

Pollinator visitation closely tracks diurnal patterns in pollen release

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Abstract

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

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

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

Conclusions: Stratifying pollen availability to pollinators during the day may be one of several mechanisms that allow coflowering plants to share their pollinators and decrease the probability of heterospecific pollen transfer.

KEYWORDS

Centaurea jacea, plant sexual reproduction, pollen presentation, pollen release schedule, pollen transfer, pollinator diurnal activity, *Succisa pratensis*, *Trifolium hybridum*

Plants pollinated by insects suffer from high pollen loss during transfer, resulting in only a tiny fraction of the produced pollen reaching conspecific stigmas (Harder and Thomson, 1989; Rademaker et al., 1997; Gong and Huang, 2014). Pollen loss due to pollen consumption (Parker et al., 2016), grooming behavior (Holmquist et al., 2012; Koch et al., 2017), or inefficient pollen transfer (Parker et al., 2016) can even lead to an almost complete depletion of pollen within a plant population (Stanghellini et al., 2002b; Parker et al., 2016; Koski et al., 2018) and consequently limit plant sexual reproduction. Facing such a

threat, plants are under strong selective pressure to develop adaptations to prevent pollen losses by limiting the amount of pollen removed by a single pollen-wasting pollinator and increasing the number of pollen carriers (Harder and Thomson, 1989; LeBuhn and Holsinger, 1998; Castellanos et al., 2004). In addition, coflowering plants are threatened by the risk of heterospecific pollen transfer from and to other plant species (Arceo-Gómez et al., 2016). Therefore, plants are expected to evolve different pollen presentation schedules to prevent pollen losses caused by pollinators by pollen wastage and heterospecific transfer.

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

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

To develop pollen presentation schedules, plants evolved various mechanisms allowing them to release pollen at different times and with different magnitudes (Lloyd and Yates, 1982), allowing optimization of the amount of pollen presented to pollinators (Harder and Wilson, 1994). For example, some plants, such as some genera of Fabaceae (e.g., *Medicago*, *Trifolium*, *Ulex*), possess explosive pollen release mechanisms (reviewed by Aluri and Reddi, 1995) to rapidly release a vast proportion of the pollen from flowers during the first pollinator visit. Other plants dispense pollen by gradual maturation of anthers within a flower and present pollen from only a fraction of anthers at each moment (Ren and Bu, 2014). A similar goal, i.e., gradual pollen release, can also be reached by the pump mechanism employed, e.g., by most Asteraceae, where pollen is pumped out by gradual pistil growth through the flower tube (Erbar and Leins, 1995; Galloni et al., 2007). Furthermore, plants often display flowers organized into inflorescences possessing more simultaneously presented flowers. Plants forming inflorescences benefit from attracting more pollinators but may suffer a higher risk of geitonogamy (i.e., self-pollination; Ishii and Sakai, 2001; Karron et al., 2004), especially when the inflorescence is compact and forms one single unit from the point of view of the pollinator (e.g., capitulum of Asteraceae or Fabaceae) (Liao and Harder, 2014). Hence, floral and inflorescence traits may act together to affect pollen transfer and pollinator behavior (Harder et al., 2004; Kudo and Harder, 2005). Overall, the mechanisms of pollen release within flowers and flower development within inflorescences enable variation in the timing of pollen presentation among species and their populations (Stone et al., 1998; Nansen and Korie, 2000; Kudo and Harder, 2005; Eisen et al., 2017), resulting in structured pollen presentation schedules.

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

specialization in particular time frames (Schwarz et al., 2021; Wang et al., 2021).

In this study, we investigated the pollen presentation schedule of three coflowering species and the diurnal visitation pattern of their pollinators, aiming to explore how closely pollinator activity tracks pollen availability. For our study, we selected three plant species exhibiting differences in their pollen release mechanism, flower and inflorescence organization, and partial overlap in their pollinator spectra. Notably, we address the following questions: (1) Does pollen availability follow a pattern during the day? (2) Does pollinator visitation activity track pollen availability? We expected that the studied plants will differ in the pattern of pollen presentation, which will be closely tracked by pollinator visitation activity.

MATERIALS AND METHODS

Study system

We used *Trifolium hybridum* L. (Fabaceae), *Centaurea jacea* L. (Asteraceae), and *Succisa pratensis* Moench (Caprifoliaceae), which differ in their pollen dispensing mechanisms. *Trifolium hybridum* flowers are closed, with explosive release of pollen toward the front part of the pollinator body as in other Fabaceae species (Galloni et al., 2007); *C. jacea* presents pollen gradually throughout the day using a so-called pump mechanism, where pollen is extruded from a closed tube by the growing style (Erbar and Leins, 1995); and *S. pratensis* releases pollen openly on four anthers sticking out from the flower and the pollen is easily accessible to pollinators. The individual flowers of all studied species are organized into inflorescences (capitula: *T. hybridum*, *S. pratensis*; flowerheads: *C. jacea*; Figure 1).

The study was conducted at the K Handrkovu meadow near Vernýřov village, Central Bohemia region, Czech Republic (49.8466N, 15.1498E). The locality was described in detail by Janovský et al. (2013). The experiment was done in stable weather conditions with no or only moderate wind and sunny weather. We checked the conditions by measuring solar radiation, relative air humidity, and temperature using Minikin RTHi/QTHi automatic dataloggers (EMS BRNO, Brno, Czech Republic). We also determined the average wind speed at 30-min intervals (Windmaster 2, Kaindl Electronics GmbH, Rohrbach, Germany). Weather conditions were comparable during the whole experiment, with moderate increases in temperature and radiation and decreases in relative humidity on the second day (Appendix S1, Figures S1, S2).

Pollen availability and flowering schedule

To answer Question 1, we collected data about pollen availability on flowers (measured as number of pollen grains

A *Trifolium hybridum*



B *Centaurea jacea*



C *Succisa pratensis*



FIGURE 1 Photographs of individual flower (left) and inflorescence (right) of (A) *Trifolium pratensis*, (B) *Centaurea jacea*, and (C) *Succisa pratensis*. Images of individual flowers illustrate differences in pollen-releasing mechanism: *T. hybridum* has an explosive pollen-release mechanism, *C. jacea* pumps pollen gradually through narrow flower tube, and *S. pratensis* presents pollen freely on the anthers. All species form a compact inflorescence, and pollinators usually visit several flowers during one visit.

available to pollinator during visit) and the proportion of active flowers within the inflorescence for all study species. This information determined the diurnal pollen presentation schedule.

Samples were collected on 15 and 16 August 2021 (Day 1 and Day 2, respectively) at hourly intervals from 8:00 until 17:00 to cover the span of main pollinator activity. At each sampling event, we placed flowers into five vials per species (hereafter called samples). Each vial contained 2 mL of an aqueous detergent solution (common kitchen detergent, 5–15% anionic surfactants; <5% non-ionic surfactants; benisothiazolinone, phenoxyethanol, perfumes, geraniol, limonene; Procter & Gamble Czech Republic, s.r.o., Rakovník, Czech Republic) and basic fuchsin to stain pollen grains (0.2 g/L of water). Flowers for each sample were collected from five randomly selected inflorescences within approximately 50 m of the weather dataloggers. From each of the five inflorescences per sample, we randomly collected three individual flowers each for *T. hybridum* and *C. jacea* and five for *S. pratensis*. The collected flowers offered pollen

(from at least one anther) and had to be actively visited by pollinators.

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

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

To estimate total pollen production, we collected flower buds with developed anthers before they were actively flowering. Collected buds were manually opened inside a vial using tweezers to avoid pollen losses. Samples were then processed as done for the samples for pollen presentation, except the sonication was extended for 10 min to ensure anther fission and pollen release. Later, the anthers were inspected under a light microscope, and pollen grains remaining within the anther tissue were counted and summed with the pollen counts from the image analysis.

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

The next day (17 August 2021; Day 3), we investigated the proportion of pollen-presenting flowers within the inflorescence of each species during the day. Flowers on each inflorescence were counted and classified as flower buds, active flowers, or old flowers. Active flowers refer to flowers with visible pollen that are visited by pollinators, i.e., those that could be sampled for pollen presentation. Flowers were counted on 20 randomly chosen inflorescences per species every hour from 8:00 to 17:00 to match the pollen presentation schedule.

Pollinator diurnal visitation activity

To compare the pollen presentation schedule with pollinator activity (Question II), we used aggregated data on plant–pollinator interactions from the same site and time of the year (approximately 6-day collection campaigns between

13 and 26 August, matched with the flowering of *S. pratensis*) for the last 10 seasons (2011–2021). These data were collected using the protocol of Janovský et al. (2013).

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

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

Data for *T. hybridum* are based on 404 plot \times year combinations (min = 2, median = 43, max = 66 plots per year), each censused for pollinators at least 20 times. *Centaurea jacea* pollinators were censused on 676 plot \times year combinations (min = 41, median = 60, max 82 plots per year) and *S. pratensis* on 182 plot \times year combinations (min = 13, median = 17, max = 22 plots per year). Mean inflorescence densities per occupied plot were 5.1, 7.0, and 7.9 occupied subplots per plot for *T. hybridum*, *C. jacea*, and *S. pratensis* respectively.

Since pollinator occurrences on studied plants within plot censuses contained large quantities of zeroes, we needed to obtain average pollinator densities at a given time interval. For all censuses originating from a given time interval, pollinator occurrences on the focal plant species were summed and then divided by the sum of inflorescence densities of the studied plant species. For *S. pratensis* and *C. jacea*, we averaged observations from all plots observed in 10-min intervals, whereas for *T. hybridum* with lower pollinator densities, the observations were averaged over 20-min intervals. We refer to this quantity as the pollinator density hereafter.

Data analyses

To answer Question 1, we did two analyses for each of the studied species. First, we modeled the amount of available pollen in individual flowers using the average number of pollen grains per sample as a response. The predictors were time of the day, sampling day, and their interaction because there were tangible differences between the days in terms of available pollen, but not in the shape of the relationship to the time of day. Second, we modeled the proportion of actively flowering flowers within an inflorescence with respect to the time of the day. We considered both linear

and quadratic terms (available pollen per flower) or even cubic terms (proportion of actively flowering flowers within the inflorescence) for time of the day, since unimodal patterns during the day could be expected (and the cubic term allows adding “asymmetry” to the resulting unimodal curve). In both analyses, we used a linear model, and to meet its assumption for homogeneity of variance, we had to transform the response variables by taking their logarithm (available pollen per flower) or square root (proportion of actively flowering flowers). We considered the cubic term only when a quadratic term was significant. In general, we viewed the use of polynomial terms in our study mainly as a way to track and test the general shape of the relationship between the response and predictor variables rather than as an assumption of the underlying polynomial functional relationship between predictor and response. Furthermore, we did use linear models, since the measured quantities in both cases (hundreds to thousands pollen grains and higher tens of flowers) and non-extreme observed proportions of open flowers within inflorescences (ca 0.2 to 0.7) allowed the approximation of these response variables by normal distribution and use of linear models.

Since the studied plant species differed in their overall pollen production per flower (Appendix S1, Table S1), to visualize the data (but not for the analysis), we standardized the data by total pollen production per flower of the species. Furthermore, we combined the two models to provide an estimate of the available amount of pollen per inflorescence for the three species under study to compare it with pollinator densities (Figure 3A).

To answer Question 2, we analyzed pollinator densities with respect to time of day. As we did for models aimed at answering Question 1, we considered a unimodal pattern of pollinator densities with respect to time of day. In models with a significant quadratic term, we considered the cubic term as well. The response variable was square-root transformed to meet the assumption of homogeneity of variance. Since we were interested mainly in the timing of maxima of pollinator density during the day, we standardized pollinator densities by their maximum for data visualization (Figure 3B, nonstandardized pollinator densities can be found in Appendix S1, Table S1, Figure S3)

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

RESULTS

Pollen presentation and flowering schedule

The amount of available pollen on the flowers varied over time in all plant species under study (Table 1). In *S. pratensis* and *T. hybridum*, the amount of available pollen per flower decreased steadily from morning onward. While the flowers of *S. pratensis* were almost depleted by 13:00, the decrease was not as steep in *T. hybridum*. However, *C. jacea* followed a unimodal pattern during the day with a peak at approximately 13:00 (Figure 1).

Centaurea jacea and *T. hybridum* also differed significantly in the amount of available pollen between the 2 days of our observation (Table 1, Figure 1), with increased pollen release on the second day of sampling. However, the pattern of release during the day remained the same; there was only a difference in magnitude.

Plant species also differed in their total pollen production: *T. hybridum* produced on average 5162 pollen grains (SD 350), *C. jacea* produced 6008 pollen grains (SD 785), and *S. pratensis* produced 991 (SD 232) (Appendix S1, Table S2). The proportion of active flowers of both *C. jacea* and *T. hybridum* varied with time (Table 2), showing a unimodal pattern with peaks at approximately 13:00 (*C. jacea*) and 15:00 (*T. hybridum*). In contrast, *S. pratensis* showed almost no change in inflorescence development with approximately 25% of flowers active from early morning until the end of our observation and without a distinctive peak (Figure 2C).

Pollinator activity

We recorded 439, 5855, and 7300 pollinators visiting *T. hybridum*, *C. jacea*, and *S. pratensis* respectively, during the 11 years of our pollinator observations. The pollinator spectrum of *T. hybridum* was strongly dominated by

TABLE 1 Linear model of amount of available pollen (log-transformed) depending on time of day and day of sampling. SS = sum of squares, Coef. = coefficients, R^2 = coefficient of determination. Coefficients for statistically nonsignificant predictors are not shown (represented by a dash).

Predictor	<i>Trifolium hybridum</i>					<i>Centaurea jacea</i>					<i>Succisa pratensis</i>				
	df	SS	Coef. ± SE	R^2	P	df	SS	Coef. ± SE	R^2	P	df	SS	Coef. ± SE	R^2	P
Day	1	9.151	0.592 ± 2.174	0.226	<0.001	1	4.852	0.592 ± 1.866	0.175	<0.001	1	1.185	1.894 ± 2.145	0.016	<0.05
Time	1	5.419	-0.273 ± 0.255	0.134	<0.001	1	0.464	—	0.017	0.13	1	47.674	-0.47 ± 0.252	0.635	<0.001
Time ²	1	0.323	—	—	0.28	1	3.442	0.007 ± 0.009	0.124	<0.001	1	1.134	0.009 ± 0.01	0.015	<0.05
Day × time	1	0.017	—	—	0.8	1	0.195	—	0.007	0.33	1	0.013	—	—	0.83
Day × time ²	1	0.001	—	—	0.96	1	0.014	—	0.001	0.79	1	0.150	—	—	0.45
Residuals	94	25.554	—	—	—	94	18.812	—	—	—	94	24.870	—	—	—

TABLE 2 Linear models of proportion of active flowers within an inflorescence (square-root transformed) depending on time of day. SS = sum of squares, Coef. = coefficients, R^2 = coefficient of determination. Coefficients for statistically nonsignificant predictors are not shown (represented by a dash).

Predictor	<i>Trifolium hybridum</i>					<i>Centaurea jacea</i>					<i>Succisa pratensis</i>				
	df	SS	Coef. \pm SE	R^2	P	df	SS	Coef. \pm SE	R^2	P	df	SS	Coef. \pm SE	R^2	P
Time	1	47.399	-0.454 \pm 0.112	0.478	<0.001	1	25.937	0.244 \pm 0.036	0.107	<0.001	1	2.548	—	0.011	0.14
Time ²	1	11.687	0.044 \pm 0.009	0.118	<0.001	1	35.883	-0.009 \pm 0.001	0.148	<0.001	—	—	—	—	—
Time ³	1	5.188	-0.001 \pm 0.0002	0.052	<0.001	—	—	—	—	—	—	—	—	—	—
Residuals	194	34.953	—	—	—	198	180.642	—	—	—	198	235.235	—	—	—

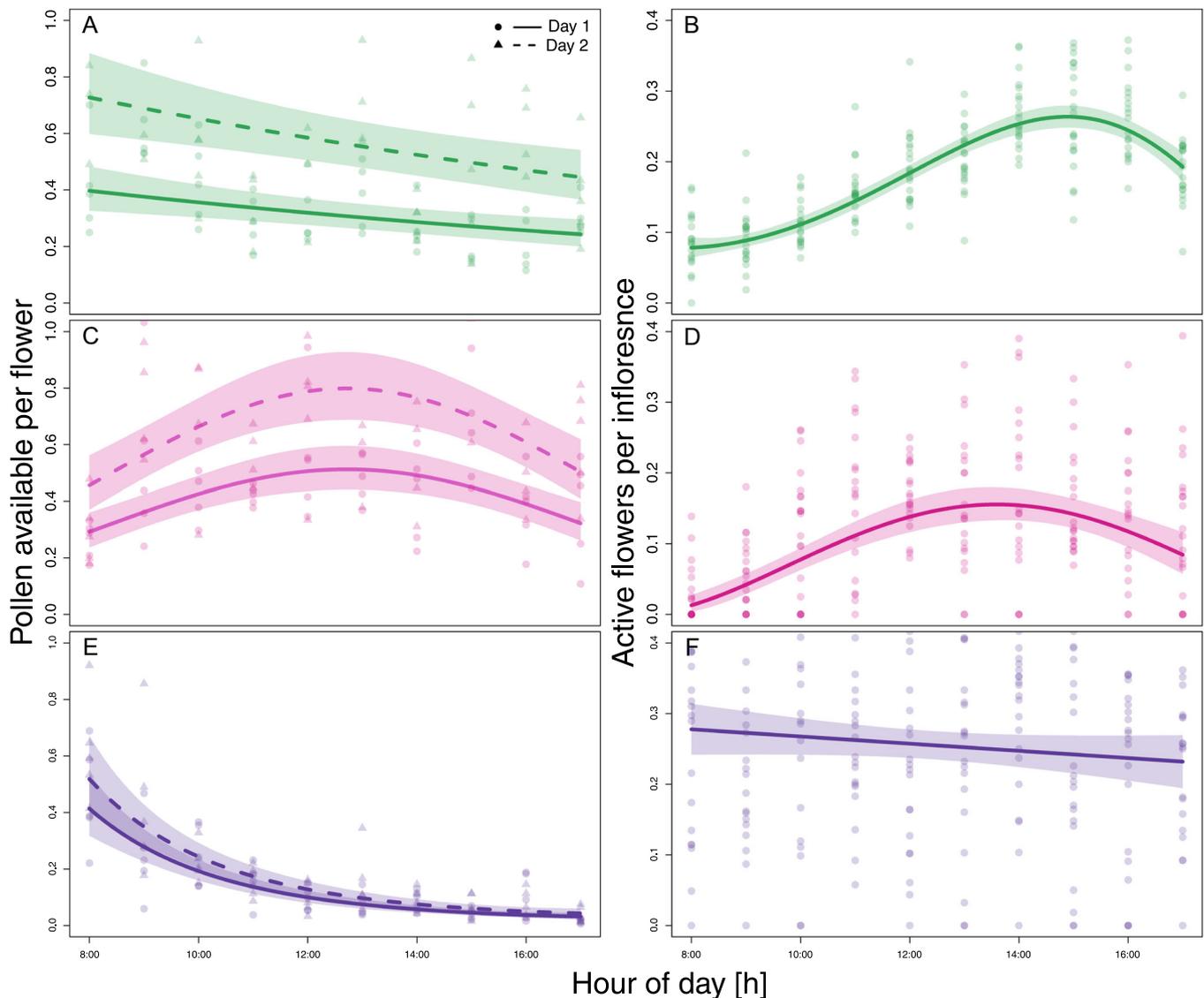


FIGURE 2 Time course of pollen availability and flowering schedule for (A, B) *Trifolium pratensis*, (C, D) *Centaurea jacea*, and (E, F) *Succisa pratensis*. In A, C, and E, points represent amount of pollen presented on flowers divided by total pollen production for each species. For graphs of absolute amount of available pollen, see Appendix S1, Figure S4. Dots stand for Day 1 and triangles for Day 2. Lines represent predictions and polygons confidence intervals obtained from minimal adequate models, solid lines stand for Day 1 and dashed lines for Day 2. In B, D, and F, points represent proportion of active flowers from whole inflorescence, lines represent predictions and shaded areas represent confidence interval obtained from minimal adequate models.

honeybees (61.5%; bumblebees, 25.7%; and hoverflies, 3.6%). The spectrum of *C. jacea* was dominated by honeybee (43.7%), bumblebees (32.6%), and hoverflies (14.6%). On the other hand, hoverflies dominated the

pollinator spectrum of *S. pratensis* making up 75.6%, while the proportion of honeybee and bumblebees was much lower (2.3% and 12.6%, respectively) (Appendix S1, Table S1).

The visits of the pollinators to all three studied plant species had a unimodal diurnal pattern (Table 3, Figure 3), but the time of the peak density of their visits differed (ca 15:45 for *T. hybridum*, 13:30 for *C. jacea*, 11:45 for *S. pratensis*) and matched the peak pollen availability in the inflorescences. There was a delay

between the peak pollen availability and the peak pollinator densities in all three studied plant species. In *C. jacea* and *T. hybridum*, the delay was only ca 30–45 min, the delay in *S. pratensis* was much higher, but still the pollinator densities peaked on *S. pratensis* earliest among the three plant species.

TABLE 3 Linear models of pollinator density (square-root transformed) depending on the time of day. SS = sum of squares, Coef. = coefficients, R^2 = coefficient of determination. Coefficients for statistically nonsignificant predictors are not shown (represented by a dash).

Predictor	<i>Trifolium hybridum</i>					<i>Centaurea jacea</i>					<i>Succisa pratensis</i>				
	Df	SS	Coef. ± SE	R^2	<i>P</i>	df	SS	Coef. ± SE	R^2	<i>P</i>	df	SS	Coef. ± SE	R^2	<i>P</i>
Time	1	5.223	0.042 ± 0.009	0.522	<0.001	1	2.369	0.113 ± 0.007	0.061	<0.001	1	2.850	0.212 ± 0.067	0.180	<0.001
Time ²	1	1.568	-0.001 ± 0.0004	0.157	<0.001	1	28.599	-0.004 ± 0.0002	0.741	<0.001	1	7.612	-0.013 ± 0.005	0.480	<0.001
Time ³	—	—	—	—	—	—	—	—	—	—	1	0.215	—	0.014	0.1
Residuals	33	3.217	—	—	—	69	7.614	—	—	—	68	5.177	—	—	—

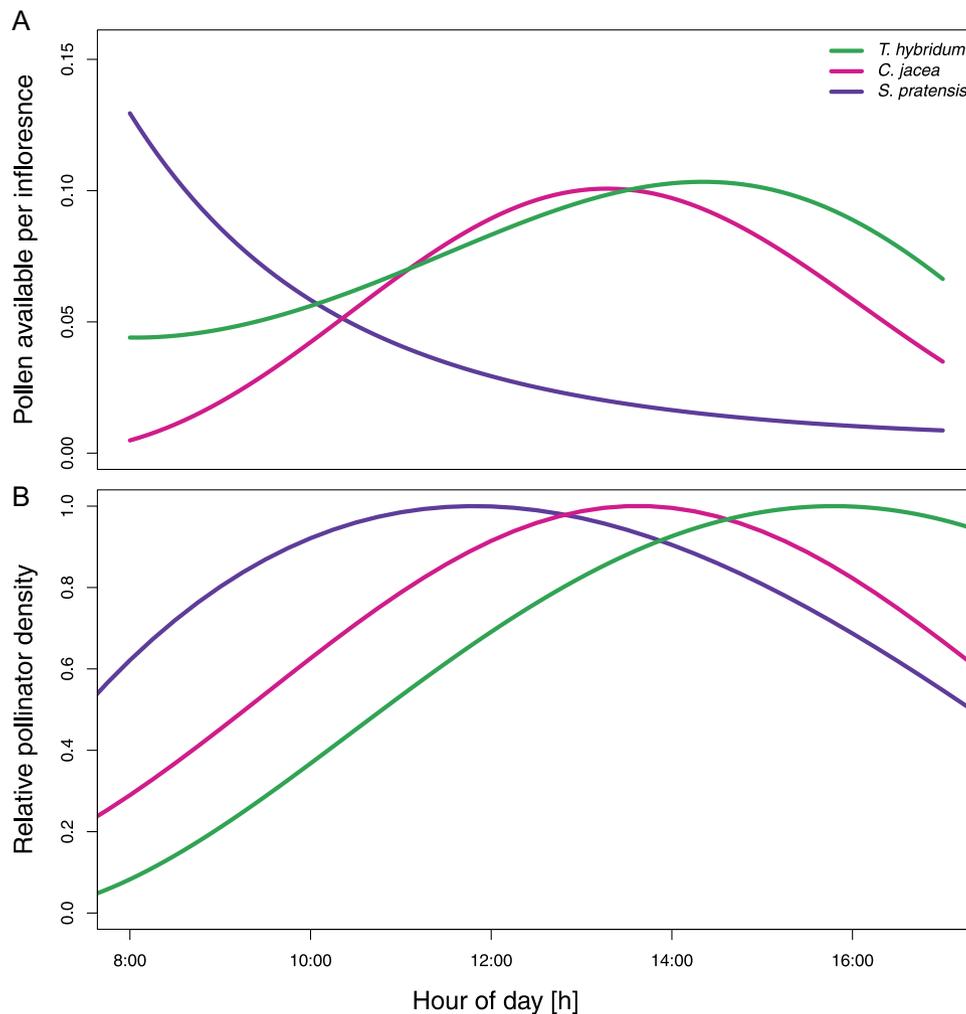


FIGURE 3 (A) Estimated relative amount of available pollen per inflorescence (predicted amount of pollen per flower × proportion of active flowers within an inflorescence) during the day. (B) Relative pollinator densities (predicted pollinator densities/maximum predicted pollinator density; i.e., peak pollinator density = 1) during the day. For graphs depicting pollinator densities and their variation, please see Appendix S1, Figure S3.

DISCUSSION

Our results showed a strong diurnal pattern in pollen availability of three coflowering plant species, closely matching the peaks in visitation by their pollinators. However, the mechanisms of the timing of the peak pollen availability differed among the studied plant species. The afternoon peak of *Trifolium hybridum* pollen availability (Figure 3A) was caused mainly by the pattern of opening of flowers within an inflorescence, while the average amount of pollen in flowers decreased only moderately during the day. This result is in line with the explosive release of pollen from anthers, which is expected to lead to the removal of most of the pollen from flowers after a single visit (Galloni et al., 2007).

On the other hand, *Centaurea jacea* released pollen gradually from 09:00 on, with a peak at approximately 13:00 and a decrease in the late afternoon (Figure 3A). Gradual pollen presentation was caused by combination of gradual release of pollen from flowers and gradual opening of individual flowers within inflorescences. Pollen in *C. jacea* flowers was present on the top of the narrow flower tubes (Figure 1B), which are accessible to pollinators for only a limited time.

Succisa pratensis released pollen in the early morning in easily accessible anthers (Figure 1C), and from that moment, the amount of available pollen decreased rapidly; pollinators had removed most pollen from flowers before 12:00. The number of open flowers remained constant throughout the day, suggesting a single cohort of flowers develops every day in the morning throughout the life of an inflorescence.

A diurnal pattern in pollen presentation in various plant species has been reported in numerous studies (Percival, 1950; Young and Stanton, 1990; Gregg, 1991; LeBuhn and Anderson, 1994; Stone et al., 1998; Nansen and Korie, 2000; Castellanos et al., 2006; Eisen et al., 2017). According to theoretical models proposed by Harder and Thomson (1989) and LeBuhn and Holsinger (1998), gradual pollen release and pollen presentation may strongly increase male fitness. Consequently, LeBuhn and Holsinger (1998) predicted strong evolutionary pressure on the development of pollen presentation schedules in plants (but see Eisen et al., 2017). However, the particular presentation schedules may differ in the amount of pollen available at a given moment, depending on the pollinator effectiveness: The presentation schedule should be more gradual with less-effective pollinators and vice versa (Harder and Thomson, 1989; Castellanos et al., 2006). The expectation for the optimization of the pollen presentation schedule according to the pollinator efficiency is in the line with our observation because studied plant species differed in their pollen presentation strategy; *C. jacea* and *T. hybridum* released pollen more gradually (Figure 2A, B), possibly because they are visited mainly by pollen-collecting bees (i.e., most pollen

collected during a visit is eaten), whereas *S. pratensis* is visited mainly by hoverflies (Appendix S1, Table S1), which are more abundant and possibly remove less pollen per visit. However, a detailed comparison of pollinator effectiveness is necessary to confirm this conclusion.

Our results also show different contributions of inflorescence development and architecture to pollen presentation. Whereas *T. hybridum* and *C. jacea* opened flowers within inflorescences gradually, *S. pratensis* did not, which resulted in a rapid decline in the amount of available pollen. This fact highlights the importance of including observations of inflorescence development in other studies of pollen presentation. Moreover, there is scarce evidence suggesting that changes in number of active flowers within inflorescence per day during the inflorescence development (Percival, 1950; Thomson et al., 1989; Young and Stanton, 1990). It would be therefore interesting to include this observation into the next experiments.

Pollinator visitation activity tracked the pattern of pollen availability. *Succisa pratensis* was the only species for which the maximum of pollinator visits did not match the actual peak in pollen availability (Figure 3). We hypothesize that the mismatch between pollen release and pollinator visitation activity is due to the overall diurnal pattern of activity of pollinators since we observed generally only a few pollinators before 8:30, then a steady increase in pollinator activity throughout the study site until approximately 11:00. Between 8:00 and 9:00, the microclimatic conditions typically change rapidly at the site; temperature and radiation increase, and relative air humidity decreases (Appendix S1, Figures S1, S2A–C), which is associated with evaporation of morning dew. Thus, we suggest that the peak in pollinator visits to *S. pratensis* is the result of an equilibrium between an overall increase in pollinator activity during the day and a concurrent decrease in the amount of available pollen on *S. pratensis*. *Succisa pratensis* also differs from the other two species in our study in also being an important nectar source, which may be the reason for the less-pronounced decrease in pollinator visits following the decrease in available pollen (compared to *C. jacea*). If such a shift in the sought-out reward indeed occurs, then the afternoon pollinator visits might contribute less to *S. pratensis* pollination than would be expected based on their still relatively high densities (Young et al., 2007). However, we did not measure the amount of available nectar or record pollinator foraging behavior in our study.

Schwarz et al. (2021) experimentally demonstrated the pollinator response to changing patterns in flower accessibility and revealed that diurnal reward availability is potentially an important driver affecting the structure of plant–pollinator interactions. Hence, overlooking the temporal structure of plant–pollinator interactions might lead to overestimates of pollinator generalization (Brosi, 2016) because plant–pollinator interactions are usually analyzed at the species level (e.g., Ballantyne et al., 2017, but see e.g.,

Dupont et al., 2011; Tur et al., 2014) without considering differences in visitation on diurnal scale. Our results indirectly suggest a strong temporal structure in the plant–pollinator interactions driven by pollen availability. A detailed investigation at a finer scale may reveal that generalized systems are temporally structured and more specialized than is expected (e.g., Lucas et al., 2018).

Differences in the timing of pollen availability and subsequent pollinator visitation between coflowering species may be advantageous for plant species sharing pollinators. While the temporal separation of pollen presentation is predicted to decrease heterospecific pollen transport and limit its negative effects on plant fitness, surprisingly little evidence supports that it takes place. Stone et al. (1998) showed that differences in pollen presentation match pollinator activity in several species that share pollinators. An investigation of heterospecific pollen transfer in pollinator-sharing plant communities showed that less heterospecific pollen is deposited on stigmas, which would be expected from the pollinator visitation network (Zhang et al., 2021). Whereas floral morphology is usually considered an important factor limiting heterospecific pollen transfer (Moreira-Hernández and Muchhala, 2019; Peuker et al., 2020; Zhang et al., 2021), the effect of differences in pollen presentation schedules is usually not investigated. Moreover, only a limited number of studies have compared pollen presentation schedules of several coflowering species (e.g., Stone et al., 1998), and to our knowledge, nobody has directly linked pollen presentation schedules in a coflowering community with pollen carry-over or pollen deposition by pollinators. Ballantyne et al. (2017) found minor differences in the peaks of pollen deposition in several plants in a coflowering community, suggesting that pollen presentation schedules differ; however, the experiment was not designed primarily to investigate this relationship. Similarly, some studies have shown diurnal variation in pollinator effectiveness in plant species, but did not further investigate causal mechanisms (Herrera, 1990; Stanghellini et al., 2002a; Rader et al., 2012; Willmer and Finlayson, 2014). Therefore, further research may reveal the relative importance of pollen presentation schedules in coflowering plant communities in comparison to other mechanisms that result in avoidance of heterospecific pollen transfer (such as pollen placement on different parts of the pollinator body, see Huang and Shi, 2013).

CONCLUSIONS

Our findings show differences in the pollen presentation schedules of three coflowering species and the response of the pollinator visitation activity in common grassland. Pollinators closely track the pollen availability of the studied plant species, which may decrease competition for pollinators and allow coflowering plants to share pollinators. Our results highlight the need to consider

temporal variation in plant–pollinator interactions even at the diurnal level.

AUTHOR CONTRIBUTIONS

Z.J. and J.Š. conceived the research idea. J.Š. and L.J. designed the method for sampling pollen and collected the pollen and inflorescence data. E.M., J.H., M.M., and Z.J. collected the data on plant and pollinator abundances. Z.J. and J.Š. analyzed the data. J.Š. and Z.J. wrote the manuscript with edits and comments from the rest of the authors.

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DATA AVAILABILITY STATEMENT

Data for this study are available at the Figshare repository: <https://doi.org/10.6084/m9.figshare.21136831>, <https://doi.org/10.6084/m9.figshare.21136834>, and <https://doi.org/10.6084/m9.figshare.21136810>.

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SUPPORTING INFORMATION

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

Appendix S1. Supporting information.

Figure S1. Microclimatic measurements 1.

Figure S2. Microclimatic measurements 2.

Table S1. Overview of pollinator visitors.

Table S2. Pollen production and average number of flowers per inflorescence.

Figure S3. Pollinator densities.

Figure S4. Absolute pollen availability during day.

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