

# Causes and consequences of variation in heterospecific pollen receipt in *Oenothera fruticosa*

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## Abstract

**Premise:** Heterospecific pollen transfer, the transfer of pollen between species, is common among co-flowering plants, yet the amount of pollen received is extremely variable among species. Intraspecific variation in heterospecific pollen receipt can be even greater, but we lack an understanding of its causes and fitness consequences in wild populations.

**Methods:** We examined potential drivers of variation in heterospecific pollen receipt in *Oenothera fruticosa*. We evaluated the relationship between heterospecific and conspecific pollen receipt and considered how visitation by different pollinator groups, local floral neighborhood composition, and flowering phenology affect the total amount and proportion of heterospecific pollen received. Finally, we tested whether variation in heterospecific pollen receipt translated into lower seed production.

**Results:** Heterospecific pollen was ubiquitous on *O. fruticosa* stigmas, but the amount received was highly variable and unrelated to conspecific pollen receipt. Heterospecific pollen receipt depended on pollinator type, the proportion of nearby conspecific flowers, and flowering date. Significant interactions revealed that the effects of pollinator type and neighborhood were not independent, further contributing to variation in heterospecific pollen. Naturally occurring levels of heterospecific pollen were sufficient to negatively impact seed set, but large amounts of conspecific pollen counteracted this detrimental effect.

**Conclusions:** Although selection could act on floral traits that attract quality pollinators and promote synchronous flowering in *O. fruticosa*, the risk of heterospecific pollen is equally dependent on local floral context. This work highlights how extrinsic and intrinsic factors contribute to intraspecific variation in heterospecific pollen receipt in wild plants, with significant fitness consequences.

## KEYWORDS

co-flowering, floral neighborhood, flowering phenology, interspecific pollen transfer, intraspecific variation, Onagraceae, pollen load, pollination, pollinator effectiveness, seed set

Over 80% of angiosperms rely on animal pollinators for successful pollination (Ollerton et al., 2011). Yet because most plants share pollinators with several to a few dozen other co-flowering species (Jordano, 1987; Waser et al., 1996), this success can be compromised by pollinator-mediated competition among plant species (Brown et al., 2002; Lopezaraiza-Mikel et al., 2007). Plants can compete directly for visitation by shared pollinators (Waser, 1978) or indirectly when shared pollinators transfer pollen between plant species, termed heterospecific pollen transfer (HPT;

Morales and Traveset, 2008). Heterospecific pollen transfer is extremely common in co-flowering communities, and its incidence and intensity vary markedly across species (McLernon et al., 1996; Fang and Huang, 2013; Arceo-Gómez et al., 2016; Tur et al., 2016), with implications for the evolution of floral morphology, species divergence, and community structure (Moreira-Hernández and Muchhala, 2019). Even as our understanding of the forces driving such interspecific variation grows (Ashman and Arceo-Gómez, 2013; Arceo-Gómez et al., 2016), investigations into

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the extent of variation in HPT within species and its underlying causes has scarcely begun (Moreira-Hernández and Muchhala, 2019; Ashman et al., 2020). Indeed, few studies sample thoroughly enough to comment on within-species variation in HPT (Arceo-Gómez et al., 2018). Strikingly, one study indicates that intraspecific variation in stigmatic loads of heterospecific pollen (HP) can be greater than interspecific variation in HP loads (Arceo-Gómez et al., 2016).

Most plant species are visited by a suite of generalist pollinator species (Waser et al., 1996). Indeed, studies examining the interactions within entire plant–pollinator communities have revealed broadly generalized interaction networks (Bascompte et al., 2003; Vázquez and Aizen, 2004). Visitation by the entire suite of pollinators need not be distributed evenly among individuals of a given plant species; instead, individuals—and even specific flowers on an individual—will be visited by some subset of pollinators (Bruckman and Campbell, 2014; Tur et al., 2014; Kuppler et al., 2016), contributing to intraspecific variation in pollination success. The effectiveness of any given pollinator depends in part on its morphology (e.g., body size), foraging behavior, and its visitation frequency (Sahli and Conner, 2007; King et al., 2013; Ballantyne et al., 2015; Koski et al., 2018; Page et al., 2019). Differences in these factors result in the common observation that floral visitors are not equally desirable as pollinators with respect to conspecific pollen (CP) deposition (King et al., 2013; Bruckman and Campbell, 2014; Koski et al., 2018). Pollinators are also known to vary in the quality of pollen they deliver (Herrera, 1987), but pollinator effectiveness with respect to HP deposition is less studied. Furthermore, fidelity to a given plant species within a foraging bout (floral constancy; Waser, 1986) is not necessarily widespread among pollinator taxa (Gross, 1992; Amaya-Márquez, 2009). Consequently, pollinators should also vary in the amount of HP and/or the proportion of HP relative to CP they transfer. In fact, the relationship between HP and CP deposited within species may be driven in part by pollinator assemblage (Arceo-Gómez et al., 2016).

The probability of HP receipt for any given plant might also be a problem of circumstance, specifically a plant's local neighborhood. Greater numbers of conspecific individuals within a local neighborhood are often associated with greater CP receipt (Ghazoul, 2005), at least up to a point (Benadi and Pauw, 2018), and can be more important than population-level abundance measures in determining reproductive success (Roll et al., 1997; Spigler and Chang, 2008). However, the impacts of co-flowering species in local floral neighborhoods on HP receipt are unclear (Morales and Traveset, 2008; Cariveau and Norton, 2009; Charlebois and Sargent, 2017; Ha and Ivey, 2017). For example, co-flowering neighbors may facilitate pollinator visitation (Mitchell et al., 2009; Morales and Traveset, 2009; Tur et al., 2016), but negatively affect the purity of pollen loads transported (Brown et al., 2002; Bell et al., 2005), leading to a positive relationship between HP and CP receipt (Thomson et al., 2019). However, precisely because pollinators diverge in foraging patterns and those patterns are often governed by the abundance and distribution of

flowering plants at small spatial scales (Feinsinger et al., 1991; Goulson, 2003; Ghazoul, 2005), the impact of the neighborhood on HP receipt likely varies with pollinator type.

Heterospecific pollen receipt might also change predictably across the flowering season or be stochastic. During the flowering period of a given species, large-scale changes in the diversity, relative abundance, and composition of plant species in the flowering community typically occur (CaraDonna et al., 2017; Kantsa et al., 2018). Meanwhile, the flowering schedules of individual plants are not evenly distributed across the season, and early versus late flowering plants could have different pollination outcomes (Stone et al., 1998; Kitamoto et al., 2006; Chen et al., 2017). In addition, synchronous flowering of individuals within a population can be positively associated with successful pollination because it increases the absolute number of available mates and can boost signals to pollinators against the backdrop of the flowering community (Elzinga et al., 2007; Bartkowska and Johnston, 2014). If this results in pollinators temporarily specializing on a given species during its peak flower, individuals may not only receive more CP but less HP. On the other hand, if HP receipt is steady or stochastic as a consequence of the background “noise” of the community, then the proportion of HP may instead change across the season with variable CP deposition. Currently, we know little about intraseasonal variation in HP receipt and the relationship between HP receipt and flowering phenology.

Interest in HPT is driven in large part because it is anticipated to have major fitness consequences for plants, though current evidence is equivocal, revealing a mixture of negative and neutral fitness effects (Morales and Traveset, 2008; Ashman and Arceo-Gómez, 2013; Moreira-Hernández and Muchhala, 2019). Most of these studies represent experimental hand pollinations of flowers with mixes of CP and HP (Moreira-Hernández and Muchhala, 2019) to test and isolate factors such as the arrival of HP relative to CP (Caruso and Alfaro, 2000; Bruckman and Campbell, 2016a), HP diversity (Ashman and Arceo-Gómez, 2011), and HP donor identity (Arceo-Gómez and Ashman, 2016; Arceo-Gómez et al., 2019). Fewer have investigated the fitness consequences of naturally occurring levels of HP receipt in wild populations (Briggs et al., 2016; Suárez-Mariño et al., 2019; Parra-Tabla et al., 2020). The documentation of potential HP impacts on pollen tube development and extreme within-species variability in HP receipt (Arceo-Gómez et al., 2016, 2018; Briggs et al., 2016; Fang et al., 2019) suggests an opportunity for natural selection on traits that better allow plants to avoid HP receipt, but only if such variation is associated with fitness differences in natural populations.

In this study, we examined variation in HP receipt and its potential causes and consequences in the perennial *Oenothera fruticosa* L. (Onagraceae). Specifically, we asked the following sets of questions. (1) How much variation is there in HP receipt among *O. fruticosa* individuals, and what is the relationship between CP and HP receipt? (2) To

what extent do pollinator identity, local floral neighborhood composition, and timing of flowering influence HP receipt? And, does the impact of local neighborhood depend on pollinator type? Finally, to address the significance of natural variation in HP receipt for plant fitness and its implications for microevolutionary dynamics, we asked (3) to what extent does this natural variation translate into variation in seed set?

## MATERIALS AND METHODS

### Study species and site

*Oenothera fruticosa* is an herbaceous, perennial native to eastern North America that occurs in open habitats such as fields and meadows. The species is hermaphroditic and blooms from June through July, producing showy yellow flowers that provide nectar as a floral reward. Individuals typically have 1 to 3 flowers open at a time; each flower contains an average of 150 ovules, opens in the morning, and lasts approximately 24 h. *Oenothera fruticosa* is self-incompatible and thus requires outcross pollination from insects (Silander and Primack, 1978). Pollinated flowers develop into dehiscent capsules that mature around 3 weeks after pollination.

We conducted the study at Fort Indiantown Gap, an active National Guard training center, in Annville, Pennsylvania, United States. Approximately 88 ha of training areas and ranges on the base have been designated for researching the plant and animal species that thrive in periodically disturbed grassland habitats. In June 2019, we tagged 122 *O. fruticosa* plants distributed across a 12-ha grassland that contains a diverse assemblage of >70 flowering plant species. Focal *O. fruticosa* plants were at least 2 m apart and tagged before flowering such that their blooming times were randomly distributed across the entire flowering season. From June to August, we randomly selected one flower per plant to follow from date of anthesis through to fruit set, quantifying pollinator visitation, pollen deposition, and seed production. We recorded diurnal insect visitation on each flower for approximately 1 h using high-definition video cameras (Sony, San Diego, CA, USA) during pollinator activity (08:00–16:00 hours) on warm, sunny days, capturing a total of 130 h of film. We note that although many *Oenothera* species are visited by both diurnal and nocturnal floral visitors, the importance of these groups as pollinators varies across species (Rhodes et al., 2017; Antón and Denisow, 2018; Krakos and Austin, 2021). Prior work has demonstrated that *O. fruticosa* is visited by diurnal pollinators including, butterflies, bees, and beetles, with no record of nocturnal visitors (Primack and Silander, 1975; Silander and Primack, 1978; Krakos and Austin, 2021). From the video footage, we identified pollinators and determined pollinator visitation rates of each, only considering insects that were observed contacting the reproductive structures of the flowers.

Pollinators captured on film were almost exclusively bees (*Bombus*, *Ceratina*, *Halictus*, *Lasioglossum*, *Augochlorini*) and flies (*Milesia virginica*, *Toxomerus*); there were no visits from lepidopterans and only two visits from coleopterans, representing 0.7% of total visits. Therefore, we considered and quantified pollinator visitation rates of four functional groups: large bees, small bees, large flies, and small flies, consistent with pollinator groupings used in other studies of HP and CP deposition (Koski et al., 2015; Bruckman and Campbell, 2016b). Large and small bees were the most common (40.8% and 41.8%, respectively), followed by small flies (11.4%) and large flies (6.0%). Details about pollinator visitation rates, including mean visitation rates and proportion of flowers visited by each pollinator group can be found in Appendix S1.

### Composition of local floral neighborhoods

We quantified the composition of the local floral neighborhood within a 1-m radius of each focal plant on the date visitation was filmed. This scale was chosen because it has been demonstrated to significantly influence pollinator visitation, conspecific pollen receipt, and seed production in a number of other grassland systems (e.g., Roll et al., 1997; Spigler and Chang, 2008; Cariveau and Norton, 2009). Within each neighborhood, we counted the number of open *O. fruticosa* flowers and open flowers (or floral units) of heterospecifics. We then calculated the proportion of conspecific flowers per neighborhood as the abundance of conspecific flowers divided by total flower number within the neighborhood. Floral units of heterospecifics were defined depending on the inflorescence structure of each species. For species with singular flowers, we were able to count individual flowers; for composite flowers or compact inflorescences with small flowers (e.g., asters), we considered each inflorescence as a floral unit. The most common co-flowering species across the focal neighborhoods were *Securigera varia* (40% of heterospecific flowers), *Erigeron annuus* (22%), and *Leucanthemum vulgare* (19%); the remaining 20 species in bloom each represented 5% or less, though they still occurred in up to 37% of neighborhoods (Appendix S2).

### Pollen deposition

We collected stigmas of all filmed flowers the day after filming, when flowers were wilted, using clean forceps and stored them in 70% ethanol until processing. Because *O. fruticosa* has relatively large stigmas, we used an acetolysis processing step (Kearns and Inouye, 1993) to remove stigmatic tissue and retain only pollen grains. We mounted post-acetolysis pollen samples in ethanol and examined under a compound light microscope (Nikon, Tokyo, Japan) to count conspecific and heterospecific grains. Pollen grains of *O. fruticosa* have a unique triangular shape and are

especially large ( $>100\ \mu\text{m}$  long), making them easily distinguishable from pollen grains of all other species in the community. We considered that pollen grains could be lost during wash steps of the acetolysis procedure. We captured the wash and quantified pollen lost in these steps for a subset of samples. The amount of pollen grains lost was minimal (mean pollen lost  $<1\%$  per sample), such that we effectively have complete counts per stigma.

## Fruit collection and seed set

Finally, we collected mature fruits approximately 3 weeks after flowering and stored them in coin envelopes until processing. In total, we were able to collect 93 fruits from the 122 flowers that were filmed. Some of the fruits went missing in the field or experienced herbivory and were not included in the analyses. Each fruit was carefully examined with a dissecting microscope to count fully developed seeds and unfertilized ovules. We calculated seed set as the number of developed seeds divided by total ovule number (the sum of developed seeds and unfertilized ovules).

## Statistical analyses

We evaluated the relationship between HP and CP deposition. We considered both linear and nonlinear relationships given these patterns may reflect different ecological mechanisms (Arceo-Gómez et al., 2016). We evaluated separate general linear models including a linear-response model (nontransformed data), log-linear model (HP was log-transformed), and log-log model (HP and CP were log-transformed), the latter two of which would indicate a nonlinear relationship between HP and CP. Due to a single zero-value for HP grains, we added one to HP grain number before log-transformation. We note that we also considered generalized linear models: a Poisson error distribution, which proved to fit poorly based on residuals and the dispersion parameter (results not shown), and a negative binomial, which proved better but with qualitatively similar results to the general linear models (results not shown).

We evaluated the contributions of different pollinator groups, local floral neighborhood composition, and flowering phenology to both the number and proportion of HP grains received using a linear model and a generalized linear model, respectively. In the former, we used the square root of HP grain number per stigma as the response variable to conform to model assumptions. In the latter, we modeled the proportion of HP received with a binomial error distribution and logit link function weighted by the total number of grains per stigma. Six of the flowers filmed were excluded from these analyses because of partially missing data ( $N = 116$ ). In both models, we included visitation by each of the four main pollinator groups (large bees, small bees, large flies, and small flies)

as independent binary predictor variables because of extreme overdispersion in the distribution of pollinator visitation rates. We also included as predictor variables: proportion of neighborhood conspecific flowers and date (Julian day) of flowering. Both predictors were  $z$ -transformed to allow for comparisons between their dissimilar scales. Finally, to test whether the impact of pollinators is context-dependent, we included interactions between each visitor group variable and proportion of conspecific flowers. However, to maximize power of our analyses to detect main effects, interactions were retained only when significant.

To test whether increasing HP receipt translates to lower reproductive output of *O. fruticosa*, we used a generalized linear model to model seed set following a binomial error distribution, weighted by total ovule number. This model included fixed effects for CP grain number, HP grain number, and their interaction. Julian day was included as a covariate to account for differences in seed set across the season. Each predictor was  $z$ -transformed to allow for comparisons between their dissimilar scales. All analyses, unless otherwise noted, were conducted using R 4.0.2 (R Core Team, 2020).

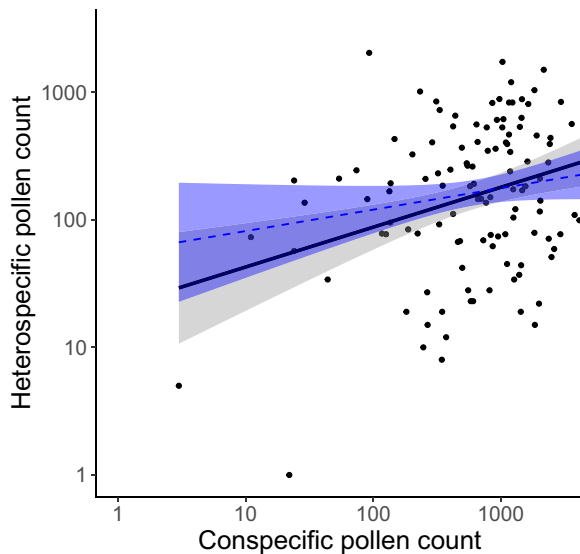
## RESULTS

### Conspecific and heterospecific pollen receipt

Deposition of HP on *O. fruticosa* flowers at our site was ubiquitous: all but one of the 122 flowers we examined received HP. On average, *O. fruticosa* flowers received 319.9 ( $\pm 362.8$  SD; range 0–2030) HP grains compared to an average of 998.7 ( $\pm 938.9$  SD; range 2–5016) CP grains. Both were highly variable; when scaled by the mean, HP receipt was more so ( $CV = 113$ ) than CP receipt ( $CV = 94$ ). Mean proportion HP per stigma was 0.30 ( $\pm 0.25$  SD) and highly variable as well (range 0–0.98). We did not detect a linear relationship between HP and CP grain number (i.e., untransformed data;  $F = 0.89$ ,  $df = 1$ ,  $P = 0.35$ ) nor a significant log-linear relationship ( $F = 3.01$ ,  $df = 1$ ,  $P = 0.09$ ). The log-log relationship between HP and CP grain number was significant ( $F = 8.10$ ,  $df = 1$ ,  $P = 0.005$ ), but this relationship was weak ( $R^2 = 0.06$ ; Figure 1) and hinged on two data points (removed:  $F = 1.34$ ,  $df = 1$ ,  $P = 0.25$ ).

Both the number of HP grains and proportion HP grains decreased significantly as the proportion of conspecific flowers increased in the neighborhood (Table 1). However, for proportion HP, significant interactions between proportion conspecific flowers and each pollinator group revealed that the effects of neighborhood composition and pollinator type depended on each other (Table 1, Figure 2). The negative relationship between proportion HP received and proportion local conspecific abundance was significantly steeper when plants were visited by large bees and small flies (Figure 2A, D). For flowers visited by large bees, this amounted to similar proportions of HP deposited

in neighborhoods where conspecific flowers ranged from low density to absent, regardless of whether large bees visited (Figure 2A). Yet as conspecific density increased, visitation by large bees becomes increasingly important,



**FIGURE 1** Linear regression of the relationship between heterospecific pollen and conspecific pollen deposited on *Oenothera fruticosa* stigmas. Both heterospecific pollen counts and conspecific pollen counts were log-transformed for analysis and are plotted here on a log scale. Shading indicates 95% confidence intervals for regression line. Black solid line and gray shading are for the entire data set ( $N = 122$ ); blue dashed line and blue shading are for the data set with two overly influential points removed ( $N = 120$ ).

such that the proportion of HP received was significantly lower for plants visited by large bees. Plants visited by small bees consistently received greater proportions of HP regardless of conspecific floral density. In this case, the interaction arises because the benefit of conspecific neighbors was substantially weakened for plants visited by small bees, i.e., proportion HP deposited was relatively constant under small bee visitation (Figure 2B). Surprisingly, visitation by large flies reversed the relationship between proportion HP receipt and percentage conspecific flowers (Figure 2C). This pattern remains significant even when we removed the data point representing the highest proportion HP received under large fly visitation (results not shown). In contrast to the results for proportion HP deposition, only small bee visitation significantly influenced the number of HP grains per stigma, increasing HP deposition independently of neighborhood composition (Table 1).

Additionally, we found that flowers opening later in the season received significantly greater proportions of HP (Table 1, Figure 3A). The number of HP grains, however, was not significantly affected by phenology (Table 1, Figure 3B). Thus, despite constant HP receipt across the season, the proportion HP increased because CP deposition declined later in the season ( $F = 25.80$ ,  $df = 1$ ,  $P < 0.001$ ; Figure 3C).

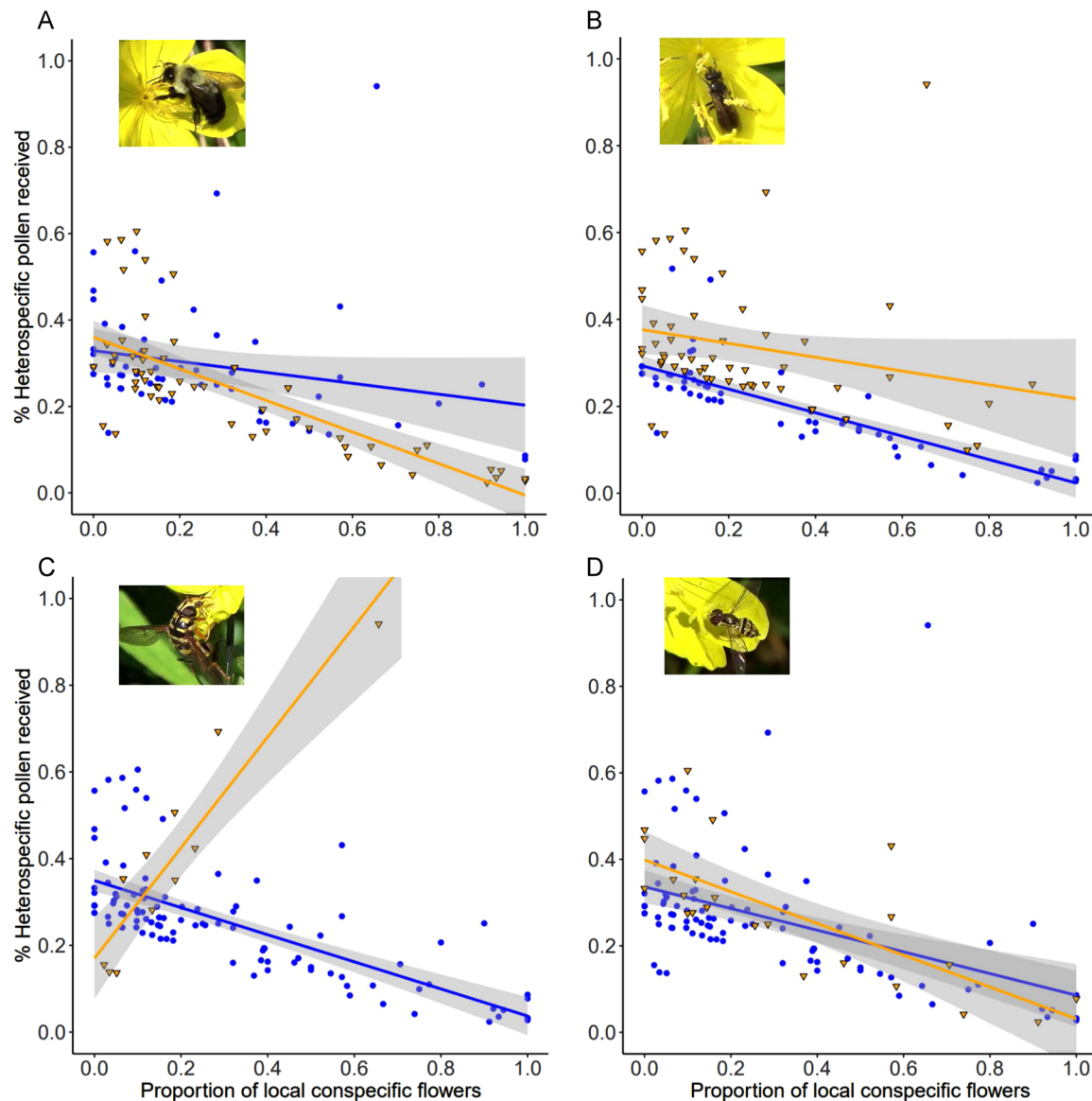
## The effect of pollen receipt on seed production

Seed set was highly variable ( $39.3\% \pm 27.5\%$ , mean  $\pm$  SD) and significantly influenced by the composition of pollen deposited and phenology (Table 2). We found that seed

**TABLE 1** Results from linear and generalized linear models on the effects of insect visitation, local neighborhood composition, and Julian day on heterospecific pollen receipt in *Oenothera fruticosa*

| Fixed effects           | Response variables   |               |              |              |                           |                |                  |
|-------------------------|----------------------|---------------|--------------|--------------|---------------------------|----------------|------------------|
|                         | Count of HP received |               |              |              | Proportion of HP received |                |                  |
|                         | df                   | Estimate      | F            | P            | Estimate                  | $\chi^2$       | P                |
| Large bee visitation    | 1                    | 0.628         | 0.151        | 0.698        | <b>-0.167</b>             | <b>155.80</b>  | <b>&lt;0.001</b> |
| Small bee visitation    | 1                    | <b>4.149</b>  | <b>5.911</b> | <b>0.017</b> | <b>0.341</b>              | <b>623.14</b>  | <b>&lt;0.001</b> |
| Large fly visitation    | 1                    | -3.502        | 1.342        | 0.249        | <b>0.642</b>              | <b>104.30</b>  | <b>&lt;0.001</b> |
| Small fly visitation    | 1                    | 0.933         | 0.213        | 0.645        | 0.033                     | 3.46           | 0.063            |
| %Con                    | 1                    | <b>-2.696</b> | <b>12.11</b> | <b>0.001</b> | <b>-0.401</b>             | <b>758.69</b>  | <b>&lt;0.001</b> |
| Julian day              | 1                    | -1.710        | 2.607        | 0.109        | <b>0.442</b>              | <b>1788.88</b> | <b>&lt;0.001</b> |
| Large bee $\times$ %Con | 1                    | —             | —            | —            | <b>-0.327</b>             | <b>464.34</b>  | <b>&lt;0.001</b> |
| Small bee $\times$ %Con | 1                    | —             | —            | —            | <b>0.198</b>              | <b>177.75</b>  | <b>&lt;0.001</b> |
| Large fly $\times$ %Con | 1                    | —             | —            | —            | <b>2.179</b>              | <b>484.12</b>  | <b>&lt;0.001</b> |
| Small fly $\times$ %Con | 1                    | —             | —            | —            | <b>-0.178</b>             | <b>102.78</b>  | <b>&lt;0.001</b> |

Notes: %Con is the proportion of conspecific flowers in local floral neighborhoods. The heterospecific pollen count model uses square-root-transformed counts of heterospecific pollen as a response with a Gaussian distribution. The proportion heterospecific pollen model uses a binomial distribution. Visitation by each pollinator group is coded as a binary predictor variable. %Con and Julian day were z-transformed. Significant model parameters are shown in bold.  $N = 116$ . Only significant interactions were retained for final models.



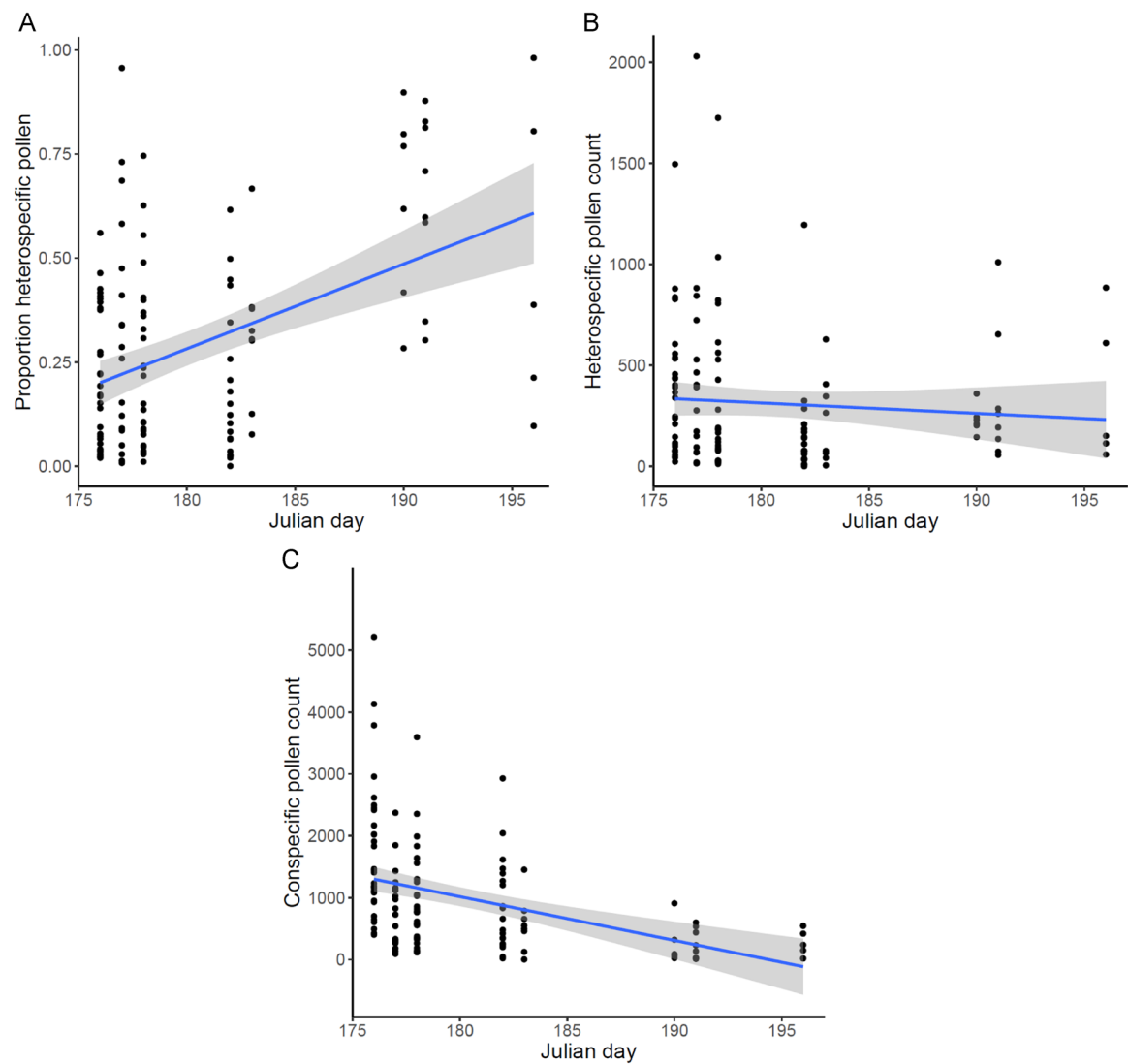
**FIGURE 2** Scatterplots of the proportion heterospecific pollen received by the proportion of conspecific flowers in local floral neighborhoods.  $N = 116$ . This relationship is mediated by the visitation of various pollinator groups: (A) large bees, (B) small bees, (C) large flies, and (D) small flies. Yellow triangles and lines represent flowers that were visited by each group; blue circles and lines represent flowers not visited by each group. Shading indicates 95% confidence intervals of each line

set was negatively related to the number of HP grains per stigma, positively related to CP grain number, and declined across the season (Table 2). However, there was also a significant interaction between HP and CP deposition (Figure 4). In particular, the negative impact of HP deposition was strongest at low levels of CP, below the mean ( $z$ -scaled mean = 0 in Figure 4) and then began to dissipate as the number of CP grains on the stigma increased. At the highest levels of CP receipt, there even appeared to be a positive impact of HP, though we caution that this area of the contour map was not well supported, based on a few data points of excessively high CP and HP. With respect to HP loads, we saw that greater CP receipt can rescue seed set of plants with heavy HP. Ultimately,

our data support that the proportion of heterospecific and the total number of CP and HP grains received influences seed set.

## DISCUSSION

Our study identified extrinsic and intrinsic factors shaping intraspecific variation in HP receipt. We revealed how the interaction between pollinator identity and local floral neighborhood, together with a plant's flowering phenology, affect HP deposition on *O. fruticosa* flowers. HP receipt was largely independent of CP receipt and sufficient to negatively impact seed set. Our study joins only one other in



**FIGURE 3** Scatterplots of the proportion of heterospecific pollen (A), count of heterospecific pollen (B), and count of conspecific pollen (C) for individual *Oenothera fruticosa* flowers through the season.  $N = 122$ . Blue lines depict linear regressions with 95% confidence intervals

**TABLE 2** Results from generalized linear model on the effects of pollen receipt and Julian day on seed set in *Oenothera fruticosa*

| Fixed effects              | Response variable |               |               |                  |
|----------------------------|-------------------|---------------|---------------|------------------|
|                            | Seed set          |               |               |                  |
|                            | df                | Estimate      | $\chi^2$      | $P$              |
| CP count                   | 1                 | <b>0.226</b>  | <b>57.62</b>  | <b>&lt;0.001</b> |
| HP count                   | 1                 | <b>-0.194</b> | <b>38.42</b>  | <b>&lt;0.001</b> |
| Julian day                 | 1                 | <b>-0.270</b> | <b>111.84</b> | <b>&lt;0.001</b> |
| CP count $\times$ HP count | 1                 | <b>0.289</b>  | <b>175.77</b> | <b>&lt;0.001</b> |

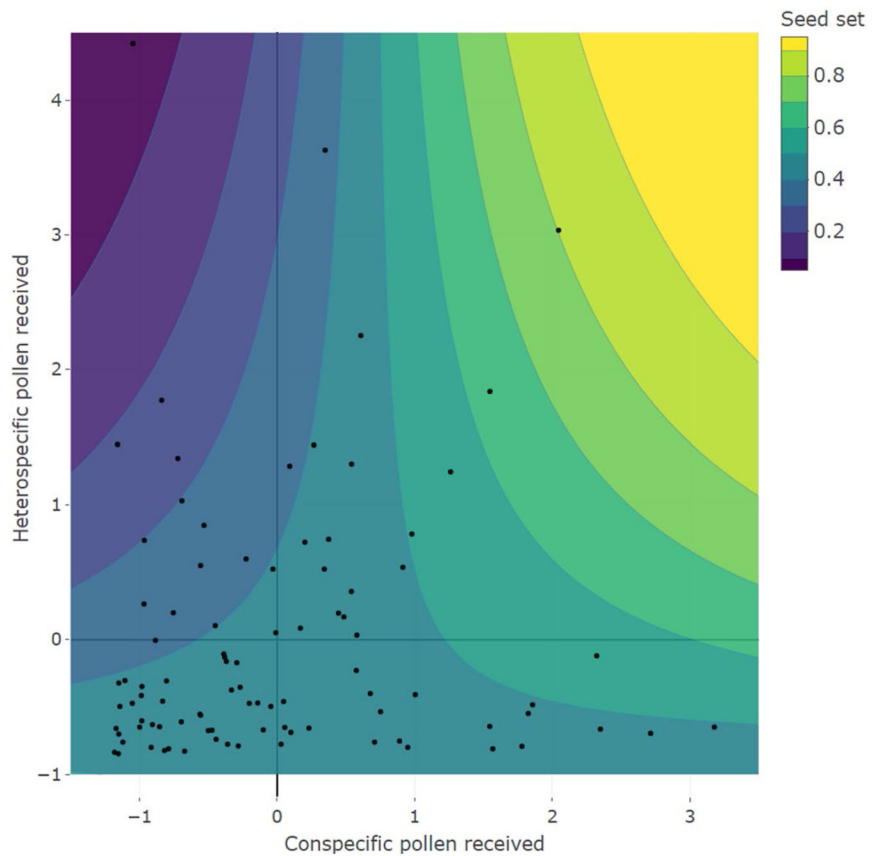
Notes: CP count and HP count are counts of conspecific and heterospecific pollen deposited on stigmas, respectively. This model uses seed set as a response with a binomial distribution. All predictor variables were z-transformed. Significant model parameters are shown in bold.  $N = 93$ .

demonstrating the consequences of HP loads for seed production in wild populations (Briggs et al., 2016). We discuss our results and their implications for natural selection to avoid HP receipt and its constraints.

### Intraspecific variation in heterospecific pollen receipt

Pollinators notoriously vary in their effectiveness at depositing CP (Herrera, 1989; King et al., 2013; Ballantyne et al., 2015), but their effectiveness with respect to CP need not correlate with their probability of depositing HP (Mitchell et al., 2009; Arceo-Gómez et al., 2016; Ashman et al., 2020). In general, we found that HP and CP deposition stigmas were not related—or at best only weakly so—in *O. fruticosa*. Arceo-Gómez et al. (2016) suggested that such independence could arise if one or few high-quality pollinators deliver nearly pure CP loads, while HP is brought stochastically by ineffective pollinators. The number of HP grains received by *O. fruticosa* varied only with visitation by small bees, but visitation by all pollinator groups influenced the *proportion* of HP received. Thus, all these pollinator groups likely bring varying amounts of CP with relatively consistent HP loads, resulting in a gradient of pollinator

**FIGURE 4** Contour plot representing predicted seed set values for given amounts of heterospecific and conspecific pollen deposited.  $N = 93$ . Pollen counts are z-transformed; 0 represents the mean observed value on each axis. Black dots represent data points used in the model. The detrimental fitness impact of receiving heterospecific pollen occurs in stigmas that received fewer than the mean value of conspecific pollen grains, but disappears for moderate/high CP values



effectiveness. Likewise, Fang et al. (2019) suggest that species receiving high average HP loads are likely to be visited by pollinators that carry relatively high, consistent amounts of HP.

Nearly half of the visits to *O. fruticosa* flowers in the population under study were by small bees, which are inefficient pollinators in some systems (Gorenflo et al., 2017; Koski et al., 2018; Konzmann et al., 2019). Indeed, two previous studies of pollinator importance in other *Oenothera* species found that small bees functioned as poor pollinators or pollen thieves (Artz et al., 2010; Rhodes et al., 2017). We showed that both the number and proportion of HP were greater for *O. fruticosa* flowers visited by small bees. In contrast, visitation by large bees, which comprised a similar proportion of visits as small bees, had no influence on the number of HP grains but was associated with a lower proportion of HP grains. Thus, the large bees deposited higher amounts or proportions of CP, relative to the less effective pollinators in our system. The comparison of HP loads from small and large bees was consistent with this interpretation; flowers that were visited only by small bees during our observation periods received slightly more HP and slightly less CP on average than those visited solely by large bees, and the proportion HP received was significantly lower for flowers visited by only large bees ( $\chi^2 = 1419.1$ ,  $df = 1$ ,  $P < 0.001$ ). The well-documented floral constancy of *Bombus* pollinators during foraging bouts may be responsible for this effect

(Goulson, 2003). Indeed, multiple studies have reported that large amounts of CP are lost when pollinators switch between species during a foraging bout (Flanagan et al., 2009; Muchhala and Thomson, 2012). For constant pollinators, the transfer of CP grains may be more strongly influenced by pollinator behavior than the transfer of HP grains, which appears to be a more consistent by-product of pollinator sharing. Nocturnal visitors have not been documented previously for *O. fruticosa*, but if present in this population, could also have contributed to HPT. Nevertheless, though we cannot empirically exclude their importance, we did see strong patterns of HPT related to diurnal visitors alone.

We further showed that HP deposition in *O. fruticosa* was context dependent, influenced by a plant's local floral neighborhood and the way pollinators interact with this neighborhood. Individuals in local neighborhoods with high proportions of conspecific flowers received fewer HP grains and a lower proportion of HP relative to CP. The strength and even the direction of impact of floral neighborhood, however, was mediated by pollinator group. Theoretical models predict changes in pollinator foraging behavior with changes in the relative densities of species in mixed plant assemblages (Goulson, 1994; Kunin and Iwasa, 1996). We found a steeper decrease in the proportion of HP received per increase in proportion of local conspecific flowers for flowers visited by large bees and small flies compared to flowers they did not visit. The difference in slope was strongest for large

bees, which might be expected for constant foragers, either because the absolute increase in local CP availability in neighborhoods with greater *O. fruticosa* relative abundance enables constant pollinators to bring in greater amounts of CP relative to HP or because their constancy (Waser, 1986) or preference (Smithson, 2001) increases with *O. fruticosa* density. The latter cases would lead to these pollinators visiting *O. fruticosa* disproportionately as its floral density increases. Flowers visited by small bees exhibited a weaker negative relationship between proportion of HP received and proportion of conspecifics in their floral neighborhoods. In neighborhoods with higher *O. fruticosa* abundance, this relationship translates to a greater proportion of HP on flowers visited by small bees compared to those that were not. This pattern is again consistent with small bees depositing pollen loads generally of poorer purity (more HP) and suggests that small bees foraging behavior is influenced less by the composition of local floral neighborhoods than that of other visitors.

Surprisingly, when large flies visited, the proportion of HP increased as local conspecific relative abundance increased. We suggest caution in interpreting this curious finding, given visits by *M. virginica* were relatively rare during our study. Still, we speculate that such a pattern could arise if large flies exhibit negative-frequency-dependent foraging (Smithson, 2001) on *O. fruticosa*, i.e., visitation to *O. fruticosa* is inversely related to *O. fruticosa* abundance. Negative-frequency-dependent foraging can occur when rarer pollinators must compete with more common pollinators for access to the more common resource, assuming that other plant species are equally rewarding (Eckhart et al., 2006). Alternatively, we observed the large fly, *M. virginica*, consuming pollen from *O. fruticosa* anthers and even the stigma. If instead these pollinators increase visitation to *O. fruticosa* when *O. fruticosa* is abundant, they might remove more CP from stigmas while still depositing HP. Consideration of neighbor identity might help unpack the interactions causing patterns involving large flies as well as those of the other pollinator groups. For example, co-flowering species with similar floral morphologies and colors (Stout et al., 1998; Bell et al., 2005; Liao et al., 2011) or substantially more attractive and rewarding flowers (Randle et al., 2018; Thomson et al., 2019) may encourage pollinators to switch between species more frequently during a given foraging bout and transfer more pollen between species.

Timing of flowering is well known to influence HPT dynamics and competition for pollinators more generally among species. In fact, these dynamics can lead to character divergence and shape community structure (Mosquin, 1971; Pleasants, 1980; Rathcke, 1983). Our results illustrate the significant role of flowering time in determining intraspecific variation in HP receipt. Specifically, the proportion of HP found on *O. fruticosa* flowers increased predictably with flowering date across the season, and the strength of this effect was as great as the effect

of local neighborhood. This increase in the proportion of HP occurred even though the number of HP grains received did not change with flowering time or at best declined weakly, because CP receipt declined significantly across the same period. Thus, as the flower season progresses, fewer individuals remained in bloom, reducing the amount of CP available for transport in the population. This shortage of CP and the consistent receipt of HP lead to increased proportions of HP for late-flowering individuals. Additionally, lower CP deposition rates at the end of the season were not due to decreased pollinator visitation ( $F = 14.88$ ,  $df = 1$ ,  $P < 0.001$ ;  $R^2 = 0.11$ ). Rather, the seasonal change in proportion HP deposition highlights the importance of flowering synchronously against a backdrop of steady HP arrival. Synchronous flowering increases the amount of CP available for transport in the population (Méndez and Díaz, 2001; Elzinga et al., 2007). Since *O. fruticosa* population-level flower abundance was positively skewed and leptokurtic in our study population (Smith et al., 2021), early flowering *O. fruticosa* plants were likely more synchronous with the population. Thus, synchronous flowering can not only benefit plants by increasing CP receipt, but also by reducing the proportion of HP received.

### Fitness consequences of heterospecific pollen receipt

Both the number and proportion of HP on stigmas influenced seed production, demonstrating a clear detrimental fitness effect of HP receipt in *O. fruticosa*. Although the fitness impacts of receiving HP have been shown in many hand-pollination experiments, recent work has shown similar negative impacts on CP tube growth (Suárez-Mariño et al., 2019; Parra-Tabla et al., 2020) and seed set (Briggs et al., 2016) in wild plant populations. There are many mechanisms by which HP can cause reproductive interference including stigma clogging (Galen and Gregory, 1989), pollen allelopathy (Murphy and Aarssen, 1995), style clogging (Randall and Hilu, 1990), and ovule usurping (Burgess et al., 2008). We suspect that interference by HP in our system likely took place on the stigmatic surface, given that HP tube growth in the style and via ovule usurpation is unlikely between distantly related species (Moreira-Hernández and Muchhala, 2019) and that no confamilial species bloomed during our study. Perhaps key is that we found that the interaction between the amount of CP and HP influences seed set; seed set was the lowest for flowers that received low amounts of CP and high amounts of HP. Low CP deposition alone, however, is unlikely the cause because seed set is relatively constant across the range of CP amounts seen in our study, so long as HP is also low (Figure 4). The proportion of HP on stigmas has been found to influence seed set in other systems as well (Thomson et al., 1981; Briggs et al., 2016) and is more suggestive of neutral dilution or allelopathic

effects of HP that reduce pollen germination rates and pollen tube growth of CP (Suárez-Mariño et al., 2019; Parra-Tabla et al., 2020). High levels of CP may be able to effectively neutralize the negative impacts of HP. Although detailed experiments are needed to identify the mechanism, our results suggest that there may be a threshold level of CP receipt *O. fruticosa* flowers can reach to avoid the detrimental impacts of HP receipt.

Given the substantial fitness impacts of HP, the question is whether and how selection could act for plants to avoid the detrimental impacts of HP receipt. It has been hypothesized that species may adopt alternative strategies of HP avoidance or tolerance to minimize the negative effects of HP receipt (Ashman and Arceo-Gómez, 2013; Fang et al., 2019). Extrinsic factors such as a plant's local floral neighborhood cannot be controlled, which combined with a constant background threat of HP deposition seen in our study, limits the ability of *O. fruticosa* to avoid HP. Instead, our findings suggest that the best defense against HP in *O. fruticosa* is to increase CP deposition. We expect to see selection for earlier flowering synchrony, which was associated with receiving lower proportions of HP relative to CP. In addition, although we did not measure stigma sizes in this study, if the large stigmas of *O. fruticosa* allow it to tolerate HP, as suggested for other species (Montgomery and Rathcke, 2012; Arceo-Gómez and Ashman, 2014), then selection could also favor plants with larger stigmas in this population. Finally, there is the opportunity for selection on floral traits in *O. fruticosa* that influence attraction of pollinators bringing low proportions of CP. For instance, Kuppler et al. (2016) found the composition of pollinators visiting individual *Sinapis arvensis* plants varied with floral phenotypes and influenced reproductive output. Other studies also found that pollinator groups discriminate between individuals based on floral traits like flower color (Briggs et al., 2018), floral scents (Parachnowitsch et al., 2012), and flower size (Conner and Rush, 1996; Mothershead and Marquis, 2000). Individual *O. fruticosa* would benefit from attracting fewer small bees because they bring more HP grains and higher proportions of HP.

## CONCLUSIONS

Our study highlights how pollinators interact with the composition of floral neighborhood at local spatial scales to influence intraspecific variation in heterospecific pollen receipt. By flowering earlier, and perhaps more synchronously, *O. fruticosa* can increase CP receipt and so offset the cost of constant HP receipt across the flowering season. The combination of intrinsic and extrinsic forces influencing HP receipt can help explain why heterospecific pollen is so ubiquitous among flowering plants (Fang and Huang, 2013; Arceo-Gómez et al., 2016; Tur et al., 2016), despite its detrimental impacts.

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## AUTHOR CONTRIBUTIONS

G.X.S. and R.B.S. conceived of and designed the study. G.X.S. conducted the study with help from M.T.S. G.X.S. analyzed the data, and G.X.S. and R.B.S. wrote the paper with contributions from M.T.S.

## DATA AVAILABILITY STATEMENT

Data used for this study available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.hqbkzkh1g9> (Smith Jr. et al., 2021).

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**Appendix S1.** Table containing details about pollinator visitation rates, including mean visitation rates and proportion of flowers visited by each group.

**Appendix S2.** Table containing the counts and presence of co-flowering heterospecific plant species across all floral neighborhoods.

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