Multi-year stigmatic pollen-load sampling reveals temporal stability in interspecific pollination of flowers in a subalpine meadow

Qiang Fang^{1,2}, Jie Gao³, W. Scott Armbruster^{4,5} and Shuang-Quan Huang¹

¹Inst. of Evolution and Ecology, School of Life Sciences, Central China Normal Univ., CN-430079 Wuhan, PR China

²College of Agriculture, Henan Univ. of Science and Technology, Luoyang, PR China

³Inst. of Horticulture, Henan Academy of Agricultural Science, Zhengzhou, PR China

⁴School of Biological Sciences, Univ. of Portsmouth, Portsmouth, UK

⁵Inst. of Arctic Biology, Univ. of Alaska, Fairbanks, AL, USA

Corresponding author: Shuang-Quan Huang, Inst. of Evolution and Ecology, School of Life Sciences, Central China Normal Univ., CN-430079 Wuhan, PR China. E-mail: hsq@mail.ccnu.edu.cn

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Abstract

Co-flowering plants may commonly experience interspecific pollination. It remains unknown, however, whether interspecific pollination is a largely stochastic process or consistent enough over years to exert selection for traits that can reduce interspecific pollination or ameliorate its deleterious effects on reproduction. To assess the likelihood of this precondition being met, stigmatic pollen loads on 17 to 34 insect-pollinated plant species over 3 consecutive years were scored in a subalpine meadow in southwestern China. Plant species varied significantly in the amount and proportion of heterospecific pollen (HP) on stigmas. Both the number of HP species and the proportion of the pollen load that was HP for each recipient species correlated positively between years (reflected in pairwise correlations for all year-by-year combinations). Although inter-annual variation was smaller for conspecific pollen (CP) than for HP loads, species tended to experience either consistently high or consistently low HP proportions across years. We found that species with higher stigmatic HP proportions generally experienced lower proportional variation in stigmatic HP, an unexpected result if high HP loads are the result of rare stochastic events. The novel finding of between-year consistency in stigmatic loads of heterospecific pollen suggests that adaption to stigmatic loads of HP is possible, and two divergent strategies may have evolved: HP avoidance and HP tolerance. The observation of temporally consistent differences among species in levels of HP supports the idea that natural selection may be operating either to increase tolerance or to minimize arrival of heterospecific pollen on stigmas in co-flowering plants. Such adaptations may be important for the maintenance of high levels of local plant diversity in biodiversity hotspots such as our study area.

Keywords: avoidance and tolerance; co-flowering species; conspecific pollen, heterospecific pollen, interspecific pollination, pollen deposition, species coexistence, temporal dynamics

Introduction

Pollinators moving among flowers in diverse plant communities may transfer not only conspecific pollen (**CP**) but also heterospecific pollen (**HP**) (Armbruster and Herzig 1984, Feinsinger et al. 1986, Waser et al. 1996, Morales and Traveset 2008). In generalized pollination systems especially, significant levels of pollinator sharing and interspecific pollinator movement often result in pollen transfer from anthers of one species to the stigmas of other species (Grabas and Lavery 1999, Flanagan et al. 2009, Huang et al. 2015, Fang and Huang 2016a). HP transfer appears to be, indeed, common in communities with multiple co-flowering species. A literature survey reported that almost no plant species was immune to HP transfer in communities with multiple co-flowering plant species, and about half of the species had HP on the stigmas of over 50% of their flowers (Ashman and Arceo-Gómez 2013). Investigations of numerous plant species within communities have demonstrated great variation in the proportions of HP (%HP), from less than 0.1% to ~70% of total stigmatic pollen loads (Montgomery and Rathcke 2012, Fang and Huang 2013). HP transfer is deleterious to the donor male function (loss of pollen that might otherwise fertilize conspecific ovules; Muchhala and Thomson 2012) and also often the female function of recipients (reduced sexual reproduction caused by stigma clogging, pollen interference, and/or increased risk of pollen limitation; Armbruster and Herzig 1984, Campbell and Motten 1985). These effects have potential ecological consequences because they may generate strong reproductive interference (including competition for pollinator services) among species, which can, in turn, structure the composition of plant communities (Armbruster et al. 1994, Armbruster 1995, Morales and Traveset 2008, Sargent and Ackerly 2008, Mitchell et al. 2009, Muchhala and Thomson 2012).

Pollen transfer by pollinators from one plant species to another might be an occasional stochastic event, as some theoretical models suggest (Kallimanis et al. 2009). Indeed, plant-pollinator networks have been observed to vary spatially and temporally with variation in local pollinator assemblages (Alarcón et al. 2008, Lázaro et al. 2009, Vázquez et al. 2009, Traveset et al. 2015, Fang and Huang 2016b, CaraDonna et al. 2017), suggesting spatial and temporal variation in rates of interspecific floral movements by pollinators. However, plants sharing pollinators do not necessarily

exchange large quantities of HP (e.g. Armbruster et al. 1994; Fang and Huang 2016a). It has been suggested that multiple possible reproductive strategies may minimize the deleterious effects of interspecific pollinator moves, thus promoting the coexistence of plant species in natural communities (Sargent and Ackerly 2008, Ashman and Arceo-Gómez 2013).

The unexpectedly high frequency of interspecific pollination in some plant communities (McLernon et al. 1996, Montgomery and Rathcke 2012, Emer et al. 2015, Fang and Huang 2016a, Arceo-Gomez et al. 2016, Johnson and Ashman 2019) has stimulated new ideas about floral adaptation in diverse communities of co-flowering plants. One hypothesis posits that plants whose stigmas consistently receive large amounts of HP may have evolved tolerance mechanisms, while those experiencing consistently lower levels may have instead evolved avoidance mechanisms, further decreasing receipt of HP (Ashman and Arceo-Gomez 2013, Arceo-Gomez et al. 2016). This is an interesting new way to look at species interactions in communities of co-flowering plants. However, we lack critical tests of this tolerance-avoidance hypothesis. For example, do among-species differences in HP receipt reflect largely stochastic processes or do the differences persist across multiple years, as the hypothesis requires? Between-year consistency in among-species variation in amounts of interspecific pollination is a precondition for, and an expected result of, the evolution of tolerance and avoidance strategies, respectively. In order to test this idea, we assembled data from a large multi-year study of the species composition of pollen loads on stigmas. We tested three specific predictions of the tolerance-avoidance hypothesis: 1) species should vary significantly from one another in the amount and proportion of heterospecific pollen on their stigmas; 2) the relative proportions of HP that species receive should be relatively consistent across years, as would be reflected in year-to-year correlations in the percents of heterospecific pollen on recipient stigmas; 3) alternatively, if HP deposition reflects purely chance events, the proportional variation in HP should vary randomly or depend on the proportion of the heterospecific species surrounding the recipient species. The null expectation is that the CV of %HP is similar in species with high and low %HP. Our 3-year study of CP and HP receipt in co-flowering plants in a diverse subalpine

meadow in southwest China is the first assessment of temporal stability in interspecific pollination, allowing us to test these hypotheses for the first time.

Materials and methods

Study sites and sampling

Species were sampled in subalpine meadows or forest edges on a hillside at a field station, the Shangri-La Alpine Botanical Garden (SABG; 27°54'5"N, 99°38'17"E, 3300m to 3350m a.s.l.), Shangri-La, Yunnan Province, southwest China, during three summer flowering seasons. Locally, over 100 plant species flower during July and August (Fang and Huang 2012). In 2011, 17 flowering species that were relatively abundant in a natural, sloped meadow (60 m × 150 m) were randomly sampled (Table S1). As flowering individuals were usually patchily distributed, we collected stigmas from numerous patches of each species, reducing confounding effects of neighbourhood context on pollen receipt. In 2012 and 2013, a further 17 species widely distributed within the same meadow in SABG were added to our sampling list (Table S1). In total, we investigated 33 hermaphrodites with bisexual flowers and one gynodioecious species (*Cyananthus delavayi*) from 15 families (Table S1).

To examine stigmatic pollen loads, we collected stigmas from flowers in late anthesis on clear days for each species during its peak blooming period (Fang and Huang 2013, 2016a), primarily during 5-10 August each year. In each species, a total of 41-59 stigmas per year were sampled. When collecting the stigmas, we did not record the individuals, which limited the use of stigmas collected from the same plant as independent replicates. Usually two, or occasionally three, stigmas were collected per plant from over 20 “random” individuals. These stigmas were stored separately in 70% ethanol in clean microcentrifuge tubes. In the laboratory, each stigma was squashed on a slide (Fang and Huang 2013, 2016a). The pollen grains that fell off during storage were centrifuged and transferred from the bottom 30 µl to a slide. Conspecific and heterospecific pollen grains on each stigma were examined under a microscope at 400x and identified on the basis of morphological features including size, shape, darkness and exine ornamentation (Fang and Huang 2016a). Pollen grains were identified to species or morphospecies (hereafter, species) using a pollen reference collection from all species

observed flowering at the same time. The numbers of conspecific and heterospecific pollen grains, and the number of species of heterospecific pollen on each stigma were recorded; data are available in an online supplemental Excel file (Table S1). The %HP was defined as $HP/(CP+HP) \times 100\%$. The mean numbers of stigmas examined per species were 51.1 ± 3.2 (Mean $\pm$ SD) in 2011, 54.1 ± 0.9 in 2012 and 54.0 ± 0.9 in 2013. The biological significance of observing no HP on stigmas differs drastically between cases where pollen is absent and where the pollen load is pure CP. We therefore excluded from the analysis the small fraction of stigmas (0.8%, $n = 34$) that received no pollen grains, but we included the stigmas that received only CP.

Data analysis

Prior to between-year correlation analyses of stigmatic pollen loads and analyses of stigmatic pollen-load variation among species and years, proportions and percents were transformed to improve normality using Zar's (1996, p. 283) modification of Freeman and Tukey's (1950) angular transformation of proportions. This method was chosen because it deals especially well with "excessively" numerous zeros and ones in a data set, as was observed in our data. After transformation, deviation from normal was assessed graphically and found to be acceptably small. Heteroscedasticity was not so easily dealt with, and caution should be applied in interpreting hypothesis tests. Note that additional caution is warranted, because the error degrees of freedom are potentially as low as half those specified in the analyses.

General Linear Model analysis (GLM) was conducted on the transformed proportion of the pollen load that was heterospecific [(no. HP pollen grains)/(no. total pollen grains per flower); %HP], treating both year and species as random factors, and including the species-by-year interaction term and, initially, the total-pollen load. The total pollen-load term was dropped in the final analysis because it had a small effect size (ca. 0.1% of the species effect size) and was not significant ($P = 0.12$), and the AICc information criterion of the model including it was marginally larger than in the simpler model ($\Delta AICc = 0.404$).

To check the robustness of this analysis, patterns of heterospecific pollen arrival were also analyzed with a generalized linear model, with HP entered as a count, the error

distribution specified as negative binomial with log link, and the effect of total pollen controlled by including conspecific pollen as a covariate. Because the statistical results were nearly identical, these results are reported in a supplemental table, but not discussed further.

To evaluate the among-species dynamic of pollen deposition, the coefficient of variation (CV) of total stigmatic pollen, CP and HP loads, number of HP species, and HP proportion were calculated for each species. The CV (s.d./mean) was calculated by treating each stigma as one estimate of HP and CP. Because one stigma was collected from each of two, or rarely three flowers per plant, the error degrees of freedom are arguably about half reported in the analysis, so caution should be employed in interpreting significance, although effect sizes should be unaffected by this design issue. Pearson correlations were used to compare the HP percents, CVs of HP load size, CP load size and HP proportions for each species between years (pairwise).

We used hierarchical cluster analysis (between-groups linkage, squared Euclidean distance) to categorize the plant species by the HP proportion, separately for each year, and also calculated the average HP proportion for the three years. The species with %HP higher than 67.0%, 58.8% or 44.5% were classified into high %HP category in 2011, 2012, and 2013, respectively. Species with %HP lower than 21.7%, 12.3% or 11.7% were classified into low %HP category in 2011, 2012, or 2013, respectively. All other species were classified into the intermediate category. Then, we calculated the average %HP in each category across years. Analyses were performed in SPSS 19.0 or SPSS 24.0 (IBM Inc., New York, NY, USA).

Results

Among-species variation in pollen receipt

Over three years, we examined stigmas of 4,479 flowers, counting a total of 611,956 pollen grains, of which 534,907 were conspecific (87.4%) and 77,049 were heterospecific (12.6 %) (Table 1, S2). From 2011 to 2013, 95.5%, 97.2% and 98.6% of stigmas received conspecific pollen, respectively, ranging from an average of 2.8 to 529.5 grains of the CP per species in 2011, from 1.4 to 2168.2 grains in 2012, and from 5.1 to 1717.1 grains in 2013. Heterospecific pollen was deposited on 81.1%, 67.6%, and 61.3% of stigmas in the

three years. Mean numbers of heterospecific pollen grains on stigmas were 8.8, 10.6 and 27.8 from 2.3, 1.9 and 2.4 other plant species, ranging from 1.0 (*Phlomis tatsienensis*) to 36.4 (*Silene asclepiadea*) in 2011, from 0.3 (*Onosma confertum*) to 146.3 (*Silene asclepiadea*) in 2012 and from 0.1 (*Lotus corniculatus*) to 400.2 (*Silene chungtienensis*) in 2013 (Table S1). The average proportion of pollen grains that were heterospecific in each year was 29.3%, 17.0% and 16.6%, respectively, and varied greatly among species, from 4.1 to 82.4% in 2011, from 0.2 to 65.4% in 2012, and from less than 0.1 to 70.1% in 2013 (Fig. 1, Table S2).

The GLM analysis indicated that species identity contributed significantly ($P < 0.001$) to variation in %HP; the among-year contribution was weaker, but still significant ($P = 0.041$), and there was a highly significant species-by-year interaction ($P < 0.001$; Table 2). The generalized linear model analysis of heterospecific pollen counts with negative binomial error distribution yielded nearly the same results (Table S3).

Among-year dynamics of pollen receipt

Although the stigmatic pollen loads and proportions of pollen loads that were heterospecific varied significantly among species and years, the among-species patterns were similar across years. The number of HP species and proportion HP load for each recipient species were both positively related across all pairwise-year combinations (Fig. 2, Table 3). The relationship of pollen receipt between consecutive years (2011-2012, 2012 -2013) was stronger than between non-consecutive years (2011-2013; Table 3). These results indicate that, across years, co-flowering plant species consistently received similar proportions of foreign pollen, even though the pollen loads fluctuated among years. For example, in all three years, *Cynoglossum amabile* received only a few HP grains per stigma, accounting for <1% of total pollen, while *Silene asclepiadea* received hundreds of HP grains accounting for over 50% of total pollen in all years.

Based on a separate hierarchical cluster analysis for each year, %HP of each species was assigned to Low, Intermediate or High categories. Low and high %HP categories were, on average, 10.7% (11 species) and 75.5% (3 species), respectively, in 2011; 5.9% (19) and 62.1% (2) in 2012; and 4.6% (21) and 55.3% (5) in 2013 (Fig. 3). Taking the results of the three years together, only three species (*Cyananthus delavayi*, *Pterocephalus hookeri* and *Silene asclepiadea*) could be categorized into the high %HP

group ($57.3\% \pm 6.4\%$), while 21 species were assigned to the low group across the three years ($6.4\% \pm 4.0\%$, Fig. 3). The average %HP varied by less than 10% (Max - Min) across years in 64.7% (22/34) of species.

Species-level variance patterns across years

The CV of HP load was higher than the CV of CP load in 2012 (paired t test, $t = 3.96$, $p < 0.01$, $df = 33$) and 2013 ($t = 5.44$, $p < 0.01$, $df = 33$), but not in 2011 ($t = -0.32$, $p = 0.75$, $df = 16$). Taking all three years together, the CV of HP was significantly greater than that of CP ($t = 6.20$, $p < 0.01$, $df = 84$), suggesting that CP receipt generally has lower variation than HP receipt. Interestingly, the CV of proportion HP was lower in the high %HP groups than it in the low %HP group (Fig 4, $t = -3.95$, $df = 59$, $p < 0.01$). The low CV in high %HP species indicated the similar %HP among individuals. The high CV in low %HP species may be due to a few incidents of HP deposition in some flowers, while most other flowers received no HP. This could represent random ‘mistakes’ by pollinators. The among-species patterns of variance in number of HP deposited were positively and significantly correlated only in comparing counts from 2012 with those from 2013 (Table 3b, the comparison with the largest sample size and power). Coefficients of variation (CV) of %HP were positively correlated in all three year-to-year comparisons (Table 3). Overall, these results indicate that proportional variations in %HP (i.e. after removing the effects of temporal variation in total pollination) are consistent across years and likely a characteristic of each species. Indeed, the correlations of the CVs of %HP were at least as strong as the correlations of the %HP.

Discussion

Our study of pollen receipt in 34 plant species across consecutive years showed that amounts of total, conspecific, and heterospecific pollen varied significantly among species and years. The number of pollen species and the proportion of total stigmatic pollen load that was heterospecific also varied among species and years. The significant species-by-year interaction shown by the GLM analysis indicates that not all species varied in exactly the same manner across years; this is unsurprising given the multiplicity of environmental factors influencing abundances and activity levels of different

pollinator species. However, plant species with higher (or lower) %HP in one year were likely to have higher (or lower) %HP in later years, respectively.

Although both CP and HP loads varied greatly among species and among years, proportional variation (CV) in CP was generally lower than in HP. Temporal variation showed a species-specific pattern: while most species usually experienced low or intermediate load proportions of HP on stigmas, others experienced a high proportion of HP across years, suggesting that variation in HP receipt among plant species is likely the result of differing species “properties” rather than the sum of occasional random events. This new finding of some degree of temporal stability in HP interference is consistent with the idea that strategies of HP-avoidance or tolerance may have evolved in some species (Ashman and Arceo-Gómez 2013). In the absence of such consistency, the evolution of HP tolerance seems unlikely. Thus, this consistency can be seen as a necessary, although, alone, not sufficient, piece of evidence for the HP-tolerance hypothesis.

In an analysis of HP receipt in 72 species from 13 published studies, 55.6% (40/72) of species received less than 10% HP, but nearly 10% (7/72) of species had over 50% HP on their stigmas (Ashman and Arceo-Gómez 2013). A previous study of the same meadow reported on here showed the overall %HP to be 16.4%, with 56.6% of species having < 10% HP, and only two *Silene* species receiving over 50% HP (Fang and Huang 2013). Overall, CP accounted for around 80% of stigmatic pollen loads for three years in our study meadow, despite the HP percent varying greatly across species from less than 1% to 64.7%, indicating that most species experienced interspecific pollinations infrequently.

Assuming that heterospecific pollen loads on stigmas are deleterious, one can hypothesize that exposure to high HP receipt would exert selection for either reduction of HP on stigmas or tolerance thereof, two alternative evolutionary strategies that could contribute to species coexistence (Ashman and Arceo-Gómez 2013). Species that have evolved tolerance of HP receipt may ameliorate deleterious effects of HP through post-pollination events, such as protective pollen-pistil interactions, thus enabling continued pollinator sharing by co-flowering species. Following this logic, multivariate cluster analysis led us to categorize 21 species as low HP receivers (candidate HP

avoiders; 61.7% of species) and 3 species as high HP receivers (candidate HP tolerators; 8.8% of species).

In our study meadow, %HP in *Pterocephalus* and *Silene* species was always relatively high, consistent with the idea that these species may have evolved tolerance mechanisms (Ashman and Arceo-Gómez 2013), such as the deleterious effects of HP becoming ameliorated during pollen-pistil interactions. Tolerance of HP receipt in *Silene* was supported by our hand pollination treatments imposing high vs. low HP loads in three *Silene* species: seed set per flower with high HP receipt was not significantly lower than in those with low HP loads (Kai Hao, Scott Armbruster and Shuang-Quan Huang, unpublished data). The rates of HP deposition may also be affected by the density of focal and surrounding species. For example, in a recent study, the size of HP loads was observed to be positively related to the flower abundance of the donor species (Arceo-Gómez et al. 2016a). This result, however, is not inconsistent with the view that %HP is influenced by intrinsic species properties such as floral symmetry, corolla openness, and degree of style exertion (Ashman and Arceo-Gómez 2013; Fang and Huang 2013). For example, HP-load size and pollen-species number varied among six *Pedicularis* species with abundant flowers. But, because these species had restrictive corollas with specialized buzz pollination, %HP was comparatively low (Tong and Huang 2016). In contrast, %HP loads were consistently much higher in *Silene* species (Fig. 1; Fang and Huang 2016)

Species receiving relatively little HP are less likely to have evolved tolerance but may have instead evolved avoidance mechanisms further reducing HP deposition on stigmas. Alternatively, species receiving low HP loads may be “preadapted” to low levels of HP arrival, in that they have previously evolved floral specializations that lower HP arrival rates, but the specializations evolved in response to completely different selective pressures. In either case, we expect species with low HP loads to be more sensitive to the negative effects of HP than putative tolerators. This expectation seems to be borne out in some species in our study meadows. For example, we observed that in six co-flowering *Pedicularis* species, species with long styles received some HP on stigmas (< 5%), but those with short styles received none (0%). When heterospecific pollen from longer-styled species was placed on the stigmas of shorter-styled *Pedicularis* species,

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pollen tubes were able to grow down the full length of the style and had a negative effect on seed set (Tong and Huang 2016). This suggests that the species with short styles may be effective “avoiders” of HP. Similarly, increased deposition of heterospecific pollen has been shown to weaken the positive effect of conspecific pollen on seed production in a putative ‘avoider’, *Delphinium barbeyi* (Briggs et al. 2016). Members of *Clarkia xantiana* populations that had histories of exposure to HP had better conspecific pollen performance and smaller HP effects after hand pollination with a pollen mixture (80% CP + 20% HP) than did members of populations lacking HP-exposure histories (Arceo-Gómez et al. 2016). This supports the idea that tolerance of HP on stigmas may often evolve in populations with histories of substantial interspecific pollination.

It has been noted that plant species often experience high turnover in the composition of the pollinator fauna and visitation frequency, likely resulting in large variation within species in the quantity and quality of pollination service (CaraDonna et al. 2017, Chacoff et al. 2018). However, large temporal or spatial variation in plant-pollinator networks does not always lead to large variation in HP deposition on stigmas (Fang and Huang 2016a). Previous studies have shown that amount of HP received is often a species-specific characteristic, showing consistency across conspecific populations and even similarity among congeners (Tang and Huang 2016). For example, in *Delphinium barbeyi*, the proportion of HP was consistently low across sites (Briggs et al. 2016). Four of the species observed in this study were also observed in a nearby community (Fang and Huang 2016a) and could be similarly categorized into the three types of HP% (see Fig. 3). Among them, *Microula sikkimensis* had a restrictive flower shape and received very little HP, and *Silene asclepiadea* had extended stigmas and received large amounts of HP, representing two distinct situations as expected. The other two, *Potentilla lancinata* and *Geranium sibiricum*, both had unrestrictive flower morphology and exposed stigma, with variable, but generally intermediate, levels of %HP.

In sum, we found that the proportion of the stigmatic pollen load that was heterospecific varied less across years than across species. Within a species, the load was relatively consistent across subsequent years. This observation of a degree of year-to-year consistency in HP load, as reflected in the among-year correlations, is both a precondition

for the evolution of HP-tolerance mechanisms and also an expected outcome of there being two evolutionarily stable strategies (tolerance and avoidance) for “dealing with” interspecific pollinator movement. Although mechanisms of avoidance of HP loads in the face of interspecific pollinator movement have received considerable attention in the literature (e.g. Armbruster and Herzig 1984, Armbruster et al. 1994, Morales and Traveset 2008, Thomson et al. 2019), mechanisms of HP tolerance have hardly been investigated (Ashman and Arceo-Gomez 2013, Arceo-Gomez et al. 2016a, b).

Documentation of patterns of variation in HP has been considered to be a key to understanding its importance in floral evolution and species coexistence (Mitchell et al. 2009, Arceo-Gomez et al. 2016a, Fang and Huang 2013, 2016b). If all pollinators carry similar amounts of CP and HP, variation in HP receipt would be small (Arceo-Gomez et al. 2016a). This suggests that the species that we observed with high HP stigmatic loads were pollinated by pollinators carrying higher proportions of HP on each visit than were the pollinators of plant species with low proportion of HP stigma loads. This difference in HP interference was observed for three consecutive years (Fig. 4), suggesting that the two opposite strategies for minimizing the effects of HP receipt (avoidance or tolerance, Ashman and Arceo-Gómez 2013) might be operating in our natural alpine meadows. Of course, the %HP experienced by a species may be influenced by the community (neighbourhood) in which it is embedded. Because the community context is likely to be more variable across a species’ range than within a single sample meadow, further studies should assess the effect of community context on HP deposition.

In conclusion, our investigation of the pattern of pollen receipt in 17 species over three years, and an additional 17 species over two years, showed striking among-year consistency in the amount of heterospecific pollen received by species. We thus suggest that HP receipt is at least partly a property of species rather than entirely the result of occasional random events. Although it is a daunting task to study many species within communities across multiple years (see Arceo-Gómez et al. 2016a), further studies of spatial and temporal variation in intraspecific and interspecific pollination could provide better insight into the mechanisms for minimizing deleterious effects of interspecific pollinator movements, particularly assessing the effects of heterospecific pollen transfer

on female and male reproductive success in plant species experiencing large amounts of interspecific pollination (Mitchell et al. 2009, Thomson et al. 2019).

The Speculations:

It has long been accepted that the evolution of floral traits is often driven by the most effective local pollinators, i.e. those best promoting conspecific pollination. For example, empirical studies have shown the mechanical correspondence between floral shape and pollinator morphology across diverse lineages. However, studies of pollination networks have shown that heterospecific pollen transfer probably occurs frequently in most natural communities, suggesting that co-flowering species may have evolved adaptive strategies to minimize the deleterious effects of heterospecific pollen transfer. By assessing the frequency heterospecific pollen (HP) deposition in 17-34 co-flowering species over three years in a subalpine meadow, we found that the %HP of a given species was temporally stable and likely to be a property of each species in the community. We speculate that species with unrestrictive corollas are usually HP “tolerators” and those with restrictive corollas are usually HP “avoiders”. However, definitive confirmation of this hypothesis awaits further investigation.

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Figure legends

Fig. 1. Proportion of pollen load that is heterospecific across 34 species in two or three years of observations. Large differences among species in the proportion of heterospecific pollen, as well as some degree of consistency in these values across years, are presented. Error bars depict the standard deviations. Species codes below x-axis are: 1, *Cynoglossum amabile*; 2, *Onosma confertum*; 3, *Microula sikkimensis*; 4, *Ixeridium biparum*; 5, *Lotus corniculatus*; 6, *Phlomis tatsienensis*; 7, *Aster vestitus*; 8, *Trifolium repens*; 9, *Aster oreophilus*; 10, *Potentilla lancinata*; 11, *Halenia elliptica*; 12, *Swertia punicea*; 13, *Allium wallichii*; 14, *Delphinium tenii*; 15, *Epilobium wallichianum*; 16, *Prunella vulgaris*; 17, *Salvia przewalskii*; 18, *Euphrasia regelii*; 19, *Allium macranthum*; 20, *Spenceria ramalana*; 21, *Picris hieracioides*; 22, *Aconitum piepunense*; 23, *Stellaria yunnanensis*; 24, *Pimpinella diversifolia*; 25, *Polygonum runcinatum*; 26, *Silene yunnanensis*; 27, *Dipsacus asper*; 28, *Geranium sibiricum*; 29, *Silene chungtienensis*; 30, *Silene gracilicaulis*; 31, *Gentiana haynaldii*; 32, *Cyananthus delavayi*; 33, *Pterocephalus hookeri*; 34, *Silene asclepiadea*.

Fig. 2. Between-year correlations of mean proportions of total stigmatic load that are heterospecific (%HP) for each species. (a) 2012 vs. 2011 ($r = 0.82$, $N = 17$, $p < 0.001$); (b) 2013 vs. 2012 ($r = 0.76$, $N = 34$, $p < 0.001$) and (c) 2013 vs. 2011 ($r = 0.55$, $N = 17$, $p = 0.022$), indicating that among-species difference in %HP persisted across years and were especially similar in consecutive years. Figure is based on raw data, but statistics are based on angular-transformed data (see Methods).

Fig. 3. Qualitative classes of heterospecific pollen loads, based on hierarchical cluster analysis of 34 plant species for heterospecific pollen (HP) proportion of each year and average HP of three years. Plant species were categorized into low (gray square), intermediate (dark gray square) or high %HP (black square) groups. Species are ordered based on their similarity of HP%. Species codes correspond to Fig. 1. The species with %HP higher than 67.0%, 58.8% and 44.5% were classified into high %HP category and the species with %HP lower than 21.7%, 12.3% and 11.7% were classified into low %HP category from 2011 to 2013 respectively. Other species were classified into intermediate category.

Fig. 4. The relationship between the coefficient of variation (CV) of the proportion heterospecific pollen (HP) and the mean proportion across three years. Closed, triangle and open symbols indicate the species classified by the cluster analysis as high, intermediate and low proportion HP group, respectively. Plant species were separated into high, intermediate and low %HP categories at breakpoints (a) 67.0% and 21.7% in 2011; (b) 58.8% and 12.3% in 2012; (c) 44.5% and 11.7% in 2013, respectively.

Table Legends

Table 1. Total numbers of species, stigmas, and pollen grains received, including number of pollen species, conspecific (CP) and heterospecific pollen (HP), and means and standard errors (SE) of pollen receipt in the community.

(A) Pollen receipt	2011	2012	2013	Total
No. species	17	34	34	34
Total no. stigmas	864	1809	1806	4479
Total no. pollen grains	64074	239773	308109	611956
Grains/stigma, Mean $\pm$ SE	74.2 $\pm$ 7.5	132.5 $\pm$ 16.0	170.6 $\pm$ 12.9	136.6 $\pm$ 13.5
Conspecific pollen	56465	220600	257842	534907
Grains/stigma, Mean $\pm$ SE	65.4 $\pm$ 7.5	121.9 $\pm$ 16.0	142.8 $\pm$ 11.8	119.4 $\pm$ 13.1
Heterospecific pollen	7609	19173	50267	77049
Grains/stigma, Mean $\pm$ SE	8.8 $\pm$ 0.6	10.6 $\pm$ 1.3	27.8 $\pm$ 4.7	17.2 $\pm$ 1.1
No. HP species/stigma (Mean $\pm$ SE)	2.3 $\pm$ 0.1	1.9 $\pm$ 0.1	2.4 $\pm$ 0.1	2.2 $\pm$ 0.1
%Heterospecific pollen (Mean $\pm$ SE)	29.3% $\pm$ 1.1%	17.0% $\pm$ 0.9%	16.6% $\pm$ 0.9%	19.2% $\pm$ 0.9%

Table 2. Results of GLM (both species and year treated as random factors) examining variation in proportion of heterospecific pollen (%HP) across species and years. (The total pollen-load term was dropped in this analysis because it was not significant and the AIC of the model including it was marginally larger; $\Delta\text{AICc} = 0.404$.) Species differed significantly from one another in their proportions of heterospecific pollen (HP) received (after controlling for year), but the relationship was complicated by the species-by-year interaction.

		Type III				Effect Size	
		Sum of	df	Mean		(partial eta	
Source		Squares		Square	F	Signif.	squared)
Intercept	Hypothesis	670.959	1	670.959	55.644	< 0.001	0.897
	Error	76.737	6.364	12.058 ^a			
year	Hypothesis	13.798	2	6.899	6.456	0.003	0.208
	Error	52.406	49.036	1.069 ^b			
species	Hypothesis	242.036	33	7.334	6.792	< 0.001	0.821
	Error	52.903	48.988	1.080 ^c			
year * species	Hypothesis	52.778	49	1.077	22.118	< 0.001	0.198
	Error	213.977	4394	.049 ^d			

a. $0.953 \text{ MS}(\text{year}) + 0.870 \text{ MS}(\text{Sp_Code}) - 0.828 \text{ MS}(\text{year} * \text{Sp_Code}) + 0.006 \text{ MS}(\text{Error})$

b. $0.992 \text{ MS}(\text{year} * \text{Sp_Code}) + 0.008 \text{ MS}(\text{Error})$

c. $1.003 \text{ MS}(\text{year} * \text{Sp_Code}) - 0.003 \text{ MS}(\text{Error})$

d. $\text{MS}(\text{Error})$

Table 3. (A) Between-year correlations of mean pollen-receipt parameters for recipient species, including conspecific and heterospecific pollen (CP and HP). (B) Between-year correlations of CV of pollen receipt parameters. In each case, a significant correlation indicates year-to-year consistency in the mean amount of pollen (A) or variation in amount of pollen (B) each species receives relative to other species. In other words, the rank orders of means of the number and proportion of pollen grains on stigmas of recipient species are generally similar across years. The same is true of the variances of %HP.

	2011-2012 (n = 17)	2012-2013 (n = 34)	2011-2013 (n = 17)
(A) Pearson correlation of mean (r, p value)			
Total	0.411, 0.102	0.809, < 0.001	0.074, 0.778
CP	0.579, 0.015	0.927, < 0.001	0.227, 0.381
HP	0.746, 0.001	0.481, 0.004	0.272, 0.290
No. HP species	0.647, 0.005	0.866, < 0.001	0.640, 0.006
%HP	0.768, < 0.001	0.698, < 0.001	0.487, 0.048
(B) Pearson correlation of CV (r, p value)			
Total	0.482, 0.050	0.013, 0.940	-0.098, 0.708
CP	0.148, 0.571	0.229, 0.192	0.187, 0.473
HP	0.122, 0.640	0.793, <0.001	0.381, 0.131
No. HP species	0.362, 0.153	0.419, 0.014	0.457, 0.065
%HP	0.752, < 0.001	0.819, <0.001	0.774, < 0.001