

BRIEF COMMUNICATION

Floral traits influence the opportunity for selection among male gametophytes: independent and combined effects of style length and petal area

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PREMISE: Strong correlations between traits can obscure their independent effects on components of reproduction. Style length (SL) and petal area (PA) vary within species, for example, but their independent effects on the opportunity for selection among pollen genotypes are poorly understood. Previous work in *Clarkia* detected a positive effect of SL on pollen receipt, potentially intensifying selection. However, this apparent effect of SL may be influenced by a correlated trait, such as PA. Here, we examine the independent effects of these two traits on pollen receipt and performance.

METHODS: We collected petals and styles from wild populations of two insect-pollinated *Clarkia* taxa and estimated the independent and combined effects of SL and PA on pollen receipt and performance.

RESULTS: In both taxa, SL and PA are positively correlated. In *C. unguiculata*, both traits positively and independently affect pollen receipt, but in *C. xantiana* ssp. *xantiana*, the two traits act only in combination to affect pollen receipt. In both taxa, pollen receipt positively affects the numbers of pollen tubes entering and penetrating the style, as well as pollen tube attrition.

CONCLUSIONS: The effects of SL and PA on pollen receipt and performance are taxon specific. In *C. unguiculata*, both traits may be independent targets of selection due to their effects on pollen receipt. In *C. xantiana* ssp. *xantiana*, by contrast, the combined (but not independent) effects of SL and PA influence pollen receipt. Ecological differences between these taxa require exploration to understand the mechanisms by which these traits affect pollinator behavior.

KEY WORDS *Clarkia*; gametophytic selection; pollen attrition; pollen tube growth rate; pollination; sexual selection; style length.

Determining the role of natural selection in the evolution of individual traits within any population or species requires demonstrating the functional significance of variation in each trait (Armbruster et al., 2014). When traits are strongly correlated, accurate assessments of how each trait influences the process of natural selection require measures of their independent effects on fitness-related

traits or on individual fitness. Here, we examine the independent and the combined effects of two floral traits—style length and petal area—on two critical early-stage reproductive phases: pollen receipt and post-pollination pollen performance. While these floral traits have each been studied individually in many investigations (citations below), to our knowledge this is the first to examine

their independent effects on both pollination and post-pollination performance.

Style length varies within and among populations of many homostylous species, and this variation has a variety of short- and long-term ecological and evolutionary consequences. Style length has been found to influence the mechanical fit between flowers and their pollinators (Newman et al., 2015), reproductive isolation (Nista et al., 2015), seed production (Yang and Wang, 2015), outcrossing rates (Medrano et al., 2012; Irwin et al., 2016), and the amount of pollen adhering to stigmas (Mazer et al., 2016, referred to here as “pollen receipt”). It is not always clear, however, whether these apparent effects are causal or indirect. In the case of pollen receipt by receptive stigmas, for example, style length may be correlated with other floral traits, such as floral longevity, stigma area, or petal size, which may also directly and positively affect pollen receipt. In theory, the apparent effect of style length on pollen receipt could be due entirely to the effects of such correlated traits.

Here, we explore this principle in a study of the effects of two floral traits—style length and petal area—on pollen receipt and pollen performance, both of which can have strong effects on seed production. We sampled flowers from wild populations of two *Clarkia* taxa first to determine whether style length and petal area are phenotypically correlated, and then to estimate their independent and combined effects on pollen receipt and performance. We then used these observations to assess whether the positive effects of style length on pollen receipt previously reported in these taxa (Mazer et al., 2016) may have been influenced by the effects of petal area.

Style length may influence several reproductive processes, including sexual selection—in this case, the differential siring success of male genotypes following pollination—when it determines the intensity of competition among male gametophytes. There are several mechanisms by which this may occur. First, style length may affect the degree of contact between the stigma and the pollen carried by pollinators, thereby influencing the amount of pollen received by receptive stigmas and, in turn, the intensity of antagonistic or positive interactions among pollen grains and pollen tubes (Bernasconi et al., 2007). Second, style length may affect the outcome of competition among pollen tubes by determining the distance across which they compete for access to the ovary; the longer the style, the greater the opportunity for rapidly growing pollen tubes to overtake slower ones (Travers and Shea, 2001). Third, even in the absence of competition among pollen genotypes for access to ovules (i.e., when there are fewer pollen tubes than ovules available for fertilization), long styles may prevent slow-growing pollen tubes from reaching ovules while they are still receptive or from fertilizing ovules located in the most favorable positions within the ovary (Mazer et al., 1986). These features of relatively long styles could favor genotypes with rapid pollen tube growth or high endurance, enabling them to contribute their genes to future generations or to produce the highest-quality seeds (Ruane, 2009).

Several components of female function may also be affected by style length. For example, if relatively long styles impose stronger selection favoring fast-growing pollen tubes than short styles, and if there is significant overlap between the genes expressed in pollen grains and in the sporophytes that they sire (Mulcahy, 1971; Hormaza and Herrero, 1994; Mulcahy et al., 1996; Honys and Twell, 2003), then seeds derived from long-styled flowers may express less genetic variation at some loci than those derived from their short-styled counterparts. In addition, if fast-growing pollen genotypes express genes that result in relatively high performance among their offspring, then style length

will be positively correlated with offspring quality (Palmer and Zimmerman, 1994; Windsor et al., 2000; Yang and Wang, 2015), particularly following the receipt of genetically diverse pollen (Yang and Wang, 2015). Even in the absence of competition among pollen genotypes, if long styles receive more pollen than short ones, this may lead to higher seed production (Németh and Smith-Huerta, 2003). Finally, due to the potential effect of style length on herkogamy or the timing of self-pollination (Jia and Tan, 2012; Irwin et al., 2016), style length may affect the proportion of self pollen received, which in turn may affect the level of genetic variation among sired seeds and seedling vigor.

The direct effects of style length on male and female fitness components can be difficult to assess for several reasons. First, style length may influence reproductive processes both before and after fertilization. Second, potential positive correlations between style length and other traits that influence pollen receipt or quality (e.g., the proportion of outcross pollen), including corolla size, may confound the detection of the direct effects of style length on pollen receipt or performance.

A previous study of wild populations of two *Clarkia* species found that style length was positively correlated with pollen receipt, which in turn was positively correlated with the rate of pollen tube attrition: the failure of pollen tubes to penetrate the stigma or to reach the style base (Mazer et al., 2016). These relationships indicate that the higher pollen receipt of long-styled flowers results in stronger antagonistic relationships among pollen tubes and/or that longer styles provide a greater opportunity for selection against poorly performing pollen. However, style length did not have a significant effect on pollen tube attrition independent of pollen receipt. In sum, we found that long styles function to increase pollen receipt in *Clarkia*, but style length itself does appear to affect the intensity of gametophytic competition in the style.

It is possible, however, that the positive effect of style length on pollen receipt observed in *Clarkia* is not due to the direct effect of style length on pollination success, but rather is due to a positive correlation between style length and other floral traits, such as petal area, which positively affects pollinator visitation or outcross pollen receipt in many species (Johnson et al., 1995; Moeller and Geber, 2005; Anton et al., 2013; Bige et al., 2017; Briscoe Runquist et al., 2017; Knauer and Schiestl, 2017; Bode and Tong, 2018; Lebel et al., 2018; but see Herrera and Balao, 2015). In the present study, we measured the independent effects of style length and petal area on pollen receipt and pollen performance in wild populations of the same two *Clarkia* taxa previously examined: *Clarkia unguiculata* and *C. xantiana* ssp. *xantiana* (Mazer et al., 2016). Our primary goal was to determine whether the effects of style length on pollen receipt previously observed may have been indirectly caused by a strong positive correlation between style length and petal area. We also examined the effects of pollen receipt on the number of pollen tubes entering and penetrating the style to determine whether selection might operate to favor longer styles during these initial phases of sexual reproduction. We collected stigmas, styles, and petals from five wild populations of each taxon to address the following questions:

1. Are style length and petal area positively correlated?
2. Do style length and petal area independently or in combination affect pollen receipt, the number of pollen tubes entering the

style, and the number of pollen tubes reaching the base of the style in both taxa? We predicted that style length would positively affect pollen receipt and the number of pollen tubes entering the style (cf. Mazer et al., 2016), and we tested whether this effect was independent of variation in petal area.

- Does pollen receipt or the number of pollen tubes entering the style affect attrition rates, independent of style length and petal area? We predicted that stigmas with relatively high pollen receipt would exhibit higher attrition rates due to antagonistic interactions among pollen grains (e.g., competition for space in the style or allelopathic interactions).
- Does style length or petal area affect attrition rates independent of pollen receipt? We tested two opposing predictions. First, if some proportion of a population's pollen grains does not have the endurance to reach the base of its relatively long styles, then attrition rates should increase with style length. By contrast, if long styles and/or large petals indicate relatively high nutrient status of the flowers that bear them, and if maternal nutrients promote pollen tube growth, then flowers with long styles or large petals may exhibit relatively low pollen attrition.

METHODS

Study species

Clarkia is a genus of self-compatible, annual herbs native to the western United States (Lewis and Lewis, 1955; Hickman, 1993), with one species in Chile (Moore and Lewis, 1966). Members of this genus are typically among the latest to flower in their local communities and have been the focus of studies of mating system variation and evolution (Mazer et al., 2007, 2009, 2010; Briscoe Runquist et al., 2014; Pettengill et al., 2016). In California, *Clarkia unguiculata* occupies oak woodland, grazed or disturbed hillsides, and road cuts in the Coastal Ranges and the Sierra Nevada foothills south to the Peninsular Ranges. *Clarkia xantiana* ssp. *xantiana* Gray is one of two subspecies of *C. xantiana* Gray; it is predominantly outcrossing and is common on xeric, rocky slopes in the southern Sierra Nevada (Lewis and Lewis, 1955; Moore and Lewis, 1965; Eckhart and Geber, 1999; Runions and Geber, 2000). Both taxa are bee-pollinated, protandrous, and dichogamous winter annuals with mixed mating systems.

Sample collection

During the spring of 2015, from each of five populations of *C. unguiculata* and *C. xantiana* ssp. *xantiana*, we sampled flowers from 26–50 individuals distributed throughout the population. See Appendix S1 for population names, collection dates, elevations, and GPS coordinates. Individuals of both taxa are patchily distributed within populations, and we aimed to collect flowers from a representative sample of plants throughout each local population. On the primary stem of each sampled plant, the pistil of the most recently senesced flower and the petals of the closest flower bearing fully expanded petals were collected. Senescing flowers are easily identified; once *Clarkia* flowers are no longer receptive, the styles begin to desiccate and droop. We recorded the sequence of each sampled flower in relation to the position

of the first flower produced on the primary stem so that we could assess whether pollen performance was influenced by floral position, potentially due to its effect on floral nutrient status. The sequence of the first flower produced was defined as 1, with successively produced flowers assigned numbers of increasing integers. Flowers of the two taxa were harvested at similar floral sequences. The mean (\pm SD) floral sequence of the sampled stigma and style was 4.32 ± 3.35 ($n = 210$) for *C. unguiculata* and 3.61 ± 2.95 ($n = 248$) for *C. xantiana*. The mean (\pm SD) floral sequence of the flower from which petals were sampled was 6.05 ± 3.50 ($n = 210$) for *C. unguiculata* and 5.94 ± 3.16 ($n = 248$) for *C. xantiana*.

Sampled pistils were stored individually in formalin-acetic acid (FAA) in labeled, 1.5 mL microcentrifuge tubes that were kept at room temperature until further processing. From each sampled flower, all four petals were taped to paper sheets with transparent tape, labeled with identifying information, and stored at room temperature. In *C. unguiculata*, each flower produces four petals of approximately equal area; in *C. xantiana* ssp. *xantiana*, each flower produces a pair of relatively large petals and a pair of relatively small petals. In both taxa, we estimated the area (to 0.01 cm²) of the largest petal per flower using the image analysis software ImageJ (<https://imagej.nih.gov/ij>).

In a previous study of natural populations of *C. unguiculata*, Németh and Smith-Huerta (2003) recorded pollen receipt and the number of pollen tubes visible in the upper and lower style at 6, 24, and 48 h after exposure of receptive stigmas to pollinators. While pollen receipt increased from 6 to 24 h and from 24 to 48 h in their study, the numbers of pollen tubes in the upper and lower style reached their maxima at 24 h. Based on these observations, the pollen grains observed on stigmas in the present study were unlikely to have been deposited simultaneously. However, given that the number of pollen tubes observed in the distal and in the basal portions of the style remained constant from 24 to 48 h following the exposure of receptive stigmas (Németh and Smith-Huerta, 2003), the pollen grains deposited after 24 h were unlikely to produce tubes that entered the style. Consequently, due to the complete senescence of the stigmas and styles collected in the present study, we assumed that the stigmas had received their maximum pollen loads and that the pollen tubes had reached their maximum distances.

Processing of stigmas and styles—Within its microcentrifuge tube, each pistil was rinsed three times with deionized (DI) water. The stigma and style were then softened and cleared for 30 h by filling the tubes with 8 M NaOH. The style was then rinsed thoroughly with DI water and placed on a labeled glass slide. Two drops of 0.1% aniline blue solution in 0.1 N K₂HPO₄ (Martin, 1959), and one drop of glycerol, were added to the slide. The style was then gently straightened and a coverslip was placed with pressure to flatten the softened style. Style length was measured (to 0.01 mm) with digital calipers from the style base to the basal edge of the stigma. The slide was refrigerated for 18–72 h, and then each style was viewed using a 4',6'-diamidino-2-phenylindole (DAPI) excitation filter on an epifluorescence microscope (Olympus BX61, 100 \times magnification).

Clarkia pollen tubes produce callose plugs at \sim 1 mm intervals as they grow down the style (S. J. Mazer, personal observation), so the number of pollen tubes at the tip and the base of the style can be estimated from the number of callose plugs visible in 1 mm intervals at these locations. After callose plugs were counted at the stigma-style junction (SSJ) and at the style base, the number of

pollen grains retained by each stigma was recorded by viewing the squashed stigma under a light microscope. The number of pollen grains observed indicates the number of pollen grains that germinated sufficiently well for their pollen tubes to anchor the pollen grain to the stigma. Therefore, we expected this method to exclude pollen grains that were inviable, that arrived too late to achieve contact with the stigma surface (due to a large number of grains being present already), or that were deposited so late that the stigma was no longer receptive. Below, the number of pollen grains observed adhering to a stigma is referred to as “pollen receipt.”

Using the counts of the number of pollen grains adhering to the stigma and the number of callose plugs visible at the SSJ and the base of the style, two pollen tube attrition rates (AR) were estimated as follows: AR from the stigma to the SSJ = $1 - (n \text{ tubes at SSJ} / n \text{ pollen grains on stigma})$; AR from the SSJ to the style base = $1 - (n \text{ tubes at style base} / n \text{ tubes at SSJ})$. Styles in which the callose plugs were indistinct were excluded from analysis.

Statistical analyses

Bivariate relationships among style length, petal area, and pollen receipt—Within each taxon, Pearson correlation coefficients were calculated for each population and for all populations pooled to determine whether style length and petal area are correlated with pollen receipt and to test for correlations between style length and petal area across sampled flowers.

For each taxon, an ordinary least squares (OLS) linear regression model was constructed to test for an effect of petal area on style length independent of variation among populations in style length. Because of the relatively small number of populations, population was tested as a fixed effect. The population \times style length interaction was nonsignificant in both taxa, so this interaction was excluded from the model presented here.

Effect of style length and petal area on pollen receipt—For each taxon, we conducted linear regressions to detect the independent effects of style length, petal area, the sequence of the sampled style (in relation to the first flower produced), and population (treated as a fixed effect) on pollen receipt. Floral sequence and the population \times style length and population \times petal area interactions were excluded from the model presented here because their effects were nonsignificant. We first used Type III sums of squares (SS) to test for the effects of style length and petal area on pollen receipt when each variable was placed last into the model; this tests for the effect of each variable independent of all others included in the model. When using Type III SS, highly correlated variables may not exhibit significant effects on the dependent variable even when they are important in combination. We then used Type I SS to conduct two sequential tests for the effects of petal area and style length to determine whether these traits contribute to pollen receipt in combination. The first test assessed the effect of petal area on pollen receipt prior to testing for the effect of style length, and the second tested for their effects in reverse order. When using Type I SS, when two variables are strongly correlated, it is possible to detect significant effects of one or both variables when placed first into the model even when Type III SS detected no significant effect of either one.

Effects of style length, petal area, and pollen receipt on pollen tube entry into the style—Analyzing each taxon separately, we conducted OLS linear regressions to detect the independent effects of

pollen receipt, style length, petal area, the sequence of the sampled style, and population (treated as a fixed effect) on the number of pollen tubes at the SSJ. To compare taxa directly with respect to the two-way interactions between the main effects and population, interactions that were statistically significant in either taxon were included in the analyses of both taxa. Floral sequence did not have a significant effect on pollen tube entry into the style in either taxon and was therefore excluded from the analyses presented here.

Effects of style length, petal area, pollen receipt, and the number of pollen tubes entering the style on the number of pollen tubes reaching the base of the style—Analyzing each taxon separately, linear regressions were conducted to detect the independent effects of pollen receipt, style length, petal area, the number of pollen tubes at the SSJ, and population (treated as a fixed effect) on the number of pollen tubes reaching the style base. Two-way interactions between the main effects and population were not statistically significant in either taxon, so they were excluded from the model.

Effects of style length, petal area, and pollen receipt on attrition from the stigma to the SSJ and from the SSJ to the style base—For each taxon, we conducted a linear regression to detect the independent effects of petal area, pollen receipt, and population (treated as a fixed effect) on the attrition rate from the stigma to the SSJ. Style length was not included in this model because we judged that it should not influence attrition rates independent of pollen receipt or prior to style entry. Similarly, we conducted linear regressions to detect the independent effects of style length, petal area, the number of pollen tubes at the SSJ, population, and the significant two-way interactions with population on the attrition rate from the SSJ to the style base. In both sets of analyses, two-way interactions that were statistically significant in either taxon were included in the analyses of both species.

RESULTS

Population sampling

From each population of each taxon, 26–50 flowers were collected from which floral traits, pollen receipt, and pollen performance were recorded (Appendix S1). All populations in each species exhibit marked phenotypic variation in style length, petal area, and pollen receipt, and in all attrition rates.

Bivariate relationships among style length, petal area, and pollen receipt

In *C. unguiculata*, in one population and across all pooled samples, style length and petal area were significantly positively correlated (Appendix S2). In addition, flowers with longer styles received more pollen than those with shorter styles in two of the five sampled populations and among pooled samples. Flowers with larger petals received significantly more pollen than small-petaled flowers in two of the five sampled populations and across the pooled sample, but in only one population (Hastings) were both floral traits positively correlated with pollen receipt.

Clarkia xantiana showed similar patterns (Appendix S2). Style length and petal area were positively correlated in three populations and among pooled samples. The relationships between floral traits and pollen receipt were not consistent among populations. Style

length was positively correlated with pollen receipt in one population, and petal area was positively correlated with pollen receipt in another population. In contrast to *C. unguiculata*, *C. xantiana* exhibited no significant correlation between floral traits and pollen receipt in the pooled samples.

These positive correlations between style length and petal area justified the use of multivariate analyses to detect their independent effects on pollen receipt and pollen performance. When controlling for variation among populations in style length, the relationship between style length and petal area was significantly positive in *C. xantiana* but was not detectable in *C. unguiculata* (Appendix S3).

Effects of style length and petal area on pollen receipt

Within both taxa, populations differed with respect to mean pollen receipt (Table 1), but the taxa differed with respect to the effects of style length and petal area. In *C. unguiculata*, both style length and petal area positively and independently affected pollen receipt (Fig. 1; Table 1; Appendix S4, Type III SS), while in *C. xantiana*, neither floral trait had a significant effect independent of the other (Table 1). The sequential significance tests detected that, in *C. unguiculata*, petal area and style length had a significant and positive effect on pollen receipt regardless of the order in which they were placed into the model (Appendix S4, Type I SS). In *C. xantiana*,

TABLE 1. Summary of linear regression conducted to detect the effects of style length, petal area, and population on pollen receipt in *Clarkia unguiculata* and *C. xantiana* ssp. *xantiana*. Two-way interactions with population were not significant in either taxon, so they were excluded from the model. Figure 1 shows the independent and positive effects of style length and mean individual petal area on pollen receipt in *C. unguiculata*. Results of significance testing using Type III SS are shown here. Results of sequential tests using Type I SS are shown in Appendix S4. *P* values < 0.05 are in bold; $0.05 \leq P < 0.10$ are in italics.

<i>C. unguiculata</i>				
Source	df	Type III SS	F ratio	<i>P</i>
Style length	1	133719	6.45	0.0118
Petal area	1	158658	7.66	0.0062
Population	4	443461	5.35	0.0004
Model	6	772559	6.21	<0.0001
Error	203	4206497		
Corrected total	209	4979056		
Adjusted $R^2 = 0.13$				
<i>C. xantiana</i> ssp. <i>xantiana</i>				
Source	df	Type III SS	F ratio	<i>P</i>
Style length	1	6380	0.49	0.4827
Petal area	1	46777	3.63	<i>0.0581</i>
Population	4	139107	2.70	0.0316
Model	6	176233	2.28	0.0372
Error	240	3096982		
Corrected total	246	3273216		
Adjusted $R^2 = 0.03$				
Parameter estimates				
Taxon and variable	Estimate	SD	<i>t</i> ratio	Prob > <i>t</i>
<i>C. unguiculata</i>				
Style length	14.07	5.54	2.54	0.0118
Petal area	173.26	62.61	2.77	0.0062
<i>C. xantiana</i>				
Style length	3.89	5.53	0.70	0.48
Petal area	57.88	30.39	1.90	<i>0.0581</i>

petal area had a significant and positive effect on pollen deposition when tested prior to the effects of style length (Appendix S4, Type I SS), indicating that the two variables have a combined effect on pollen receipt that was not detected using Type III SS.

Effects of floral traits and pollen receipt on pollen tube entry into the style

Within both species, pollen receipt had a positive effect on the number of pollen tubes visible at the SSJ (Appendix S5; Fig. 2A, B). In neither species did style length or petal area have a significant effect on the number of pollen tubes entering into the style independent of pollen receipt. In both species, populations differed with

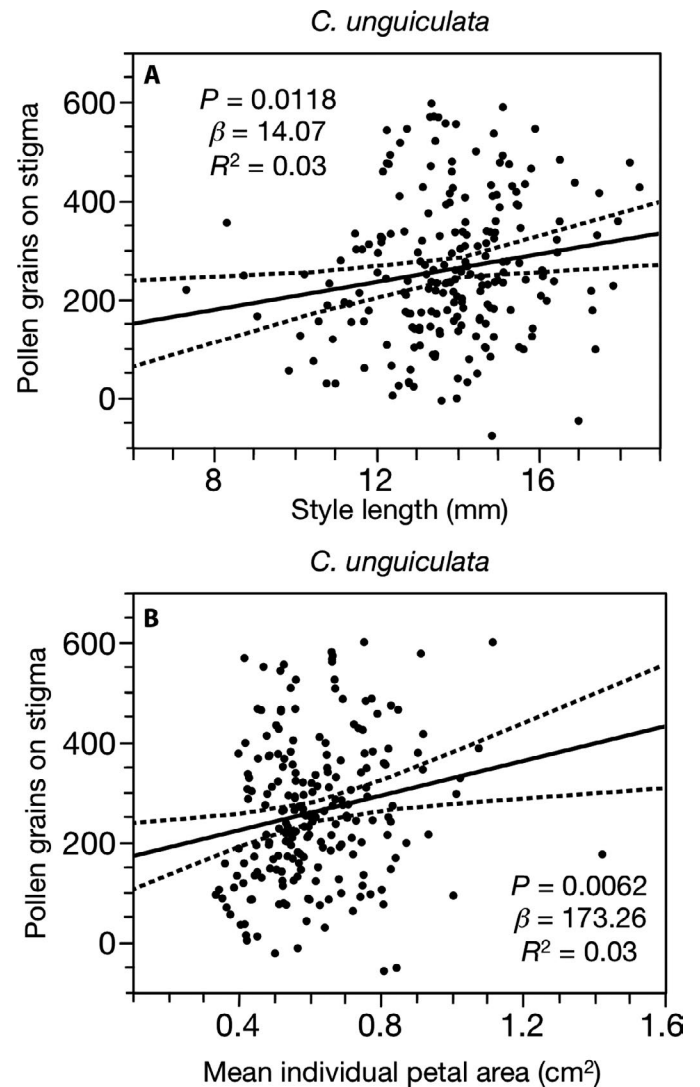


FIGURE 1. Bivariate plots illustrating the relations in *Clarkia unguiculata* between (A) pollen receipt (the number of pollen grains adhering to a stigma) and style length, independent of variation in petal area and among populations and (B) pollen receipt and petal area, independent of variation in style length and among populations. Table 1 summarizes the results of the linear regressions constructed to estimate these relationships. β = the regression coefficient associated with the independent variable in each panel. *P* = the *P* value associated with the regression coefficient.

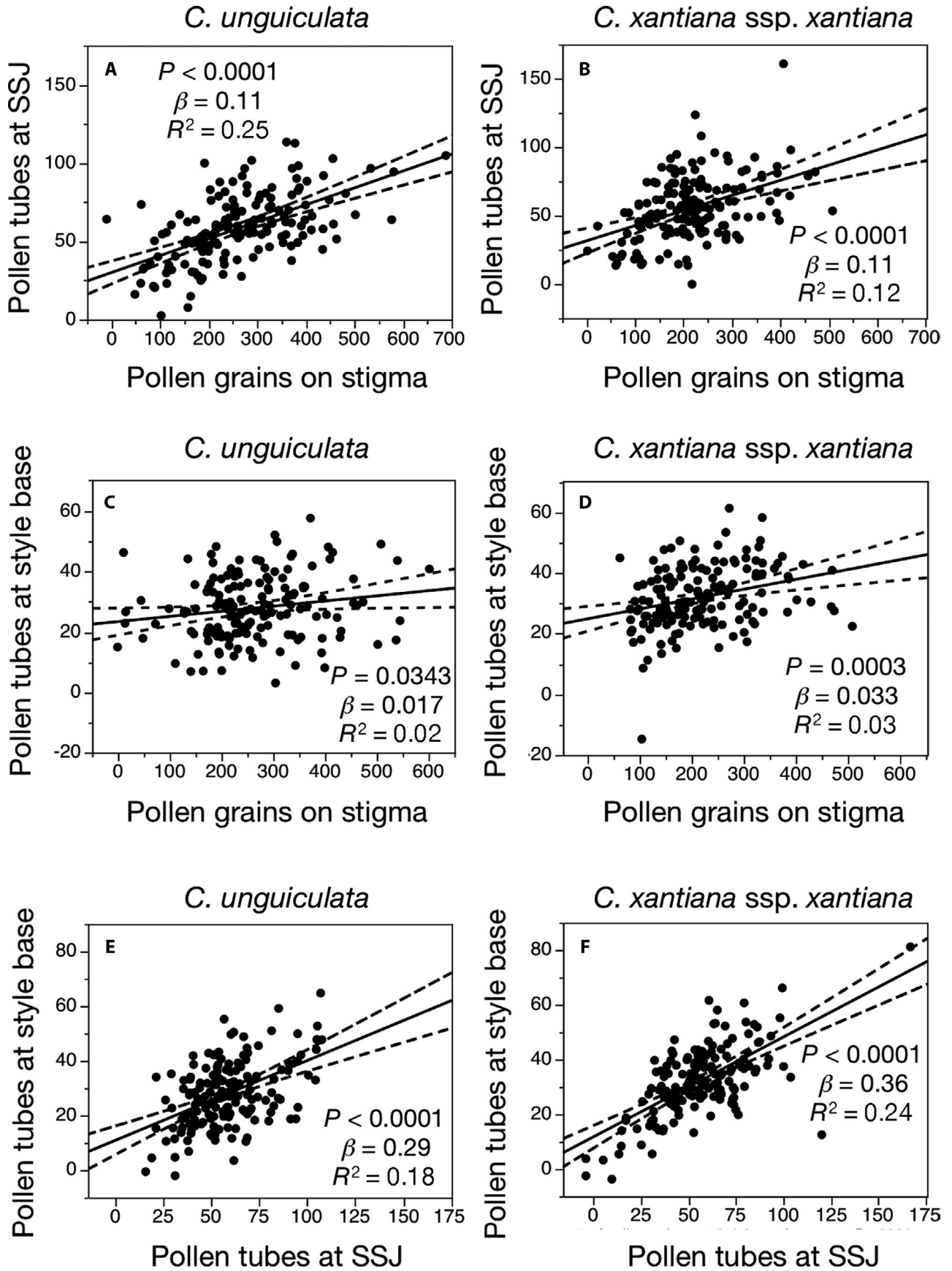


FIGURE 2. Bivariate plots illustrating the relations between components of pollen receipt and pollen tube penetration of the stigma and style. (A, B) Relation between the number of pollen tubes entering the style and pollen receipt, independent of variation in style length, in petal area, among populations, and two-way interaction terms, in (A) *Clarkia unguiculata* and (B) *C. xantiana* ssp. *xantiana*. (C, D) Relation between the number of pollen tubes at the base of the style and pollen receipt, independent of variation in the number of pollen tubes at the stigma-style junction (SSJ), style length, petal area, and among populations, in (C) *C. unguiculata* and (D) *C. xantiana* ssp. *xantiana*. (E, F) Relation between the number of pollen tubes at the base of the style and the number of pollen tubes entering the style, independent of variation in pollen receipt, style length, petal area, and among populations, in (E) *C. unguiculata* and (F) *C. xantiana* ssp. *xantiana*. Appendix S5 summarizes the results of the general linear model constructed to estimate the slopes of the regressions shown in A and B. Appendix S6 summarizes the results of the linear regressions constructed to estimate the slopes shown in C–F. β = the regression coefficient associated with the independent variable in each panel, which equals the slope of the line. P = the P value associated with the regression coefficient.

respect to the effect of pollen receipt on pollen tube entry (indicated by the population \times pollen receipt interaction). The significant two-way interactions between population and floral traits indicated that, in *C. unguiculata*, populations differed with respect to the effect of style length on the number of pollen tubes entering the style, while in *C. xantiana*, populations differed with respect to the effects of petal area on pollen tube entry into the style.

Effects of floral traits, pollen receipt, and pollen tube entry into the style on the number of pollen tubes visible at the style base

Within both species, populations differed with respect to the number of pollen tubes reaching the base of the style (Appendix S6); all two-way interactions including population were nonsignificant, so they were excluded from the model presented here. In both species, pollen receipt (Fig. 2C, D) and the number of tubes at the SSJ (Fig. 2E, F) had positive and independent effects on the number of pollen tubes reaching the style base (Appendix S6). In neither species did style length or petal area have a significant effect on the number of pollen tubes reaching the base of the style (Appendix S6).

Independent effects of petal area and pollen receipt on the attrition rate from the stigma to the stigma-style junction

In both taxa, populations differed in mean attrition rate from the stigma to the SSJ, and pollen receipt had a significant and positive effect on this attrition rate (Appendix S7; Fig. 3A). In neither taxon did petal area influence this attrition rate independent of pollen receipt.

Effects of the number of pollen tubes at the SSJ, petal area, and style length on the attrition rate from the SSJ to the base of the style

In both taxa, the number of pollen tubes at the SSJ had a positive effect on the attrition rate from the SSJ to the style base, independent of petal area and style length (Appendix S8; Fig. 3B). In *C. unguiculata*, no other variable had a significant effect on this attrition rate, nor were the two-way interactions with population significant. In *C. xantiana*, the two-way interactions indicate that populations differed with respect to the effects of the number of callose plugs at the SSJ and style length on this attrition rate.

DISCUSSION

The results presented here underscore the need to design studies of selection on floral traits to rule out the potential effects of

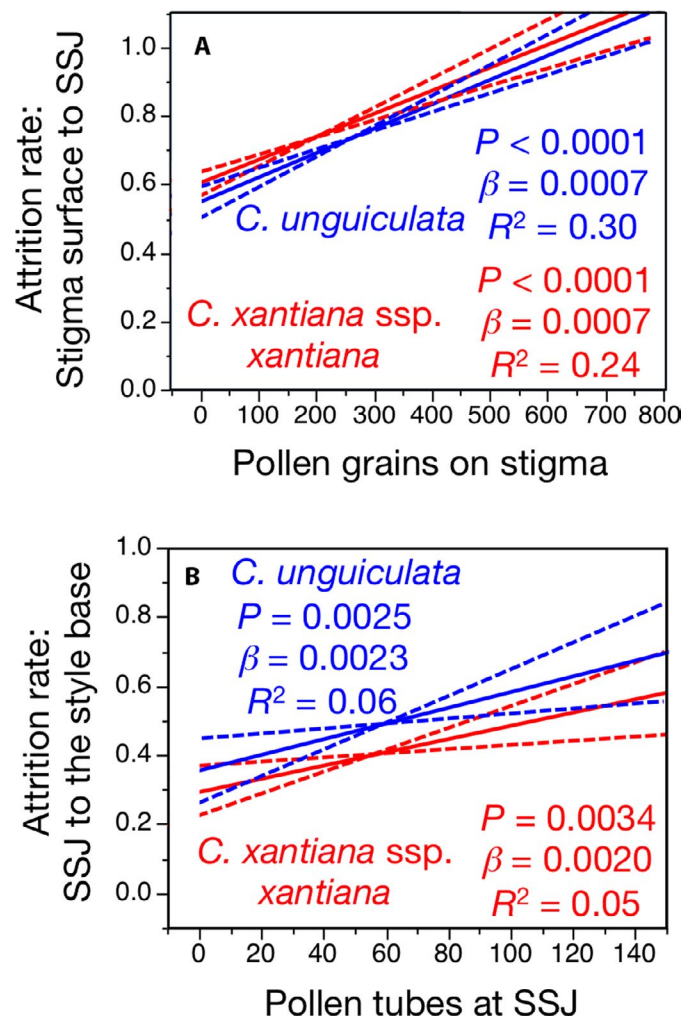


FIGURE 3. Bivariate plots illustrating the relation in *Clarkia unguiculata* and *C. xantiana* ssp. *xantiana* between (A) the attrition rate from the stigma surface to the stigma-style junction (SSJ) and pollen receipt, independent of variation in mean individual petal area, among populations, and the population \times pollen receipt interaction; and (B) the attrition rate from the SSJ to the style base, independent of variation in the number of pollen tubes at the SSJ, style length, petal area, population, and the population \times pollen tubes at the SSJ and the population \times style length interactions. Appendices S7 and S8 summarize the results of the linear regressions constructed to estimate the slopes illustrated in A and B, respectively. β = the regression coefficient associated with the independent variable in each panel. P = the P value associated with the regression coefficient.

correlations among traits. In the present study, our primary goal was to determine whether the positive effect of style length on pollen receipt previously detected in *C. unguiculata* and *C. xantiana* (Mazer et al., 2016) was due to a direct effect of style length or mediated by a positive correlation between style length and petal area. Both species exhibit a positive phenotypic correlation between petal area and style length within one or more of the sampled populations and among pooled samples (Appendix S2), so the effects of these floral traits on pollen receipt and performance need to be separated to detect their independent functions. A complete picture of the process and outcome of natural selection on style length and petal area would require the evaluation of additional, potentially correlated traits that may also contribute positively to pollen receipt, such as stigma surface area, anther size, herkogamy, and the duration of stigma receptivity. Moreover, petal or corolla size may also affect water loss and survivorship (Galen, 2000) and the probability of florivory (Teixido et al., 2011) and therefore may not always be positively correlated with individual fitness, so fitness-related traits other than pollen receipt and pollen performance must also be measured.

The independent effects and the combined effects of style length and petal area on pollen receipt

In *C. unguiculata*, both style length and petal area had positive, independent effects on pollen receipt (Table 1; Fig. 2), consistent with results reported in Mazer et al. (2016). In *C. unguiculata*, these effects were significant regardless of the order in which these variables were tested (see Type I SS in Appendix S4). By contrast, in *C. xantiana*, neither trait had a significant effect on pollen receipt independent of the other (Table 1), raising the possibility that the positive effect of style length on pollen receipt reported in Mazer et al. (2016) was mediated by the effects of petal area on pollen receipt. Moreover, the significant and positive effect of petal area on pollen receipt detected using Type I SS (Appendix S4) indicates that petal area in combination with style length has a significant effect on pollen receipt in *C. xantiana*. This analysis also supports the interpretation that the failure to detect a significant, independent effect of each trait on pollen receipt when using the Type III SS is due to the correlation between them. The ecological reasons for the difference between taxa with respect to the strength of the effects of these traits on pollen receipt are unclear and merit further investigation. Extrinsic factors that could determine the relationships between pollen receipt and style length or petal area include pollinator responses to petal area, the mechanical fit between pollinator bodies and flowers with styles of different lengths, and the taxonomic composition and structure of pollinator communities, but these possibilities are all speculative.

Factors influencing pollen performance

In addition to examining the effects of petal area and style length on pollen receipt, we measured the effects of pollen receipt on pollen performance (independent of other traits). Pollen receipt positively affected pollen attrition rates in two ways. First, in both taxa, the number of pollen grains adhering to the stigma had a positive effect on the attrition rate from the stigma to the SSJ (Appendix S7), indicating that pollen tube entry into the style is limited either by space, maternal resources, or antagonistic interactions among pollen grains or tubes. Second, in both taxa,

pollen receipt positively affected the absolute number of pollen tubes that entered the style (Appendix S5), and this number, in turn, positively affected the attrition rate from the tip to the base of the style (Appendix S8). One explanation for the positive relationship between pollen tube number and attrition is that maternal resources per pollen tube may decline with the number of tubes. If the style provides resources that pollen tubes need to reach the ovules, then a large number of pollen tubes at the SSJ may result in depletion of these resources, resulting in higher attrition (Haileselassie et al., 2005; Smith-Huerta et al., 2007; Rounds et al., 2011; Losada and Herrero, 2014).

High pollen receipt was also associated with higher attrition between the SSJ and the style base (Appendix S8). Nevertheless, for both species, when controlling for the number of pollen tubes at the SSJ, higher pollen receipt *positively* affects the absolute number of pollen tubes reaching the base of the style (Fig. 2C, D), increasing the opportunities for fertilization. This result is consistent with the interpretation that high pollen receipt results in higher-quality pollen reaching the SSJ due to stronger selection among pollen genotypes within the stigma. Even as higher pollen receipt results in a higher rate of pollen failure in these taxa, the net effect of high pollen receipt is to increase the potential for high fertilization rates. In the absence of opposing selection on floral attractiveness due to water loss, florivory, and/or trade-offs between corolla size and flower production (or other fitness-related traits), floral traits that promote pollinator visitation should contribute to higher reproductive success in these taxa.

The use of field-collected flowers for studies of pollen receipt and its relation to pollen performance provides realistic estimates of variation in pollen receipt, pollen tube entry into the style, and attrition in wild populations, but there are drawbacks to this approach. Post-pollination pollen performance may also depend on the timing of pollen receipt; once a stigma becomes receptive, early-arriving pollen may have a much greater chance of germination and stigma penetration than late-arriving pollen. If so, then the number of pollen grains adhering to a stigma does not alone determine the intensity of gametophytic competition; late-arriving pollen simply may not be able to contribute to this process. Experiments in which the number of pollen grains deposited on stigmas is manipulated, and in which pollen grains are deposited simultaneously, can more precisely reveal the effects of gametophytic competition on the numbers and proportions of pollen tubes that enter and penetrate the style.

CONCLUSIONS

Here, we extended previous work (Mazer et al., 2016) by examining the relationships between style length, petal area, and pollen receipt and their independent effects on both the number of pollen tubes entering and penetrating the style and their attrition rates. This study corroborates earlier results while also demonstrating that, in one of the two taxa investigated (*C. unguiculata*), style length positively affects pollen receipt independent of petal area. Although style length and petal area are positively correlated in some populations of each taxon, the positive effects of style length on pollen receipt previously detected in *C. unguiculata* were not likely to have been caused by the effects of petal area alone because each trait independently contributes positively to pollen receipt. By contrast, the positive effects of style length on pollen receipt previously observed in *C. xantiana* ssp. *xantiana* may have been mediated by petal area.

Based on the patterns observed here and in Mazer et al. (2016), we conclude that the amount of pollen deposited on receptive stigmas in *Clarkia* has both an immediate and a cascading effect on pollen performance. In both species, pollen receipt positively affects the number of pollen tubes observed both at the SSJ and at the style base. In addition, pollen receipt has a positive effect on the attrition rate from the stigma surface to the SSJ, indicating that at high densities, pollen tubes compete for space, exert antagonistic effects on each other, and/or suffer reduced maternal provisioning, reducing their ability to enter the style.

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DATA ACCESSIBILITY

The raw data used in the analyses presented here are available from the following website: UC Santa Barbara Dash, Dataset, <https://doi.org/10.25349/D9KW2J>.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. (A) Species, population name, collection date, elevation, and GPS coordinates. (B) Population means, standard deviation, and sample sizes for each recorded attribute.

APPENDIX S2. Bivariate correlation coefficients (and *P* values; $P \leq 0.05$ in bold; $0.05 < P < 0.10$ in italics) between floral traits and pollen receipt within populations and in pooled samples within each species.

APPENDIX S3. Summary of general linear model designed to detect the effects of petal area and population on style length in *C. unguiculata* and *C. xantiana* ssp. *xantiana*.

APPENDIX S4. Summary of linear models conducted to test for (A) the independent effects of population, style length, and petal area on pollen receipt, tested using Type III sums of squares (SS) and (B) the sequential effects of style length and petal area on pollen receipt (using Type I SS).

APPENDIX S5. Summary of linear models conducted to detect the effects of style length, petal area, pollen receipt, population, and two-way interactions with population on the number of pollen tubes visible at the stigma-style junction (SSJ) in *C. unguiculata* and *C. xantiana* ssp. *xantiana*.

APPENDIX S6. Summary of linear models conducted to detect the effects of style length, petal area, pollen receipt, the number of pollen tubes at the stigma-style junction (SSJ), and population on the number of pollen tubes at the style base in *C. unguiculata* and *C. xantiana* ssp. *xantiana*.

APPENDIX S7. Summary of linear models to detect the effects of pollen receipt, petal area, population, and two-way interactions with population on the attrition rate from the stigma to the SSJ in *C. unguiculata* and *C. xantiana* ssp. *xantiana*.

APPENDIX S8. Summary of analyses of variance to detect the effects of number of callose plugs at the SSJ, style length, petal area, and population on attrition rate from the SSJ to the style base in *C. unguiculata* and *C. xantiana* ssp. *xantiana*.

LITERATURE CITED

- Anton, K. A., J. R. Ward, and M. B. Cruzan. 2013. Pollinator-mediated selection on floral morphology: evidence for transgressive evolution in a derived hybrid lineage. *Journal of Evolutionary Biology* 26: 660–673.
- Armbruster, S. W., C. Pélabon, G. H. Bolstad, and T. F. Hansen. 2014. Integrated phenotypes: understanding trait covariation in plants and animals. *Phil. Trans. R. Soc. B* 369: 20130245.
- Bernasconi, G., D. J. Lang, and B. Schmid. 2007. Microgametophyte population sizes and plant reproductive output in the insect-pollinated *Prunella grandiflora* (Lamiaceae). *New Phytologist* 173: 393–400.
- Bigio, L., M. Lebel, and Y. Sapir. 2017. Do different measures of maternal fitness affect estimation of natural selection on floral traits? A lesson from *Linum pubescens* (Linaceae). *Journal of Plant Ecology* 10: 406–413.
- Bode, R. F., and R. Tong. 2018. Pollinators exert positive selection on flower size on urban, but not on rural Scotch broom (*Cytisus scoparius* L. Link). *Journal of Plant Ecology* 11: 493–501.
- Briscoe Runquist, R. D., E. Chu, J. L. Iverson, J. C. Kopp, and D. A. Moeller. 2014. Rapid evolution of reproductive isolation between incipient outcrossing and selfing *Clarkia* species. *Evolution* 68: 2885–2900.
- Briscoe Runquist, R. D., M. A. Geber, M. Pickett-Leonard, and D. A. Moeller. 2017. Mating system evolution under strong pollen limitation: evidence of disruptive selection through male and female fitness in *Clarkia xantiana*. *American Naturalist* 189: 549–563.
- Eckhart, V. M., and M. A. Geber. 1999. Character variation and geographic distribution of *Clarkia xantiana*. I. Sources of trait variation across a subspecies border. *Madroño* 46: 177–125.
- Galen, C. 2000. High and dry: drought stress, sex-allocation trade-offs, and selection on flower size in the alpine wildflower *Polemonium viscosum* (Polemoniaceae). *American Naturalist* 156: 72–83.
- Haileselassie, T., M. Mollel, and I. Skogsmyr. 2005. Effects of nutrient level on maternal choice and siring success in *Cucumis sativus* (Cucurbitaceae). *Evolutionary Ecology* 19: 275–288.
- Herrera, J., and F. Balao. 2015. A test of phenotypic selection on petal form in the wild carnation, *Dianthus inoxianus*. *Plant Biology* 17: 1227–1232.
- Hickman, J. C. 1993. The Jepson Manual: Higher Plants of California. Berkeley: University of California Press.
- Honys, D., and D. Twell. 2003. Comparative analysis of the *Arabidopsis* pollen transcriptome. *Plant Physiology* 132: 640–652.
- Hormaza, J. I., and M. Herrero. 1994. Gametophytic competition and selection. In E. G. Williams, A. E. Knox and R. B. Clarke [eds.], Genetic Control of Self-incompatibility and Reproductive Development in Flowering Plants, 372–400. Kluwer Academic, Dordrecht.
- Irwin, J. A., P. A. Ashton, F. Bretagnolle, and R. J. Abbot. 2016. The long and the short of it: long-styled florets are associated with higher outcrossing rates in *Senecio vulgaris* and result from delayed self-pollen germination. *Plant Ecology and Diversity* 9: 159–165.

- Jia, J., and D. Y. Tan. 2012. Variation in style length and anther-stigma distance in *Ixiolirion songaricum* (Amaryllidaceae). *South African Journal of Botany* 81: 19–24.
- Johnson, S. G., L. F. Delph, and C. L. Elderkin. 1995. The effect of petal-size manipulation on pollen removal, seed set, and insect-visitor behavior in *Campanula americana*. *Oecologia* 102: 174–179.
- Knauer, A. C., and F. P. Schiestl. 2017. The effect of pollinators and herbivores on selection for floral signals: a case study in *Brassica rapa*. *Evolutionary Ecology* 31: 285–304.
- Lebel, M., U. Obolski, L. Hadany, and Y. Sapir. 2018. Pollinator-mediated selection on floral size and tube color in *Linum pubescens*: Can differential behavior and preference in different times of the day maintain dimorphism? *Ecology and Evolution* 8: 1096–1106.
- Lewis, H., and M. E. Lewis. 1955. The genus *Clarkia*. Publications in Botany, University of California. Berkeley: University of California Press.
- Losada, J. M., and M. Herrero. 2014. Glycoprotein composition along the pistil of *Malus × domestica* and the modulation of pollen tube growth. *BMC Plant Biology* 14: 1.
- Martin, F. W. 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technology* 34: 125–128.
- Mazer, S. J., A. A. Snow, and M. L. Stanton. 1986. Fertilization dynamics and parental effects upon fruit development in *Raphanus raphanistrum*: consequences for seed size variation. *American Journal of Botany* 73: 500–511.
- Mazer, S. J., V. A. Delesalle, and H. Paz. 2007. Evolution of mating system and the genetic covariance between male and female investment in *Clarkia* (Onagraceae): selfing opposes the evolution of trade-offs. *Evolution* 61: 83–98.
- Mazer, S. J., L. S. Dudley, V. A. Delesalle, H. Paz, and P. Galusky. 2009. Stability of pollen-ovule ratios in pollinator-dependent versus autogamous *Clarkia* sister taxa: testing evolutionary predictions. *New Phytologist* 183: 630–648.
- Mazer, S. J., L. S. Dudley, A. A. Hove, S. K. Emms, and A. S. Verhoeven. 2010. Physiological performance in *Clarkia* sister taxa with contrasting mating systems: do early-flowering autogamous taxa avoid water stress relative to their pollinator-dependent counterparts? *International Journal of Plant Sciences* 171: 1029–1047.
- Mazer, S. J., A. Moghaddasi, A. K. Bello, and A. A. Hove. 2016. Winning in style: longer styles receive more pollen, but style length does not affect pollen attrition in wild *Clarkia* populations. *American Journal of Botany* 103: 408–422.
- Medrano, M., R. Requerey, J. D. Karron, and C. M. Herrera. 2012. Herkogamy and mate diversity in the wild daffodil *Narcissus longispathus*: beyond the selfing-outcrossing paradigm in the evolution of mixed mating. *Plant Biology* 14: 801–810.
- Moeller, D. A., and M. A. Geber. 2005. Ecological context of the evolution of self-pollination in *Clarkia xantiana*: population size, plant communities, and reproductive assurance. *Evolution* 59: 786–799.
- Moore, D. M., and H. Lewis. 1965. The evolution of self-pollination in *Clarkia xantiana*. *Evolution* 19: 104–114.
- Moore, D. M., and H. Lewis. 1966. Variation and evolution in South American *Clarkia*. *Heredity* 21: 37–56.
- Mulcahy, D. L. 1971. A correlation between gametophytic and sporophytic characteristics in *Zea mays* L. *Science* 171: 1155–1156.
- Mulcahy, D. L., M. Sari-Gorla, and G. B. Mulcahy. 1996. Pollen selection – past, present and future. *Sexual Plant Reproduction* 9: 353–356.
- Németh, M. B., and M. L. Smith-Huerta. 2003. Pollen deposition, pollen tube growth, seed production, and seedling performance in natural populations of *Clarkia unguiculata* (Onagraceae). *International Journal of Plant Sciences* 164: 153–164.
- Newman, E., J. Manning, and B. Anderson. 2015. Local adaptation: mechanical fit between floral ecotypes of *Nerine humilis* (Amaryllidaceae) and pollinator communities. *Evolution* 69: 2262–2275.
- Nista, P., A. N. Brothers, and L. F. Delph. 2015. Differences in style length confer prezygotic isolation between two dioecious species of *Silene* in sympatry. *Ecology and Evolution* 5: 2703–2711.
- Palmer, T. M., and M. Zimmerman. 1994. Pollen competition and sporophyte fitness in *Brassica campestris*: does intense pollen competition result in individuals with better pollen? *Oikos* 69: 80–86.
- Pettengill, J. B., R. D. Briscoe Runquist, and D. A. Moeller. 2016. Mating system divergence affects the distribution of sequence diversity within and among populations of recently diverged subspecies of *Clarkia xantiana* (Onagraceae). *American Journal of Botany* 103: 99–109.
- Rounds, C.M., L. J. Winship, and P. K. Hepler. 2011. Pollen tube energetics: respiration, fermentation and the race to the ovule. *Annals of Botany Plants* 10: plr019.
- Ruane, L. G. 2009. Post-pollination processes and non-random mating among compatible mates. *Evolutionary Ecology Research* 11: 1031–1051.
- Runions, C. J., and M. A. Geber. 2000. Evolution of the self-pollination flower in *Clarkia xantiana* (Onagraceae). I. Size and development of floral organs. *American Journal of Botany* 87: 1439–1451.
- Smith-Huerta, N. L., S. R. Carrino-Kyker, and A. J. Huerta. 2007. The effects of maternal and paternal nutrient status on pollen performance in the wild-flower *Clarkia unguiculata* Lindley (Onagraceae). *J. Torrey Bot. Soc.* 134: 451–457.
- Teixido, A. L., M. Méndez, and F. Valladares. 2011. Flower size and longevity influence florivory in the large-flowered shrub *Cistus ladanifer*. *Oecologia* 37: 418–421.
- Travers, S. E., and K. Shea. 2001. Individual variation, gametophytic competition and style length: does size affect paternity? *Evolutionary Ecology Research* 3: 729–745.
- Windsor, J. A., S. Peretz, and A. G. Stephenson. 2000. Pollen competition in a natural population of *Cucurbita foetidissima* (Cucurbitaceae). *American Journal of Botany* 87: 527–532.
- Yang, C. F., and Q. F. Wang. 2015. Nectarless flowers with deep corolla tubes in *Pedicularis*: does long pistil length provide an arena for male competition? *Botanical Journal of the Linnean Society* 179: 526–532.