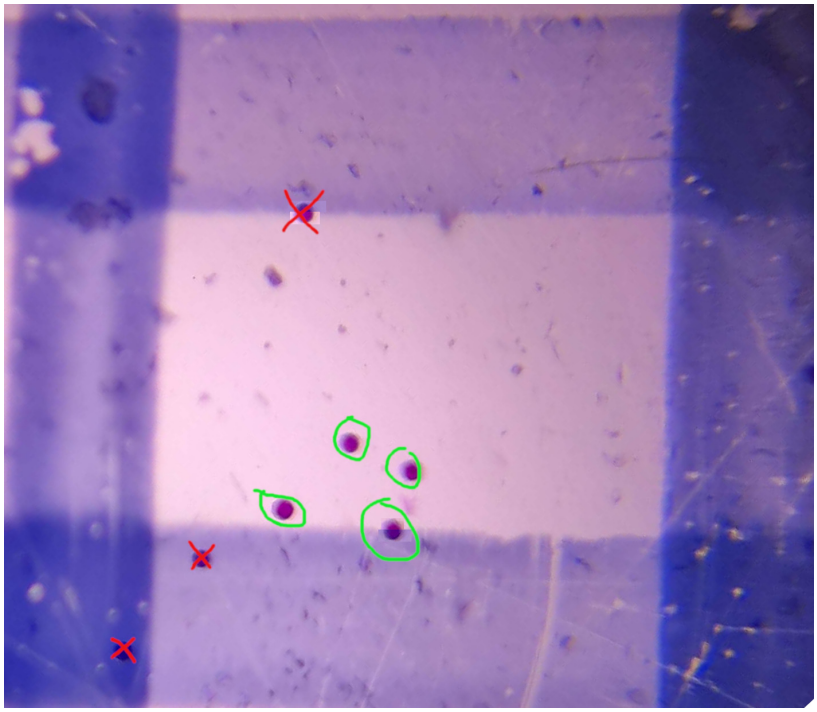

Fig 9: Green circled grains are counted. Red crossed grains are not counted. This cell should be scored in category 2.

Pollen grains are identified to species as follows:

Subset of 200 pollen grains is determined to species. Cells in categories are multiplied by following centres of intervals:

0 – multiple by 0
1 – multiple by 1
2 – multiple by 6
11 – multiple by 18
26 – multiple by 40

After multiplication, calculate the number of cells for each category proportionally by their frequency in the grid. In calculated number of cells per category determine grains into species and multiple it by the centre of interval for each category.

8.3 Supplement 3: Dataset to Experiment 1: UV-dye

In the file **Uvdye_DT_Stenc.csv**

The file is available in online database or on request on an email address: *jakubstenc@gmail.com*

8.4 Supplement 4: Dataset to Experiment 2: Pollinator behaviour

In the file **pollinator_behaviour_DT_Stenc.csv**

The file is available in online database or on request on an email address: *jakubstenc@gmail.com*

8.5 Supplement 5: Dataset to Experiment 3: Pollen carry-over

In the file **pollen_DT_Stenc.csv**

The file is available in online database or on request on an email address: *jakubstenc@gmail.com*