UC Santa Barbara
UC Santa Barbara Previously Published Works

Title

Winning in style: Longer styles receive more pollen, but style length does not affect pollen attrition in wild Clarkia populations

Permalink

https://escholarship.org/uc/item/3pp1f70n

Journal

American Journal of Botany, 103(3)

ISSN

0002-9122

Authors

Mazer, Susan J
Moghaddasi, Arrash
Bello, Alexandra K
et al.

Publication Date

2016-03-01

DOI

10.3732/ajb.1500192

Peer reviewed

Winning in style: Longer styles receive more pollen, but style length does not affect pollen attrition in wild *Clarkia* populations[1]

Susan J. Mazer[2], Arrash Moghaddasi, Alexandra K. Bello[3], and Alisa A. Hove

PREMISE OF THE STUDY: One proposed function of long styles is to intensify selection among male gametophytes relative to short styles. If so, given sufficient competition, longer styles will have higher rates of pollen tube attrition (failure to reach the style base) within the style than shorter ones. Alternatively, style length may influence pollen receipt, which itself may affect attrition rates.

METHODS: We tested these predictions by collecting senescing styles from wild populations of two insect-pollinated *Clarkia* species. We examined the number of pollen grains adhering to the stigma, length of styles, and rates of attrition from the stigma surface to the stigma–style junction (SSJ), from the SSJ to the style base, and from the stigma surface to the style base. Multivariate analyses estimated the independent effects of pollen grains per stigma, the number of pollen tubes at the SSJ, and style length on attrition.

KEY RESULTS: Style length was generally positively correlated with pollen receipt, and the number of pollen grains per stigma was positively correlated with all three attrition rates. In neither species was any attrition rate affected by style length independent of the number of pollen grains per stigma.

CONCLUSIONS: Pollen attrition was mediated by style length, but the function of style length was primarily to increase the number of germinating pollen grains, which affected attrition rates either through stigma clogging or pollen–pollen interactions. Style length may have a direct effect on pollen receipt due to the stigma's position relative to pollinator body parts, but traits correlated with style length may also directly affect pollen receipt.

KEY WORDS   *Clarkia unguiculata*; *Clarkia xantiana* subsp. *xantiana*; gametophytic competition; Onagraceae; pollen attrition; pollen competition; pollen deposition; sexual selection; style length

Differences in floral structure and morphology among taxa and between genders have been interpreted as adaptations to alternative mating systems, pollination syndromes, and gender-specific reproductive costs, and as evolutionary responses to sexual selection on traits associated with increases in male fitness (Bell, 1985; Conner et al., 1996; Barrett, 2002; Barrett and Harder, 2005; Jacquemyn et al., 2005; Carlson and Harms, 2006; Ruan et al., 2011; Temeles et al., 2012; Barrett and Hough, 2013). Similar to many floral traits, style length exhibits notable phenotypic variation within natural populations of many homomorphic plant species (i.e., excluding heterostylous species), as well as among taxa (Elmqvist et al., 1993;

Ganeshaiah et al., 1999; Klinkhamer and van der Veen-van Wijk, 1999; Sarkissian and Harder, 2001; Travers and Shea, 2001; Bernasconi et al., 2007; Nattero and Cocucci, 2007; Kulbaba and Worley, 2008; Nattero et al., 2010; Forrest et al., 2011; Medrano et al., 2012; Jia and Tan, 2012; Wright et al., 2012). This variation has motivated a number of experimental, observational, and comparative approaches to explain its ecological function and the evolutionary significance of variation both within and among species.

Experimental work has identified reproductive and ecological functions of style length in several species (Mulcahy and Mulcahy, 1975; Johnston, 1993; Forrest et al., 2011). Within-population surveys have examined phenotypic correlations between style length and other traits, such as functional gender, herkogamy, the number of pollen tubes within the style, pollen deposition by different pollinators, and corolla depth (Mulcahy, 1979; Campbell, 1989; Klinkhamer and van der Veen-van Wijk, 1999; Bernasconi et al., 2007; Nattero and Cocucci, 2007; Forrest et al., 2011; Medrano et al., 2012). Comparisons between species that differ in style length have

been conducted to detect associated differences in other traits that may explain its function, such as pollen attrition rate, mating system, flower size, pollen size, and pollen tube growth rate (Plitmann, 1993, 1994; Smith-Huerta, 1996; Bigazzi and Selvi, 2000; Johnson et al., 2010; Ramesha et al., 2011; Taylor and Williams, 2012). Simulations have been used to test hypotheses concerning the intensity of gametophytic competition and selection as a function of style length (Lankinen and Skogsmyr, 2001; Travers and Shea, 2001). In addition, genetic correlations between style length and other traits that influence individual fitness have been identified, which may constrain its independent evolution (Sarkissian and Harder, 2001; Kulbaba and Worley, 2008).

The ubiquity of style length variation within and among taxa has generated a good deal of speculation concerning its adaptive significance. The proposed functions of long relative to short styles include (1) greater exsertion of the stigma from the corolla tube to provide access to the pollen-bearing portions of pollen vectors' bodies, facilitating pollen receipt by the stigma (Bernasconi et al., 2007); (2) increased herkogamy, thereby reducing autogamous self-pollination and increasing the diversity of pollen donors (Barrett, 2002); (3) the reduction or prevention of fungal spore receipt (Elmqvist et al., 1993); (4) increased pollen deposition, thereby increasing gametophytic competition during the autotrophic stages of pollen germination and pollen tube growth (sensu Stephenson et al., 2003; Erbar, 2003), and increasing the genetic quality of pollen tubes that reach the style, the ovary, and receptive ovules; and (5) increasing the distance across which pollen tubes must compete after they enter the style, allowing small differences among pollen genotypes in pollen tube growth rate to result in significant differences in siring success (Mazer et al., 2010; Ramesha et al., 2011). Both (4) and (5) propose that style length will influence the probability that relatively low-quality pollen will reach the ovary.

If long styles intensify prefertilization selection as pollen tubes grow through them, then, within species, relatively long styles may exhibit higher rates of pollen failure (or pollen attrition, sensu Waser et al., 1987; Cruzan, 1989) than short styles. While selection against inferior pollen genotypes can occur independent of variation in style length or the number of conspecific pollen grains that have been deposited on a stigma, the opportunity for selection to operate on variation among male gametophytes in lifespan or in pollen tube growth rates may be stronger in relatively long styles for at least three reasons. First, pollen genotypes with short lifespans may be unable to reach the base of a long style before they die. Second, in relatively long styles, slow-growing pollen genotypes may fail to reach the ovary before the style itself senesces and stops provisioning pollen tubes during the heterotrophic growth stage (i.e., while navigating the style). Third, any differences in pollen tube growth rates between relatively fast- and slow-growing pollen genotypes will be enhanced in longer relative to shorter styles, providing the opportunity for selection to distinguish more effectively between relatively small differences in growth rate.

We tested the prediction that style length is positively correlated with pollen attrition by collecting senescing styles from multiple populations of two predominantly insect-pollinated and outcrossing *Clarkia* taxa: *C. unguiculata* and *C. xantiana* subsp. *xantiana*. Stigmas and styles were assessed for the number of pollen grains that adhered to the stigma and for three measures of pollen attrition: the proportion of pollen grains per stigma that failed to reach the base of the stigma (which was not a priori expected to be associated with style length); the proportion of pollen tubes that entered the style but failed to reach the base of the style; and the proportion of pollen grains that adhered to the stigma but failed to reach the base of the style (i.e., total attrition rate). Given that the number of pollen grains deposited and adhering to a stigma might influence attrition rates due to positive or negative interactions among germinating pollen grains or competing pollen tubes, we controlled statistically for variation in pollen grains per stigma and in the number of pollen tubes entering the style when examining the direct effect of style length on each measure of attrition.

Collectively, our observations were used to address the following questions: (1) Does style length influence the number of pollen grains that arrive and adhere to a stigma? (2) Does the number of

TABLE 1. Name, harvest date, sample size (*n*), location, and elevation of the populations of *Clarkia unguiculata* and *C. xantiana* subsp. *xantiana* sampled for the current study. Harvest date categories were used in the ANCOVAs reported here.

Species	Population	Harvest date	Harvest date category	*n*	GPS coordinates	Elevation (m a.s.l.)
C. xantiana	Camp 3	23 May 2009	2009	31	35°48.65'N, 118°27.24'W	865
		1 June 2010	2010-Early	31		
		11 June 2010	2010-Late	37		
C. xantiana	Borel Road	21 May 2009	2009	38	35°35.04'N, 118°31.30'W	775
		27 May 2009	2009	20		
		5 June 2010	2010-Late	28		
		11 June 2010	2010-Late	31		
C. xantiana	Sawmill Road 3.3	22 May 2009	2009	38	35°40.48'N, 118°30.60'W	1221
		8 June 2009	2009	34		
		11 June 2010	2010-Early	43		
		25 June 2010	2010-Late	33		
C. xantiana	Greenhorn Mountain Road	8 June 2009	2009	32	35°43.29'N, 118°30.04'W	1205
C. unguiculata	Stark Creek	20 May 2009	2009	22	35°28.46'N, 118°43.53'W	457
		16 May 2010	2010-Early	38		
		22 May 2010	2010-Early	33		
C. unguiculata	Live Oak	15 May 2010	2010-Early	35	35°28.81'N, 118°44.89'W	475
		3 June 2010	2010-Late	28		
C. unguiculata	Mill Creek	8 May 2009	2009	29	35°32.30'N, 118°36.80'W	732
		19 May 2010	2010-Early	35		
		3 June 2010	2010-Late	43		
C. unguiculata	China Gardens	19 May 2010	2010-Early	38	35°31.96'N, 118°38.73'W	643

pollen grains per stigma influence pollen attrition rates independent of style length? (3) Is style length positively associated with any of the focal measures of pollen attrition independent of the number of pollen grains per stigma? (4) Is there evidence for resource- or space-limitation within the stigma that limits pollen tube entry into the style when pollen receipt reaches a threshold level?

MATERIALS AND METHODS

Study species—*Clarkia* (Onagraceae) is a genus of winter annual herbs that includes approximately 41 taxa that flower in late spring (Dudley et al., 2007; Delesalle et al., 2008; Mazer et al., 2010). The species observed in this study, *Clarkia unguiculata* and *Clarkia xantiana* subsp. *xantiana* (hereafter "*unguiculata*" and "*xantiana*", respectively) are commonly found on steep slopes, trailsides, and road embankments of the oak woodlands, juniper–pinyon pine woodlands, and chaparral of the California Coastal Ranges and southern Sierra Nevada. Their distribution was more extensively described by Mazer et al. (2010). Both of these bee-pollinated taxa produce protandrous and herkogamous flowers that generally avoid autogamous self-pollination (Delesalle et al., 2008). Mating system estimates obtained from natural populations indicate that both taxa are primarily outcrossing, with *xantiana* exhibiting slightly lower outcrossing rates near its eastern range limit (Vasek, 1965; Moeller et al., 2012). Stigmas remain receptive for 2–5 d, depending on the timing and intensity of pollination.

Sample collection—During the flowering seasons of 2009 and 2010, we collected senescing flowers from haphazardly sampled individuals within four wild populations of each taxon. See Table 1 for population names, GPS coordinates, style harvest dates, and sample sizes for each collection. In 2009, most populations were sampled only once, and 22–38 styles were collected on each visit. Two of six populations, however, were sampled twice in that year (*xantiana* at Borel Road and at Sawmill Road), but the data were pooled to create a single harvest date category (2009).

In 2010, six of seven sampled populations were visited twice during flowering. When the length of time between visits exceeded 7 d, the samples were designated as 2010-Early and 2010-Late. When the length of time between visits was less than 7 d, the samples from the 2 d were pooled and included in the same harvest date category (e.g., *xantiana* styles collected at Borel Road in 2010 were all assigned to the 2010-Late harvest date; $n = 59$. The number of styles collected from each population per harvest date category ranged from 22 to 72.

On the day of sampling, from each population, one senescing stigma and style per plant (along with the attached petals) was collected by severing the ovary ~3 mm below the point where the style entered its tip. Because *Clarkia* styles begin to desiccate and to droop once stigmas are no longer receptive, spent flowers were easily distinguishable from receptive female-phase flowers. Each flower was placed into a 1.5 mL microcentrifuge tube containing formalin–acetic acid to arrest pollen tube growth. Microcentrifuge tubes from each population were labeled and sealed in a plastic bag. These bags were stored at room temperature before subsequent processing.

The results of a previous field study of *unguiculata* suggest that by the time that we collected the fully senesced styles ($\geq$24 h after their stigmas had become receptive), all pollen tube growth that would lead to fertilization had already occurred. Németh and Smith-Huerta (2003) recorded pollen deposition and the number of pollen tubes in

field collected styles at 6, 24, and 48 h following exposure to open pollination. They found that pollen deposition increased significantly throughout this period. The number of pollen tubes in the upper style and lower style, however, did not increase between the 24 and 48-h treatments. By the time we collected the styles in this study, no additional entry of pollen tubes into the ovary would occur.

Sample processing—From each sampled flower, the intact style was removed from the terminal end of the ovary by first opening the tip of the ovary to expose the style base. All floral parts other than the style were discarded, and the style and stigma were then rinsed twice within the microcentrifuge tube with deionized (DI) water. Pipette tips did not contact the style, and water was released slowly to minimize pollen removal from the stigma. Water was removed from each tube in the same manner. Rinsed styles were then softened in 8 M NaOH within the microcentrifuge tubes for 30–40 h. After this period, styles were rinsed twice with DI water and then submerged in a 0.1 N K_2HPO_4 solution with 0.1% aniline blue fluorescent stain for 2 h in the dark (Martin, 1959).

Following fluorescent staining, each style was placed onto a labeled microscope slide. Each style was straightened, and its length was measured to the nearest 0.5 mm with digital calipers. The stigma was severed from its style by slicing it with a razor blade beneath the stigma lobes; the stigma and style were then gently squashed with a coverslip. Completed slides were then viewed
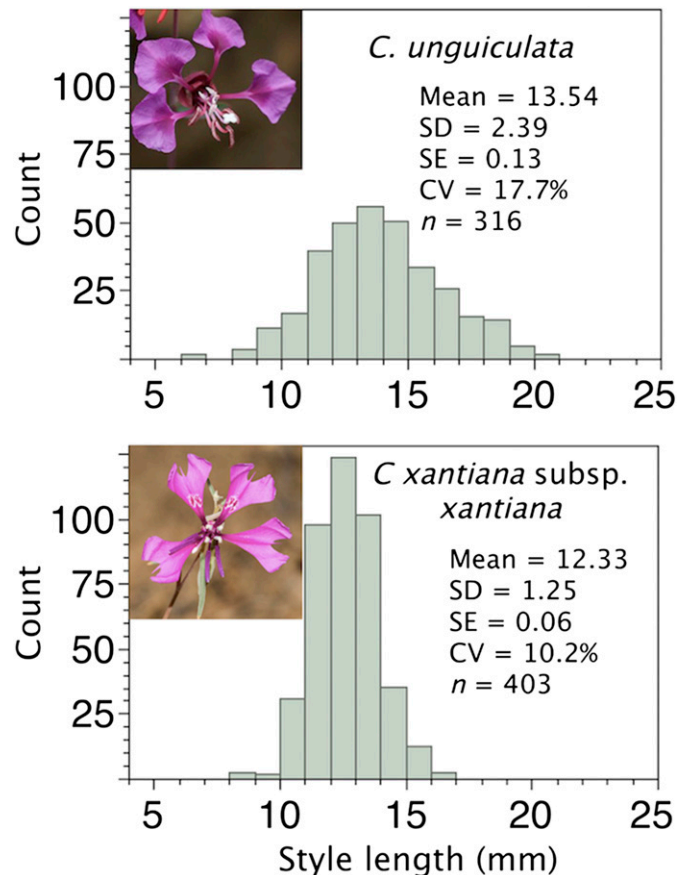

FIGURE 1 Frequency distributions of style length for *Clarkia unguiculata* and *C. xantiana* subsp. *xantiana* flowers, pooled across populations and harvest dates.

(within an hour) using a 4′,6′-diamidino-2-phenylindole (DAPI) excitation filter on an epifluorescence microscope (Olympus BX61, 40× magnification) (Franklin-Tong, 1999).

Attrition rates—In *Clarkia*, pollen tubes produce callose plugs at approximately 1-mm intervals along their entire length. We were therefore able to estimate how many pollen tubes had entered each style and how many had reached the ovary by counting the number of callose plugs within the distal and proximal 1-mm ends of the style, respectively.

We used Eq. (1) to estimate the attrition rate of pollen grains between the stigma surface and the base of the stigma (the stigma–style junction, or SSJ):

Attrition rate$\left(\text{stigma to SSJ}\right)$

$$= \frac{\left(\text{No. pollen grains on stigma} - \text{No. callose plugs at SSJ}\right)}{\text{No. pollen grains on stigma}} \quad (1)$$

Eq. (2) was used to estimate the attrition rate of pollen tubes as they grew from the stigma-style junction to the style base:

Attrition rate$\left(\text{SSJ to style base}\right)$

$$= \frac{\left(\text{No. callose plugs at SSJ} - \text{No. callose plugs at style base}\right)}{\text{No. callose plugs at SSJ}} \quad (2)$$

To estimate the attrition rate from the stigma to the style base, we used Eq. (3):

Attrition rate$\left(\text{stigma to style base}\right)$

$$= \frac{\left(\text{No. pollen grains on the stigma} - \text{No. callose plugs at style base}\right)}{\text{No. pollen grains on stigma}}$$

$$(3)$$

Pollen deposition—After counting the callose plugs at the tip and base of the style, microscope slides were refrigerated; within 2 weeks of slide preparation, slides were viewed with a dissecting microscope to record the number of pollen grains visible on the stigma. A hemocytometer was placed under the slide to facilitate pollen counting. The pollen grains on each stigma were counted two or three times until a consistent count was achieved; when counts diverged slightly, the mean of three counts was calculated. The number of pollen grains per stigma counted here may be an underestimate of the total number deposited because fixation in FAA and style processing in the laboratory may have dislodged some pollen. Here, we infer that the number of pollen grains observed per stigma represents the number that were well anchored to the stigma, perhaps because they had germinated before style collection.

Testing for interference among male gametophytes—In field populations of *unguiculata*, Delesalle et al. (2008) found that plants produce a mean of 86 ovules per flower among the first flowers they produce and 68 ovules per flower among the last flowers produced; *xantiana* flowers produce a mean of 96 and 68 ovules, respectively, among the first and last flowers produced. Of the *unguiculata* flowers sampled here, 34 stigmas received ≤100 pollen grains; in these flowers, pollen grains and pollen tubes experienced relatively weak competition for access to ovules. This result provided the opportunity to assess whether the effects of style length on pollen attrition in *unguiculata* were related to the potential for intense gametophytic competition, which was estimated as the number of pollen grains adhering to the stigma.

Statistical analyses—*Pollen grains per stigma: Effects of harvest date and style length*—A two-way fixed effect ANCOVA was conducted to evaluate the effects of harvest date, style length, and their interaction on the number of pollen grains adhering to the stigmas of *unguiculata* and *xantiana*. Each species was analyzed separately; the number of pollen grains per stigma was $\log_{10}$-transformed to approach a normal distribution. Harvest date categories included 2009 (all populations pooled because most were sampled only once), 2010-Early, and 2010-Late.

Stigmas in each species were also classified by whether they contained ≤100 or >100 pollen grains, and then one-way ANOVAs were performed to detect significant differences between these classes with respect to style length.

Pollen attrition rates: Effects of harvest date, style length, pollen grains per stigma, and the number of pollen tubes entering the style—To detect the effects of style length on pollen attrition rates from the stigma surface to the SSJ, a three-way fixed effect ANCOVA was conducted that included harvest date, style length, pollen grains/stigma, and their interactions. To detect the effects of style length on pollen attrition rates from the SSJ to the style base and from the stigma surface to the style base, we used a four-way fixed effect ANOVAs that included harvest date, style
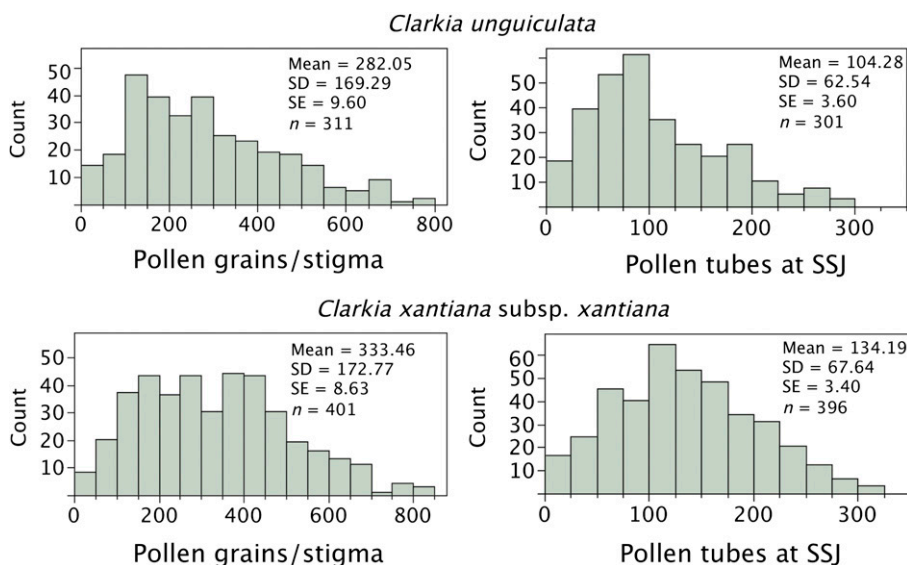

FIGURE 2 Frequency distributions of the number of pollen grains per stigma and the number of pollen tubes observed at the stigma–style junction (SSJ) among field-collected *Clarkia unguiculata* and *C. xantiana* subsp. *xantiana* flowers, pooled across populations and harvest dates.
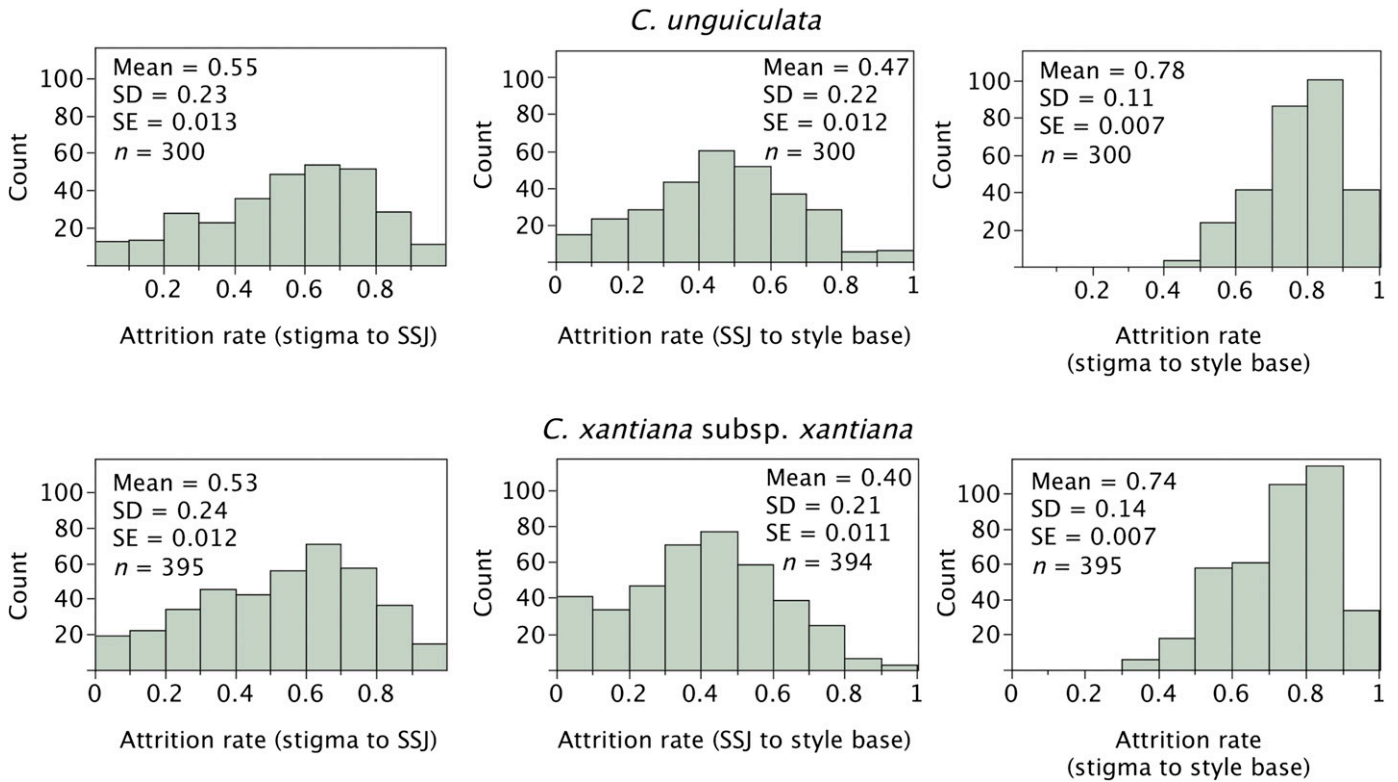
FIGURE 3 Frequency distributions for pollen attrition rates observed in *Clarkia unguiculata* and *C. xantiana* subsp. *xantiana* flowers, pooled across populations and harvest dates. SSJ: stigma–style junction.

length, pollen grains/stigma, the number of pollen tubes at the SSJ and their interactions. Each species was analyzed separately. The number of pollen grains per stigma was $\log_{10}$-transformed to approach a normal distribution.

To detect the effect of pollination intensity on pollen attrition rates from the SSJ to the style base and from the stigma surface to the style base, we used one-way ANOVAs to detect differences in attrition rates between stigmas with ≤100 or >100 pollen grains.

In field populations of *unguiculata*, we repeated the four-way model described above after excluding the samples that received >100 pollen grains. The number of stigmas of *xantiana* with ≤100 pollen grains ($n = 26$) was not sufficient to conduct this analysis.

Testing for interference among male gametophytes—Within each taxon and pollination intensity class, we conducted linear regressions of the number of pollen tubes at the SSJ on the number of pollen grains per stigma. Differences between the two pollination intensity classes (≤100 vs. >100 pollen grains/stigma) with respect to the slopes of these lines were assessed to detect evidence of interference or antagonistic interactions among germinating pollen grains. If the slope of the relationship is more positive among lightly pollinated flowers than among heavily pollinated flowers, this would indicate that, at high numbers, interference among germinating pollen grains reduces the per capita benefits of increased pollen deposition.
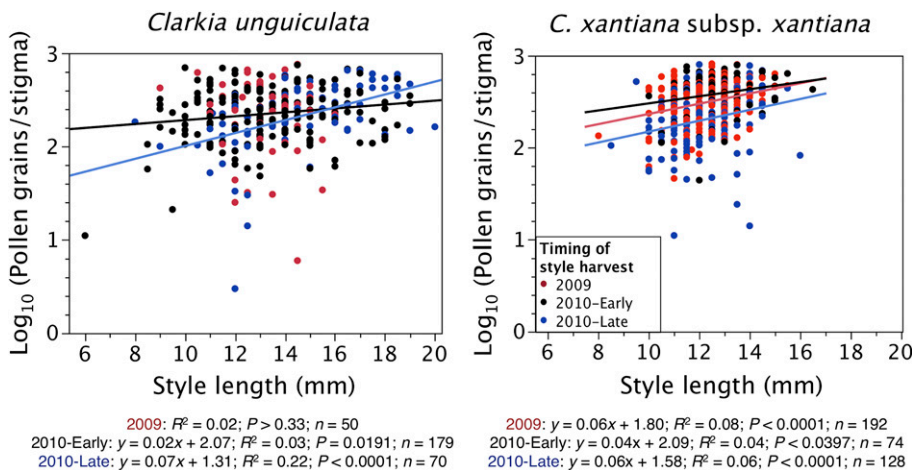
Absolute number of pollen tubes entering the style and reaching its base: Effects of harvest date, style length and pollen grains per stigma—Three-way fixed effect ANCOVAs were conducted to detect the independent effects of harvest date, style length, pollen grains per stigma and their interactions on the number of tubes that reached the SSJ in each species. Four-way fixed effect ANCOVAs were conducted

2009: $R^2 = 0.02$; $P > 0.33$; $n = 50$
2010-Early: $y = 0.02x + 2.07$; $R^2 = 0.03$; $P = 0.0191$; $n = 179$
2010-Late: $y = 0.07x + 1.31$; $R^2 = 0.22$; $P < 0.0001$; $n = 70$

2009: $y = 0.06x + 1.80$; $R^2 = 0.08$; $P < 0.0001$; $n = 192$
2010-Early: $y = 0.04x + 2.09$; $R^2 = 0.04$; $P < 0.0397$; $n = 74$
2010-Late: $y = 0.06x + 1.58$; $R^2 = 0.06$; $P < 0.0001$; $n = 128$

FIGURE 4 Linear regressions of pollen grains/stigma on style length among field collected, senesced flowers collected on each of three harvest date classes (2009; 2010-Early; 2010-Late). See Table 1 for assignments of populations and sampling dates to these categories.

to detect the independent effects of these variables and the number of pollen tubes entering the SSJ on the number of pollen tubes reaching the base of the style. In these four-way ANCOVAs, none of the interactions were significant in either species, so the interactions were excluded from the model presented here.

All analyses were conducted using the program JMP Pro 11.0.0 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Phenotypic variation in pollen deposition, style length, and attrition rates—Style length showed a high degree of phenotypic variation in both species (Fig. 1), ranging from 6 to 20 mm in *unguiculata* and from 8 to 16.5 mm in *xantiana*. The mean number of pollen grains per stigma also varied greatly among individuals and exceeded the mean number of ovules per flower in field populations, as reported by Delesalle et al. (2008). In *unguiculata* and *xantiana*, stigmas received a mean of 282.05 (±9.60 SE) and 333.46 (±8.63 SE) pollen grains, respectively (Fig. 2).

All three estimates of pollen attrition rates exhibited high phenotypic variation among flowers, spanning from 0 to 1 (Fig. 3). On average, 53–55% of the pollen grains on the sampled stigmas failed to reach the stigma–style junction (mean attrition rate for *unguiculata*: 0.55 ± 0.013 SE; *xantiana*: 0.53 ± 0.012 SE), and 40–47% of the pollen tubes that were visible at the SSJ failed to reach the base of the style (mean attrition rate for *unguiculata*: 0.47 ± 0.012 SE; *xantiana*: 0.40 ± 0.011 SE). The mean total pollen attrition rate—from the stigma surface to the base of the style—in *unguiculata* was 0.78 (±0.007 SE, $n = 300$) and in *xantiana* was 0.74 (±0.007 SE, $n = 395$).

Pollen grains per stigma: Effects of harvest date and style length—In both species, the bivariate relationship between the number of pollen grains per stigma and style length was generally positive, although pollen grains per stigma in *unguiculata* was influenced by a significant style length × harvest date interaction (Fig. 4 and Table 2A). Among *unguiculata* samples collected in 2009, pollen grains per stigma was not associated with style length, while among samples collected both Early and Late in 2010, style length was significantly, positively correlated with pollen grains per stigma. As a result of this interaction, independent of harvest date, style length had a marginally nonsignificant effect on pollen grains per stigma in *unguiculata* ($P > 0.0785$; Table 2A). By contrast, style length positively affected pollen grains per stigma in all three harvest date classes in *xantiana*; we observed a highly significant effect of style length on pollen grains per stigma and no significant style length × harvest date interaction (Table 2B). In both species, stigmas with >100 pollen grains were associated with significantly longer styles than those with ≤100 pollen grains (Table 3).

Pollen attrition rates: Effects of harvest date, style length, pollen grains/stigma, and the number of pollen tubes entering the style—The number of pollen grains per stigma had a significant and positive effect on all three measures of attrition in both taxa (Tables 4 and 5; Fig. 5). By contrast, style length had no significant effect on any estimate of pollen attrition independent of the effects of harvest date and pollen deposition (Tables 4 and 5).

In *unguiculata*, consistent with the positive effects on attrition of pollen grains per stigma detected by the ANCOVAs (Table 4;

TABLE 2. Summary of multivariate model to detect the independent effects of harvest date category and style length on the number of pollen grains per stigma among flowers of (A) *Clarkia unguiculata* and (B) *C. xantiana* subsp. *xantiana*.

A) *Clarkia unguiculata*

Source	df	Sum of squares	F ratio	Prob > F
Harvest date	2	0.98	4.33	**0.0140**
Style length	1	0.35	3.12	*0.0785*
Style length × Harvest date	2	1.35	5.93	**0.0030**
Model	5	3.39	5.95	**0.0001**
Error	309	35.16		
Total	314			

$R^2 = 0.09$

	Least squares means log₁₀ (No. pollen grains/Stigma)		No. pollen grains/Stigma (back-transformed)
Harvest date	Least squares means	SE	
2009	2.42	0.043	263.03
2010-Early	2.36	0.025	229.09
2010-Late	2.25	0.043	177.83

B) *Clarkia xantiana*

Source	df	Sum of squares	F ratio	Prob > F
Harvest date	2	4.02	30.29	**<0.0001**
Style length	1	1.52	22.87	**<0.0001**
Style length × Harvest date	2	0.02	0.15	0.8611
Model	5	6.55	19.76	**<0.0001**
Error	396	26.27		
Total	401			

$R^2 = 0.20$

	Least squares means log₁₀ (No. pollen grains/Stigma)		Pollen grains/Stigma (back-transformed)
Harvest date	Least squared mean	SE	
2009	2.50	0.018	316.23
2010-Early	2.58	0.032	380.19
2010-Late	2.31	0.023	204.17

Fig. 5A and C), stigmas with >100 pollen grains exhibited significantly higher rates of attrition from the stigma surface to the SSJ and from the stigma surface to the style base than stigmas with ≤100 pollen grains (Table 3). By contrast, stigmas with >100 pollen grains exhibited a significantly lower rate of attrition from the SSJ to the style base than did stigmas with ≤100 pollen grains. In *xantiana*, the one-way ANOVA detected no significant effect of pollen grains per stigma on the attrition rate within the style (Table 3), in contrast to the ANCOVA (Table 5; Fig. 5D).

In *unguiculata*, the relationships between attrition rates and pollen deposition differed between the entire data set and the subset of flowers expected to experience comparatively weak competition among pollen donors (i.e., stigmas that received ≤100 pollen grains). Among the latter, the ANCOVA detected significant effects of pollen grains per stigma on only one attrition rate (from the SSJ to the style base) (Appendix S1 in Supplemental Data with the online version of this article), and the sign of the pollen deposition effect differed from the complete data set. Within these flowers, in contrast to the complete data set (Fig. 5C), increased pollen deposition had a negative effect on the attrition rate from the SSJ to the style base (Fig. 6).

In *xantiana*, similar to *unguiculata*, stigmas with >100 pollen grains exhibited significantly higher rates of attrition from the stigma surface to the SSJ and from the stigma surface to the style

TABLE 3. Mean pollen deposition (number of pollen grains/stigma), style length, pollen tubes, and attrition rates (SD) among flowers of (A) *Clarkia unguiculata* and (B) *C. xantiana* subsp. *xantiana* receiving ≤100 vs. >100 pollen grains. For each trait within each species, means that differ significantly between stigmas receiving ≤100 grains vs. >100 grains (as detected by one-way ANOVAs conducted on pooled stigmas in each species) are indicated by distinct superscripts. The values for pollen grains/stigma were $\log_{10}$-transformed before analysis.

	Stigmas with ≤100 pollen grains		Stigmas with >100 pollen grains	
Variable	Mean (SD)	*n*	Mean (SD)	*n*
A) *C. unguiculata*				
Style length (mm)	12.43[a] (2.07)	35	13.71[b] (2.41)	266
Number of pollen grains/stigma	55.56[a] (30.95)	35	303.52[b] (154.46)	266
Number of pollen tubes at stigma–style junction	35.80 (24.61)	35	113.29 (60.40)	266
Number of pollen tubes at style base	16.5[a] (13.58)	35	56.38[b] (29.42)	266
Attrition: Stigma to stigma–style junction	0.38[a] (0.23)	34	0.58[b] (0.22)	266
Attrition: Stigma–style junction to style base	0.56[b] (0.25)	34	0.46[a] (0.21)	266
Attrition: Stigma to style base	0.74[a] (0.15)	34	0.79[b] (0.11)	266
B) *C. xantiana* spp. *xantiana*				
Style length (mm)	11.78[a] (1.44)	28	12.37[b] (1.23)	367
Number of pollen grains/stigma	63.05[a] (26.77)	28	352.08[b] (161.64)	368
Number of pollen tubes at stigma–style junction	39.25 (23.74)	28	141.42 (64.36)	368
Number of pollen tubes at style base	22.71[a] (15.54)	28	79.88[b] (37.36)	368
Attrition: Stigma to stigma–style junction	0.38[a] (0.29)	27	0.54[b] (0.23)	368
Attrition: Stigma–style junction to style base	0.41 (0.22)	26	0.40 (0.21)	368
Attrition: Stigma to style base	0.65[a] (0.20)	27	0.74[b] (0.13)	368

base than did stigmas with ≤100 pollen grains (Table 3). There was no difference, however, between pollination intensities in the attrition rate from the SSJ to the style base.

Evidence for interference among germinating pollen grains—In both species, the slope of the regression of the number of pollen tubes that succeeded in reaching the SSJ on pollen grains per stigma differed between lightly vs. heavily pollinated stigmas (Fig. 7). Although in both groups of flowers, the number of pollen tubes entering the style was positively correlated with pollen deposition, among stigmas with ≤100 pollen grains, the slope of the regression was twice that of the slope exhibited among stigmas with >100 pollen

TABLE 4. Summary of three-way (for the attrition rate from the stigma surface to the stigma–style junction [SSJ]) and four-way multivariate models (for other attrition rates) to detect the independent effects of harvest date, style length, the number of pollen grains/stigma, the number of pollen tubes at the SSJ, and their interactions on attrition rates in *Clarkia unguiculata*. The number of pollen tubes at the SSJ was not included in the model for the attrition rate from the stigma to the SSJ, as it is not a useful predictor variable for this step in the attrition process. The number of pollen grains/stigma was $\log_{10}$-transformed prior to analysis. SS = sum of squares.

	Attrition rate: Stigma surface to SSJ				Attrition rate: SSJ to style base				Attrition rate: Stigma surface to style base			
Source	df	SS	*F* ratio	*P* > *F*	df	SS	*F* ratio	*P* > *F*	df	SS	*F* ratio	*P* > *F*
Harvest date	2	0.35	4.59	**0.0109**	2	0.33	4.56	**0.0113**	2	0.00	0.49	0.6122
Style length	1	0.09	2.30	0.1307	1	0.05	1.38	0.2407	1	0.00	0.42	0.5157
Pollen grains/stigma	1	2.37	62.66	**<0.0001**	1	0.48	13.22	**0.0003**	1	2.00	1309.51	**<0.0001**
Pollen tubes at SSJ					1	1.78	48.53	**<0.0001**	1	1.94	1268.97	**<0.0001**
Harvest date × Style length	2	0.12	1.63	0.1975	2	0.18	2.41	0.0918	2	0.01	2.89	**0.0573**
Harvest date × Pollen grains/stigma	2	0.43	5.69	**0.0038**	2	0.11	1.57	0.2098	2	0.03	8.46	**0.0003**
Harvest date × Pollen tubes at SSJ					2	0.06	0.78	0.4599	2	0.00	0.78	0.4615
Style length × Pollen grains/stigma	1	0.27	7.02	**0.0085**	1	0.04	1.03	0.3109	1	0.00	2.69	0.1023
Style length × Pollen tubes at SSJ					1	0.00	0.13	0.7218	1	0.00	0.27	0.6062
Pollen grains/stigma × Pollen tubes at SSJ					1	1.06	28.87	**<0.0001**	1	0.94	611.89	**<0.0001**
Harvest date × Style length × Pollen grains/stigma	2	0.07	0.91	0.4040	2	0.11	1.52	0.2210	2	0.03	9.45	**<0.0001**
Harvest date × Style length × Pollen tubes at SSJ					2	0.03	0.45	0.6397	2	0.00	0.77	0.4661
Harvest date × Pollen grains/stigma × Pollen tubes at SSJ					2	0.39	5.30	**0.0055**	2	0.02	6.34	**0.0020**
Style length × Pollen grains/stigma × Pollen tubes at SSJ					1	0.04	1.05	0.3076	1	0.01	5.81	**0.0166**
Harvest date × Style length × Pollen grains/stigma × Pollen tubes at SSJ					2	0.01	0.15	0.8633	2	0.03	9.03	**0.0002**
Model	11	4.52	10.87	**<0.0001**	23	3.83	4.54	**<0.0001**	23	3.59	101.95	**<0.0001**
Error	288	10.88			276	10.10			276	0.42		
Corrected total	299	15.40			299	13.93			299	4.01		
				$R^2 = 0.29$				$R^2 = 0.27$				$R^2 = 0.89$

TABLE 5. Summary of three-way (for the attrition rate from the stigma surface to the stigma–style junction [SSJ]) and four-way multivariate models (for other attrition rates) to detect the independent effects of harvest date, style length, the number of pollen grains/stigma, the number of pollen tubes at the SSJ, and their interactions on attrition rates in *Clarkia xantiana* subsp. *xantiana*. The number of pollen tubes at the SSJ was not included in the model for the attrition rate from the stigma to the SSJ, as it is not a useful predictor variable for this step in the attrition process. Pollen grains/stigma was $\log_{10}$-transformed prior to analysis. SS = sum of squares.

Source	Attrition rate: Stigma surface to SSJ				Attrition rate: SSJ to style base				Attrition rate: Stigma surface to style base			
	df	SS	F ratio	P > F	df	SS	F ratio	P > F	df	SS	F ratio	P > F
Harvest date	2	0.25	2.95	*0.0537*	2	0.21	2.79	*0.0628*	2	0.00	1.35	0.2599
Style length	1	0.00	0.01	0.9392	1	0.01	0.32	0.5744	1	0.00	1.41	0.2360
Pollen grains/stigma	1	4.95	117.29	**<0.0001**	1	0.33	8.51	**0.0037**	1	3.84	2472.89	**<0.0001**
Pollen tubes at SSJ					1	1.17	30.59	**<0.0001**	1	2.24	1441.57	**<0.0001**
Harvest date × Style length	2	0.07	0.85	0.4300	2	0.02	0.27	0.7649	2	0.00	0.01	0.9915
Harvest date × Pollen grains/stigma	2	0.74	8.80	**0.0002**	2	0.07	0.86	0.4224	2	0.04	13.62	**<0.0001**
Harvest date × Pollen tubes at SSJ					2	0.13	1.71	0.1821	2	0.01	2.87	*0.0577*
Style length × Pollen grains/stigma	1	0.04	0.90	0.3432	1	0.01	0.19	0.6638	1	0.02	9.89	**0.0018**
Style length × Pollen tubes at SSJ					1	0.02	0.65	0.4196	1	0.00	2.17	0.1415
Pollen grains/stigma × Pollen tubes at SSJ					1	0.09	2.29	0.1314	1	0.65	420.77	**<0.0001**
Harvest date × Style length × Pollen grains/stigma	2	0.02	0.30	0.7443	2	0.03	0.39	0.6749	2	0.00	0.36	0.6968
Harvest date × Style length × Pollen tubes at SSJ					2	0.03	0.35	0.7018	2	0.00	0.12	0.8859
Harvest date × Pollen grains/stigma × Pollen tubes at SSJ					2	0.05	0.70	0.4950	2	0.05	15.95	**<0.0001**
Style length × Pollen grains/stigma × Pollen tubes at SSJ					1	0.02	0.44	0.5074	1	0.01	3.85	*0.0504*
Harvest date × Style length × Pollen grains/stigma × Pollen tubes at SSJ					2	0.01	0.11	0.8917	2	0.01	1.97	0.1408
Model	11	6.21	13.37	**<0.0001**	23	3.63	4.13	**<0.0001**	23	6.95	194.68	**<0.0001**
Error	382	16.12			369	14.11			370	0.57		
Corrected total	393	22.33		$R^2 = 0.28$	392	17.74		$R^2 = 0.20$	393	7.53		$R^2 = 0.92$

grains (*unguiculata* slopes: 1.12 ± 0.10 SE vs. 0.51 ± 0.07 SE; *xantiana* slopes: 0.90 ± 0.26 SE vs. 0.45 ± 0.06 SE).

Effect of pollen deposition and style length on absolute pollen success: Number of pollen tubes entering the style and reaching its base—In both taxa, style length had no effect on the absolute number of tubes that reached the SSJ (Table 6) or the base of the style (Table 7), but the number of pollen grains per stigma was significantly and positively associated with the number of pollen tubes reaching the SSJ and the style base (Fig. 8; Tables 6 and 7), independent of harvest date, style length, and the number of pollen tubes at the SSJ.

Effect of pollen entry into the style on subsequent attrition and on the number of tubes reaching the style base—In both species, the number of pollen tubes at the SSJ had a negative effect on attrition from the SSJ to the style base, independent of variation in style length, harvest date, and pollen grains per stigma (Fig. 9A and B; Tables 4 and 5). This negative effect contributed to the positive relationship observed between the number of pollen tubes at the SSJ and the number of pollen tubes at the style base (Fig. 9C and D; Table 7).

DISCUSSION

The high phenotypic variation observed in style length, pollen deposition, and the three measures of pollen and pollen tube attrition in natural populations of both *unguiculata* and *xantiana* indicate that these taxa are well suited to studies of the causes and

consequences of variation in these floral traits. Accordingly, *Clarkia* has been the subject of several other studies investigating the factors influencing pollen deposition and pollen tube attrition. In a survey of four wild *unguiculata* populations from which stigmas and styles were collected 6, 24, and 48 h after receptive flowers were exposed to pollinators, Németh and Smith-Huerta (2003) observed that levels of pollen deposition at 24 and 48 h (215 ± 11.2 SE and 322.8 ± 15.3 SE grains per stigma, respectively) were similar to the mean levels reported here (282 ± 9.60 SE for *unguiculata*; 333 ± 8.63 SE for *xantiana*), with pollen deposition increasing with the length of time that flowers were available for visitation. They then examined each group of flowers (6-, 24- and 48-h collections) and found that, across these three groups, mean pollen deposition was positively correlated with the mean number of pollen tubes reaching the stigma–style junction and the base of the style, similar to our observations (Fig. 8). They found no relationship among these groups between mean pollen deposition and the mean pollen tube attrition rate from the stigma–style junction to the base of the style, contrasting with the positive relationships between the number of pollen grains per stigma and the attrition rate that we observed in both *unguiculata* and *xantiana* (Fig. 5). The difference between their results and ours may have been due, in part, to the fact that they reported associations among the means of the three groups (6 vs. 24 vs. 48 h) rather than by conducting regressions among individual flowers. Németh and Smith-Huerta (2003) did not investigate the influence of style length on pollen deposition or performance, but they did examine seedling performance and detected no relationship between pollen deposition and seedling vigor, which they reasonably interpreted to mean that the intensity of pollination
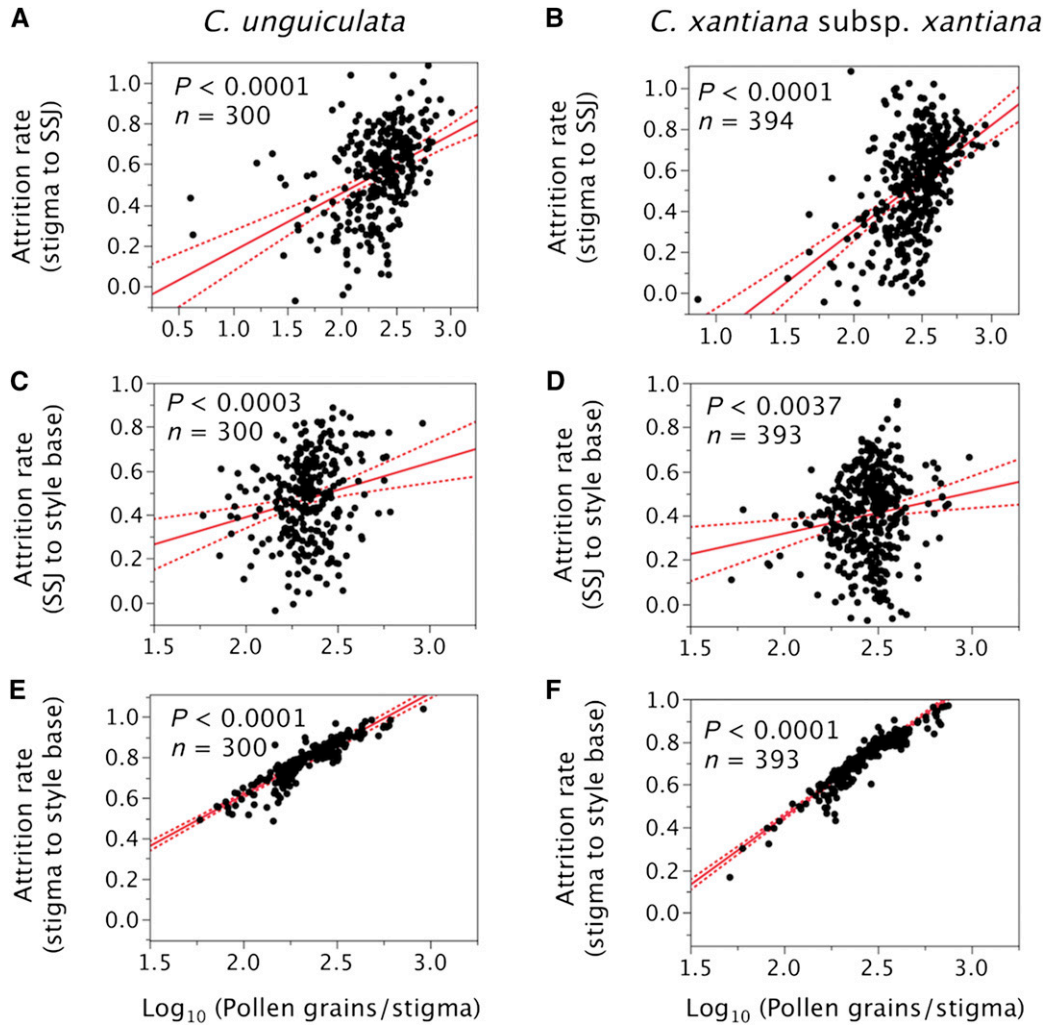
FIGURE 5 Relationships between attrition rates and the number of pollen grains per stigma among field-collected senesced flowers of *Clarkia unguiculata* and *C. xantiana* subsp. *xantiana*. In (A) and (B), the variables are adjusted for the effects of harvest date, style length, and the interaction between them. In (C–F), the variables are adjusted for the effects of harvest date, style length, the number of pollen tubes at the stigma–style junction (SSJ), and the interactions among them. Tables 4 and 5 report the independent effects of each variable on attrition rates. In each panel, the solid line represents the slope of the direct relationship between the attrition rate and pollen deposition; the dotted lines represent the 95% confidence interval.

experienced over 48 h did not affect the efficacy of gametophytic selection.

In another study of the factors influencing pollen performance in *Clarkia*, Smith-Huerta (1997) observed pollen tube attrition in the stigmas and styles of a selfing and an outcrossing population of *C. tembloriensis*; both populations exhibited high attrition rates from the stigma to the stigma–style junction (59% and 83.5% attrition in the selfing vs. outcrossing populations, respectively). Only the outcrossing population, however, exhibited additional attrition as pollen tubes grew from the style's tip to its base (as reported here for both *unguiculata* and *xantiana*).

The evolutionary significance of variation in style length—In the *Clarkia* populations sampled here, long styles functioned to increase pollen deposition, which could have been achieved in several ways. First, long styles might place the stigmatic surface in a location that increases contact between stigmas and the pollen-bearing parts of insect visitors' bodies. Second, style length may be positively correlated

with other floral traits that promote insect visitation and pollen deposition, such as petal size. Third, style length could be positively correlated with the duration of stigma receptivity and/or with the number of pollinator visits received before style senescence, both of which may increase pollen receipt. Given the positive relationship in both taxa between the number of pollen grains per stigma and the number of pollen tubes reaching the style base (Fig. 8C and D), increases in pollen deposition in these taxa may in turn increase fertilization rates (especially since the mean number of tubes reaching the style base did not exceed the mean number of ovules per ovary).

Although, based on attrition rates, we found no evidence that style length directly influenced the intensity of competition or selection among male gametophytes in *unguiculata* or *xantiana*, style length was positively associated with pollen deposition which, in turn, was positively associated with all three attrition rates observed in both taxa (Fig. 5). The positive correlation between style length and the number of pollen grains adhering to the stigma may result in a greater opportunity for selection among male gametophytes in
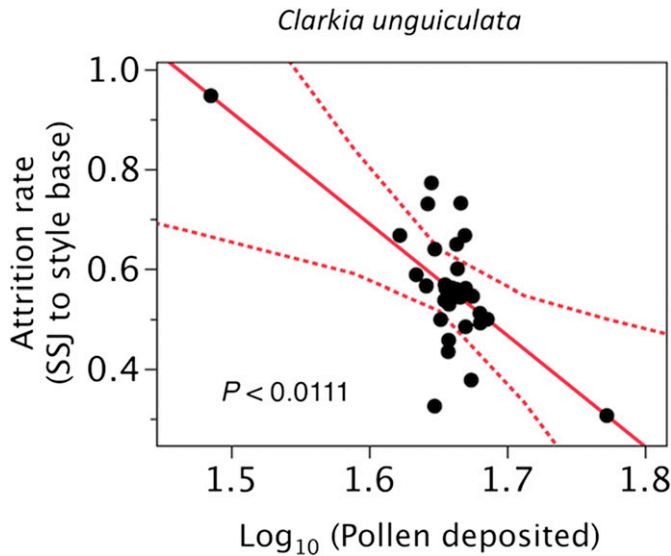
FIGURE 6 Relationship between the attrition rate within the style and the number of pollen grains deposited per stigma among *Clarkia unguiculata* flowers receiving ≤100 pollen grains, controlling for the effects of harvest date, style length, the number of pollen tubes at the SSJ, and the interactions among them. The solid line represents the slope of the direct relationship between pollen deposition and the attrition rate, independent of the other variables in the model; the dotted lines represent the 95% confidence interval.

flowers with relatively long styles. Large pollen loads may result in higher rates of pollen germination success than small ones (e.g., Brewbaker and Majumder, 1961), and subsequent selection among male gametophytes may be particularly strong as pollen tubes grow from the stigma to the upper style (Erbar, 2003; Lubliner et al.,
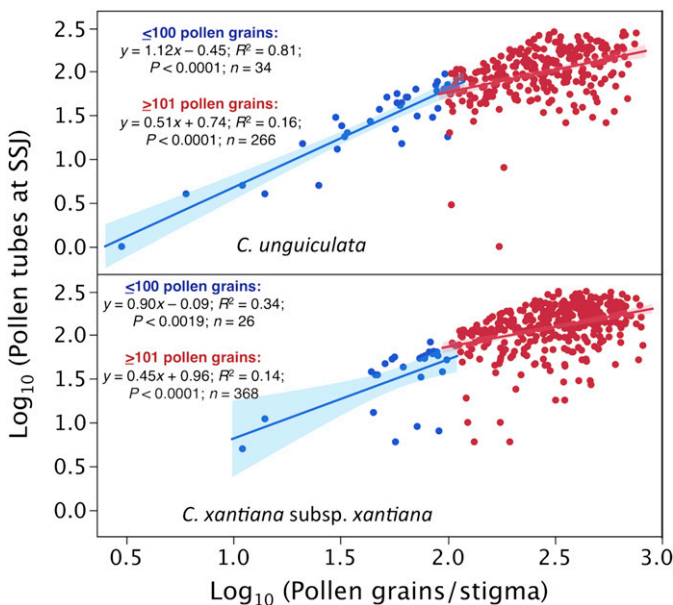

FIGURE 7 Linear regressions of the number of pollen tubes reaching the stigma–style junction (SSJ) on the number of pollen grains per stigma, for stigmas receiving ≤100 pollen grains (blue points) and >100 pollen grains (red points). Solid lines represent the least square regression slopes; shaded areas represent the 95% confidence intervals of each regression line.

2003; Stephenson et al., 2003). Consequently, plants with relatively long styles could benefit if receiving more pollen increases seed quality by increasing selection against poor quality male gametophytes during pollen germination and penetration of the stigma. Even in the absence of such selection, in both species we detected a concrete advantage to receiving more pollen; the number of pollen grains adhering to the stigma is positively correlated with the number of pollen tubes reaching the style entrance and the style base (Fig. 8). Moreover, as the number of tubes reaching the style entrance increased, the attrition rate within the style significantly declined independent of variation in style length (Fig. 9A and B); either the genetic quality of the pollen tubes entering the style or maternal resources for pollen tube growth may be elevated with increasing numbers of pollen tubes entering the style.

In both taxa, flowers that received >100 pollen grains had longer styles and higher attrition rates from the stigma to the SSJ and from the stigma to the style base than those receiving ≤100 grains (Table 3). This pattern is consistent with the inference that the opportunity for gametophytic selection is a function of style length. Interestingly, in *unguiculata*, the attrition rate within the style—from the SSJ to the style base—was *higher* in flowers that received ≤100 pollen grains than in flowers that received >100 pollen grains, in spite of the fact that the styles were shorter in the former (Table 3). There are two nonmutually exclusive explanations for this relationship. First, in stigmas with >100 pollen grains, selection may have been sufficiently strong before the pollen tubes entered the style that their genetic quality at the stigma–style junction was higher than those entering the style of lightly pollinated stigmas, resulting in lower attrition rates. Second, the provisioning of pollen tubes by the maternal tissue may have been of higher quality when more pollen tubes entered the style. In *xantiana*, however, there was no difference between the two classes of pollination intensity in the attrition rate from the SSJ to the style base (Table 3).

The independence between style length and attrition rates observed here could also have been a result of where in the style most attrition occurs. If most attrition occurs before the pollen tubes reach 10 mm in length, for example, then the remaining length of the styles would not play a direct role in gametophytic selection, and longer styles could not be supposed to provide this function. Nevertheless, while attrition rates were not directly affected by style length in this study, we cannot rule out the possibility that the genetic quality of the pollen tubes that reached the style base was related to style length. Examining the quality of seeds produced as a function of style length would be necessary to explore this possibility.

Evidence for competition among male gametophytes for access to the style—In the current study, in both species, the mean number of pollen grains per stigma and the mean number of pollen tubes observed in the SSJ exceeded the mean number of ovules per flower reported by Delesalle et al. (2008) (Fig. 2). Moreover, in 2010, flowers produced fewer than 100 ovules on average (mean = 74.13 ± 0.80 SE, $n = 456$; and 81.06 ± 0.89 SE, $n = 406$ in *unguiculata* and *xantiana* populations, respectively; A. A. Hove et al., unpublished manuscript), indicating that competition among male gametophytes for access to ovules may regularly occur in these field populations. The numbers of pollen grains per stigma reported here may underestimate the number of pollen grains deposited, as some may have washed off during the style preparation process. Any grains that did wash off, however, probably either had not germinated or had not produced pollen tubes that were well anchored in the

TABLE 6. Summary of 3-way multivariate model to detect the independent effects of harvest time, style length, pollen grains per stigma, and their interactions on the number of tubes that reach the stigma–style junction (SSJ) among flowers of (A) *Clarkia unguiculata* and (B) *C. xantiana* subsp. *xantiana*. Pollen grains/stigma was log$_{10}$-transformed prior to analysis. SS = sum of squares.

Source	df	SS	F ratio	P > F
A) *C. unguiculata*				
Harvest date	2	75,950	17.03	**<0.0001**
Style length	1	1082	0.49	0.4866
Pollen grains/stigma	1	319,615	143.32	**<0.0001**
Harvest date × Style length	2	1432	0.32	0.7271
Harvest date × Pollen grains/stigma	2	33,442	7.50	**0.0007**
Style length × Pollen grains/stigma	1	2705	1.21	0.2717
Harvest date × Style length × Pollen grains/stigma	2	11,001	2.47	*0.0867*
Model	11	520,075	21.20	**<0.0001**
Error	288	642,251		
Total	299	1,162,326		
				$R^2 = 0.45$
B) *C. xantiana* subsp. *xantiana*				
Harvest date	2	21,787	3.36	**0.0357**
Style length	1	1240	0.38	0.5367
Pollen grains/stigma	1	238,781	73.68	**<0.0001**
Harvest date × Style length	2	3867	0.60	0.5512
Harvest date × Pollen grains/stigma	2	4395	0.68	0.5082
Style length × Pollen grains/stigma	1	1193	0.37	0.5444
Harvest date × Style length × Pollen grains/stigma	2	3214	0.50	0.6095
Model	11	541,708	15.20	**<0.0001**
Error	382	1,238,006		
Total	393	17,797		
				$R^2 = 0.30$

stigma and therefore would not have contributed significantly to competitive interactions involving access to the style. Conversely, if groups of pollen grains were deposited in successive events as a result of multiple pollinator visits, with the first ~100 pollen grains arriving an hour or more before the remaining pollen, then the pollen tubes that reached the base of the style would have experienced

TABLE 7. Summary of multivariate model to detect the independent effects of harvest date, style length, the number of pollen grains per stigma, and the number of pollen tubes at the stigma–style junction (SSJ) on the number of tubes that reach the base of the style among flowers of (A) *Clarkia unguiculata* and (B) *C. xantiana* subsp. *xantiana*. Only main effects were included because none of the interactions were significant. Number of pollen grains per stigma was log$_{10}$-transformed before analysis. SS = sum of squares.

Source	df	SS	F ratio	P > F
A) *C. unguiculata*				
Harvest date	2	1044	1.38	0.2536
Style length	1	548	1.45	0.2299
Pollen grains/stigma	1	12,986	34.30	**<0.0001**
Pollen tubes at SSJ	1	56,679	149.71	**<0.0001**
Model	5	170,796	90.23	**<0.0001**
Error	294	111,306		
Total	299	282,102		
				$R^2 = 0.61$
B) *C. xantiana* subsp. *xantiana*				
Harvest date	2	5045	3.68	**0.0263**
Style length	1	30	0.04	0.8341
Pollen grains/stigma	1	13,800	20.11	**<0.0001**
Pollen tubes at SSJ	1	142,075	207.09	**<0.0001**
Model	5	331,678	96.69	**<0.0001**
Error	388	266,193		
Total	393	597,871		
				$R^2 = 0.55$

much less competition than implied by the ratio of the mean number of pollen grains per stigma to the mean number of ovules per ovary. In short, the number of pollen grains observed per stigma may not be a good measure of the realized intensity of gametophytic competition (Mulcahy and Mulcahy, 1987). The number of pollen tubes that reach the SSJ is a more reliable indicator of the potential strength of pollen competition (Herrera, 2002, 2004), and in both taxa this exceeded the number of ovules available for fertilization reported by Delesalle et al. (2008) and A. A. Hove et al. (unpublished data) (Fig. 2).

Pollen attrition rates vs. pollen grains per stigma—We consistently found that the number of pollen grains adhering to the stigma positively affects all three rates of attrition (Fig. 5), independent of variation in style length and the number of pollen tubes entering the style (Tables 4 and 5). The timing of pollen deposition, however, may have played a role in generating these relationships. If stigmas with relatively high pollen loads received pollen over a longer time period than lightly pollinated stigmas, then some portion of their pollen may have arrived after the stigma began to wilt or the stylar resources had been depleted. This pattern of pollen receipt would generate a positive association between pollen grains per stigma and subsequent attrition rates. In this study, however, the number of pollen grains per stigma was also positively associated with the number of pollen tubes entering the SSJ and the number arriving at the style base. So, the higher attrition rates observed in heavily pollinated flowers could not have been due entirely to the late arrival of "extra" but ineffective pollen to unreceptive stigmas or styles. Moreover, the positive relationship between pollen grains per stigma and attrition in the style was independent of the number of pollen tubes entering the style (Tables 4 and 5); the quality of the male gametophytes entering the style and/or the quality of the transmitting tissue in the style appeared to be influenced by the number of pollen grains deposited.

Interference among male gametophytes and the importance of the stigma–style junction—We observed that the positive effect of the number of pollen grains per stigma on the number of pollen tubes entering the style was weaker among flowers with high pollen loads (>100 grains) than among those with ≤100 pollen grains. As the number of pollen grains adhering to the stigma exceeded 100, the per capita success rate in entering the style diminished (Fig. 7). This pattern suggests that crowding and/or antagonistic interactions among pollen grains or tubes limit the benefits of increased pollen deposition among the more heavily pollinated flowers. Antagonistic interactions among mixtures of *unguiculata* pollen from different donors were detected by Németh and Smith-Huerta (2002), corroborating this possibility. Alternatively, the composition of the
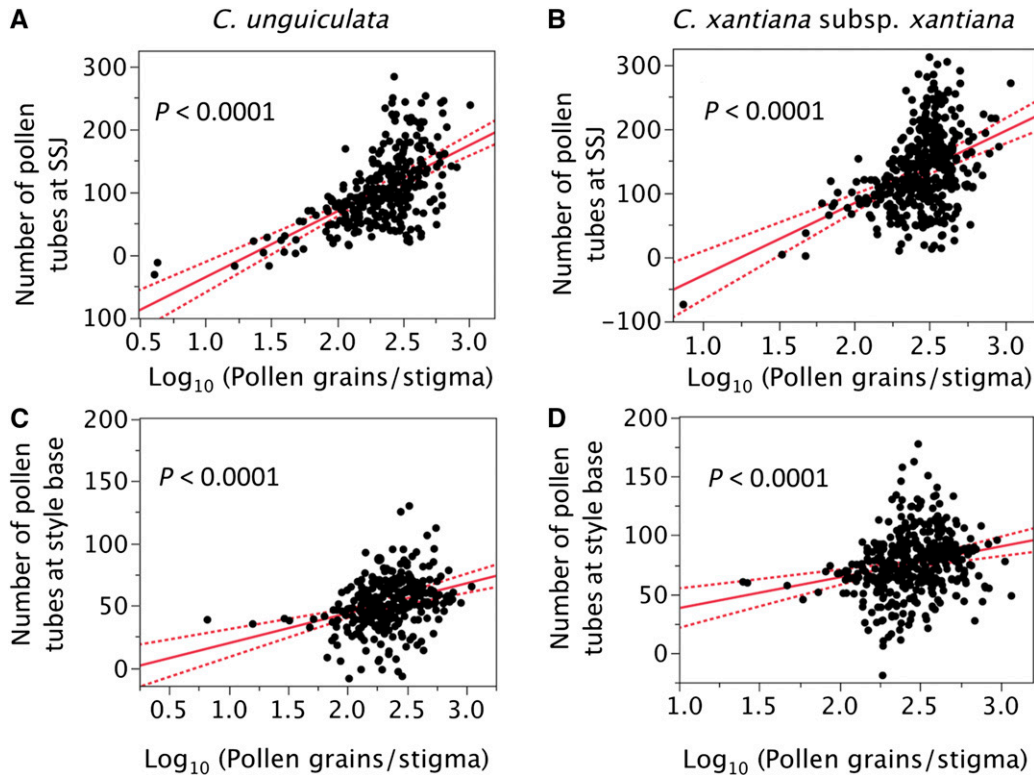
FIGURE 8 Effects of pollen grains per stigma on pollen tube success among flowers of *Clarkia unguiculata* (A, C) and *C. xantiana* subsp. *xantiana* (B, D). (A, B) The relationship between the number of pollen tubes reaching the stigma–style junction (SSJ) and the number of pollen grains per stigma. (C, D) The relationship between the number of pollen tubes reaching the base of the style and the number of pollen grains per stigma. In each panel, the solid line represents the slope of the direct relationship between the number of pollen tubes and pollen grains/stigma, independent of the other variables in the model (Tables 6 and 7); the dotted lines represent the 95% confidence interval.

pollen load, with respect to the proportions of self pollen and/or pollen from donors at different distances from the recipient plant, may have varied with the size of the pollen load, which could have influenced attrition rates (Cruzan, 1990). In either case, successful penetration of the stigma–style junction is clearly an important barrier to overcome for pollen that has arrived on a stigma, and this achievement appeared to become less likely per pollen grain as pollen deposition exceeded 100 grains. A caveat, however, is that this pattern could simply be a result of heavily pollinated stigmas receiving a higher proportion of their pollen relatively late, when the stigma was no longer maximally receptive.

Style length and pollen receipt in other taxa—Positive effects of style length on pollen receipt or on the number of pollen tubes found in the style have been reported in other species. In a study of natural populations of *Prunella grandiflora*, Bernasconi et al. (2007) found that style length and the number of pollen tubes in the style were positively correlated; moreover, in one of the eight populations surveyed, the number of pollen tubes was positively correlated with seed set, demonstrating an indirect positive effect of style length on fertility per fruit. In a manipulative experiment, Cresswell (2000) investigated the relationship between stigma height and pollen receipt in *Brassica napus*; he increased phenotypic variation in stigma height by replacing a portion of the pistil with fine wire, and then measured pollen receipt across the enhanced range of stigma heights that he created. This method enabled him to detect stabilizing

selection on stigma height (using pollen deposition as a fitness estimate), a pattern of selection that can maintain phenotypic variation in stigma height, whether it is controlled by pistil length or by style length.

The observation that relatively long styles receive more pollen than comparatively short ones does not necessarily mean that seed production or offspring quality will be higher as well, although numerous manipulative studies have found that high pollen deposition can have this effect (see Baskin and Baskin, 2015 for a recent review and for counter-examples). For example, Björkman (1995) found that high pollination intensity in *Fagopyrum esculentum* resulted in more successful pollen tube development, in higher seed set, and in larger, more vigorous seedlings (but not larger seeds). Jóhannsson and Stephenson (1997) found that higher pollen loads improved progeny vigor in *Cucurbita texana*. Mitchell (1997) reported that, in *Lesquerella fendleri*, high pollen loads resulted in higher seedling emergence and survival than low pollen loads did. In *Pyrus pyrifolia*, higher pollen density on the stigma surface gave rise to higher rates of pollen germination and faster pollen tube growth and also resulted in higher proportions of viable seed, higher fruit growth rates, higher fruit set, and larger fruit size (Zhang et al., 2010). Marshall et al. (2007) reported that, following hand pollinations of *Raphanus sativus* with mixed-donor pollen loads (all of which greatly exceeded the number of ovules per pistil), the smallest pollen loads resulted in lower seed production per fruit than did larger loads, but pollen load size did not influence the relative success of different pollen donors.
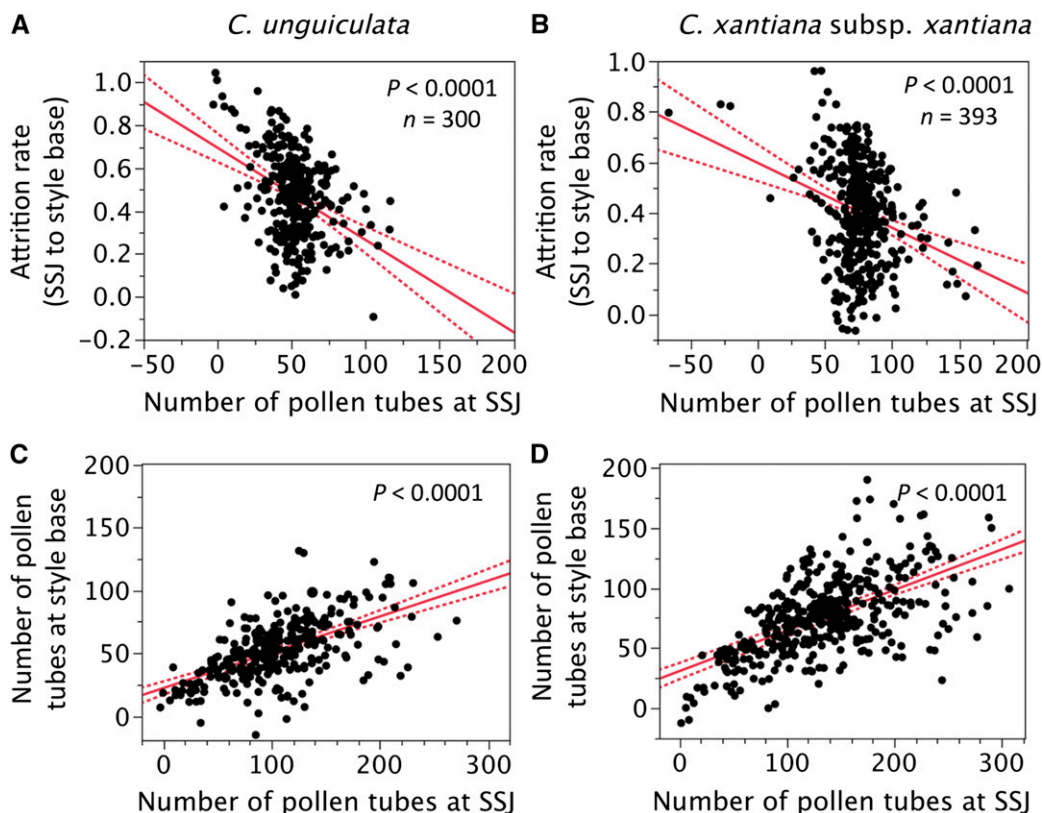
FIGURE 9 Effects of the number of pollen tubes entering the style on attrition rates within the style and on the number of pollen tubes reaching the style base among flowers of *Clarkia unguiculata* (A, C) and *C. xantiana* subsp. *xantiana* (B, D). (A, B) The relationship between the attrition rate within the style and the number of pollen tubes at the style base. (C, D) The relationship between the number of pollen tubes at the style base and the number of pollen tubes at the stigma–style junction (SSJ). Variables are adjusted for the effects of harvest date, style length, the number of pollen grains per stigma, and the interactions between them. In each panel, the solid line represents the slope of the direct relationship between the number of pollen tubes at the SSJ and the dependent variable, independent of the other variables in the model; the dotted lines represent the 95% confidence interval. Tables 4 and 5 report the independent effects of the number of pollen tubes at the SSJ on attrition rates and Table 7 reports the independent effects of the number of pollen tubes at the SSJ on the number of tubes at the style base.

While larger pollen loads may allow fertilization by only the fastest pollen tubes and result in higher quality seeds than smaller pollen loads, this does not inevitably mean that the fastest-growing pollen tubes express genes that directly affect seed quality. An alternative explanation is that maternal provisioning favors ovules and seeds that are fertilized first (Delph et al., 1998). If heavily pollinated stigmas include a higher number of fast-growing tubes than lightly pollinated ones, then the resulting ovaries may, on average, contain more ovules that are fertilized relatively early and receive the benefits of preferential maternal provisioning.

Limitations of the current study—Three aspects of the current study constrain our ability to interpret the direct effect of style length on pollen attrition and on the intensity of male gametophytic competition. First, while longer styles generally received more pollen than shorter ones in the *Clarkia* populations surveyed here, we do not know the schedule of pollen arrival or germination. Late-arriving or late-germinating pollen grains that initiate pollen tube growth much later than early-arriving ones may not, in a practical sense, directly compete with the latter for access to ovules (Thomson, 1989). When, on a given stigma, variation in the time of

pollen arrival or germination is sufficiently high, the number of pollen grains observed on the stigma provides an inaccurate estimate of the intensity of pollen competition and of the strength of selection on germination speed or pollen tube growth rate (Mulcahy and Mulcahy, 1987). Second, we did not measure seed production or seed quality among the fruits that developed from the flowers sampled here; this is an essential step for making unambiguous inferences regarding the putative fitness benefits of both high pollen receipt and strong competition among pollen tubes before or after entry into the style.

Even in the absence of an effect of style length on attrition rates, style length may affect the strength of selection. That is, the quality of the pollen genotypes that reached the ovary or fertilized the ovules—neither of which were measured here—could have been a function of style length. Other studies have detected positive relationships between pollen loads and offspring quality (e.g., Björkman, 1995; Johannson and Stephenson, 1997; Mitchell, 1997; Kalla and Ashman, 2002; Baskin and Baskin, 2015), as well as positive associations between the distance traveled by pollen tubes and progeny vigor (Mulcahy and Mulcahy, 1975; McKenna and Mulcahy, 1983).

A third limitation is that we did not measure other floral traits that may be correlated with style length and have direct effects on

pollen deposition. For example, petal area and the total number of open flowers are both likely to positively influence pollinator visitation rates, thereby affecting pollen deposition (e.g., Johnson et al., 1995). In addition, stigma surface area and the duration of stigma receptivity are likely to be positively correlated with the number of pollen grains observed on senescent stigmas. If these traits are positively correlated with style length, then the putative effects of style length on pollen deposition presented here may actually be caused by these correlated traits.

CONCLUSIONS

Longer styles received more pollen than shorter ones in natural populations of the two *Clarkia* taxa examined here, yet the longer distance traveled by pollen tubes in the former did not directly cause higher rates of pollen attrition. Nevertheless, style length could affect offspring quality if the strength of selection among pollen genotypes is stronger in long styles, even in the absence of direct effects on attrition rates. Furthermore, given that style length may influence other processes such as the timing of pollen deposition relative to the loss of stigma receptivity or the availability of resources within the style, observations of the timing of pollen receipt in wild populations can shed further light on the functional significance of style length. Finally, experimental work should be designed to detect both the direct and the antagonistic or synergistic effects of petal area (and other traits that may affect pollinator visitation rate or efficacy), style length and pollen deposition on the quality of the resulting offspring. While stronger gametophytic selection has been predicted to result in higher quality offspring, the adaptive significance of variation in style length under field conditions can only be detected when its influence is isolated from the direct and indirect effects of correlated traits.

ACKNOWLEDGEMENTS

LITERATURE CITED

Barrett, S. C. H. 2002. The evolution of plant sexual diversity. *Nature Reviews. Genetics* 3: 274–284.

Barrett, S. C. H., and L. D. Harder. 2005. The evolution of polymorphic sexual systems in daffodils (*Narcissus*). *New Phytologist* 154: 45–53.

Barrett, S. C. H., and J. Hough. 2013. Sexual dimorphism in flowering plants. *Journal of Experimental Botany* 64: 67–82.

Baskin, J. M., and C. C. Baskin. 2015. Pollen (microgametophyte) competition: An assessment of its significance in the evolution of flowering plant diversity, with particular reference to seed germination. *Seed Science Research* 25: 1–11.

Bell, G. 1985. On the function of flowers. *Proceedings of the Royal Society of London, B, Biological Sciences* 224: 223–265.

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.

Bigazzi, M., and F. Selvi. 2000. Stigma form and surface in the tribe Boragineae (Boraginaceae): Micromorphological diversity, relationships with pollen, and systematic relevance. *Canadian Journal of Botany* 78: 388–408.

Björkman, T. 1995. The effect of pollen load and pollen grain size competition on fertilization success and progeny performance in *Fagopyrum esculentum*. *Euphytica* 83: 47–52.

Brewbaker, J. L., and S. K. Majumder. 1961. Cultural studies of the pollen population effect and the self-incompatibility inhibition. *American Journal of Botany* 48: 457–464.

Campbell, D. R. 1989. Measurements of selection in a hermaphroditic plant: Variation in male and female pollination success. *Evolution* 43: 318–334.

Carlson, J. E., and K. E. Harms. 2006. The evolution of gender-biased nectar production in hermaphroditic plants. *Botanical Review* 72: 179–205.

Conner, J. K., S. Rush, S. Kercher, and P. Jennetten. 1996. Measurements of natural selection on floral traits in wild radish (*Raphanus raphanistrum*). 2. Selection through lifetime male and total fitness. *Evolution* 50: 1137–1146.

Cresswell, J. E. 2000. Manipulation of female architecture in flowers reveals a narrow optimum for pollen deposition. *Ecology* 81: 3244–3249.

Cruzan, M. B. 1989. Pollen tube attrition in *Erythronium grandiflorum*. *American Journal of Botany* 76: 562–570.

Cruzan, M. B. 1990. Pollen tube and pollen–style interactions during pollen tube growth in *Erythronium grandiflora* (Liliaceeae). *American Journal of Botany* 77: 116–122.

Delesalle, V. A., S. J. Mazer, and H. Paz. 2008. Temporal variation in the pollen:ovule ratios of *Clarkia* (Onagraceae) taxa with contrasting mating systems: field populations. *Journal of Evolutionary Biology* 21: 310–323.

Delph, L. F., C. Weinig, and K. Sullivan. 1998. Why fast-growing pollen tubes give rise to vigorous progeny: The test of a new mechanism. *Proceedings of the Royal Society of London, B, Biological Sciences* 265: 935–939.

Dudley, L. S., S. J. Mazer, and P. Galusky. 2007. The joint evolution of mating system, floral traits and life history in *Clarkia* (Onagraceae): Genetic constraints vs. independent evolution. *Journal of Evolutionary Biology* 20: 2200–2218.

Elmqvist, T., D. Liu, U. Carlsson, and B. Giles. 1993. Anther–smut infection in *Silene dioica*: Variation in floral morphology and patterns of spore deposition. *Oikos* 68: 207–216.

Erbar, C. 2003. Pollen tube transmitting tissue: Place of competition of male gametophytes. *International Journal of Plant Sciences* 164: S265–S277.

Forrest, J. R. K., J. E. Ogilvie, A. M. Goriscek, and J. D. Thomson. 2011. Seasonal change in a pollinator community and the maintenance of style length variation in *Mertensia fusiformis* (Boraginaceae). *Annals of Botany* 108: 1–12.

Franklin-Tong, V. E. 1999. Signaling and the modulation of pollen tube growth. *Plant Cell* 11: 727–738.

Ganeshaiah, K. N., P. Kathuria, and R. Uma Shaanker. 1999. Does optimal packing of flowers in syconia shape style length variation in monoecious figs? *Biotropica* 31: 312–320.

Herrera, C. M. 2002. Censusing natural microgametophyte populations: Variable spatial mosaics and extreme fine-graininess in winter-flowering *Helleborus foetidus* (Ranunculaceae). *American Journal of Botany* 89: 1570–1578.

Herrera, C. M. 2004. Distribution ecology of pollen tubes: Fine-grained, labile spatial mosaics in southern Spanish Lamiaceae. *New Phytologist* 161: 473–484.

Jacquemyn, H., C. Micheneau, D. L. Roberts, and T. Pailler. 2005. Elevational gradients of species diversity, breeding system and floral traits of orchid species on Réunion Island. *Journal of Biogeography* 32: 1751–1761.

Jia, J., and D. Y. Tan. 2012. Variation in style length and anther–stigma distance in *Isolirion songaricum* (Amaryllidaceae). *South African Journal of Botany* 81: 19–24.

Jóhannsson, M. H., and A. G. Stephenson. 1997. Effects of pollination intensity on the vigor of the sporophytic and gametophytic generation of *Cucurbita texana*. *Sexual Plant Reproduction* 10: 236–240.

Johnson, M. T. J., S. D. Smith, and M. D. Rausher. 2010. Effects of plant sex on range distributions and allocation to reproduction. *New Phytologist* 186: 769–779.

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.

Johnston, M. O. 1993. Tests of two hypotheses concerning pollen competition in a self-compatible, long-styled species (*Lobelia cardinalis*, Lobeliaceae). *American Journal of Botany* 80: 1400–1406.

Kalla, S. E., and T.-L. Ashman. 2002. The effects of pollen competition on progeny vigor in *Fragaria virginiana* (Rosaceae) depend on progeny growth environment. *International Journal of Plant Sciences* 163: 335–340.

Klinkhamer, P. G. L., and C. A. M. van der Veen-van Wijk. 1999. Genetic variation in floral traits of *Echium vulgare*. *Oikos* 85: 515–522.

Kulbaba, M. W., and A. C. Worley. 2008. Floral design in *Polemonium brandegei* (Polemoniaceae): Genetic and phenotypic variation under hawkmoth and hummingbird pollination. *International Journal of Plant Sciences* 169: 509–522.

Lankinen, A., and I. Skogsmyr. 2001. Evolution of pistil length as a choice mechanism for pollen quality. *Oikos* 92: 81–90.

Lubliner, N., D. T. Singh-Cundy, and A. Singh-Cundy. 2003. Characterization of the pollen growth transition in self-incompatible *Petunia inflata*. *Sexual Plant Reproduction* 15: 243–253.

Marshall, D. L., M. G. M. Shaner, and J.-P. Oliva. 2007. Effects of pollen load size on seed paternity in wild radish: The roles of pollen competition and mate choice. *Evolution* 61: 1925–1937.

Martin, F. W. 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain Technology* 34: 125–128.

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.

McKenna, M. A., and D. L. Mulcahy. 1983. Ecological aspects of gametophytic competition in *Dianthus chinensis*. *In* D. L. Mulcahy and E. Ottaviano [eds.], Pollen: Biology and implications for plant breeding, 419–424. Elsevier, New York, New York, USA.

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.

Mitchell, R. J. 1997. Effects of pollination intensity on *Lequerella fendleri* seed set: Variation among plants. *Oecologia* 109: 382–388.

Moeller, D. A., M. A. Geber, V. M. Eckhart, and P. Tiffin. 2012. Reduced pollinator service and elevated pollen limitation at the geographic range limit of an annual plant. *Ecology* 93: 1036–1048.

Mulcahy, D. L. 1979. The rise of the angiosperms: A genecological factor. *Science* 206: 20–23.

Mulcahy, D. L., and G. B. Mulcahy. 1975. Influence of gametophytic competition on sporophytic quality in *Dianthus chinensis*. *Theoretical and Applied Genetics* 46: 277–280.

Mulcahy, D. L., and G. B. Mulcahy. 1987. The effects of pollen competition. *American Scientist* 75: 44–50.

Nattero, J., and A. A. Cocucci. 2007. Geographical variation in floral traits of the tree tobacco in relation to its hummingbird pollinator fauna. *Biological Journal of the Linnaean Society* 90: 657–667.

Nattero, J., A. N. Sersic, and A. A. Cocucci. 2010. Patterns of contemporary phenotypic selection and flower integration in the hummingbird-pollinated *Nicotiana glauca* between populations with different flower–pollinator combinations. *Oikos* 119: 852–863.

Németh, B. M., and N. L. Smith-Huerta. 2002. Effects of pollen load composition and deposition pattern on pollen performance in *Clarkia unguiculata* (Onagraceae). *International Journal of Plant Sciences* 163: 795–802.

Németh, B. M., and N. 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.

Plitmann, U. 1993. Pollen tube attrition as related to breeding systems in Brassicaceae. *Plant Systematics and Evolution* 188: 65–72.

Plitmann, U. 1994. Assessing functional reproductive traits from herbarial material: The test case of pollen tubes in pistils of Polemoniaceae. *Taxon* 43: 63–69.

Ramesha, B. T., M. D. Yetish, G. Ravikanth, K. N. Ganeshaiah, J. Ghazoul, and R. Uma Shaanker. 2011. Stylish lengths: Mate choice in flowers. *Journal of Biological Sciences* 36: 229–234.

Ruan, C.-J., C. Shi-Chao, Q. Li, and J. A. Teixeira da Silva. 2011. Adaptive evolution of context-dependent style curvature in some species of the Malvaceae: A molecular phylogenetic approach. *Plant Systematics and Evolution* 297: 57–74.

Sarkissian, T. S., and L. D. Harder. 2001. Direct and indirect responses to selection on pollen size in *Brassica rapa* L. *Journal of Evolutionary Biology* 14: 456–468.

Smith-Huerta, N. L. 1996. Pollen germination and tube growth in selfing and outcrossing populations of *Clarkia tembloriensis* (Onagraceae). *International Journal of Plant Sciences* 157: 228–233.

Smith-Huerta, N. L. 1997. Pollen tube attrition in *Clarkia tembloriensis* (Onagraceae). *International Journal of Plant Sciences* 158: 519–524.

Stephenson, A. G., S. E. Travers, J. I. Mena-Ali, and J. A. Winsor. 2003. Pollen performance before and during the autotrophic–heterotrophic transition of pollen tube growth. *Philosophical Transactions of the Royal Society of London, B, Biological Sciences* 358: 1009–1018.

Taylor, M. L., and J. H. Williams. 2012. Pollen tube development in two species of *Trithuria* (Hydatellaceae) with contrasting breeding systems. *Sexual Plant Reproduction* 25: 83–96.

Temeles, E. J., Y. J. Rah, J. Andicoechea, K. L. Byanova, G. S. J. Giller, S. B. Stolk, and W. J. Kress. 2012. Pollinator-mediated selection in a specialized hummingbird–*Heliconia* system in the Eastern Caribbean. *Journal of Evolutionary Biology* 26: 347–356.

Thomson, J. D. 1989. Germination schedules of pollen grains: Implications for pollen selection. *Evolution* 43: 220–223.

Travers, S. E., and K. Shea. 2001. Individual variation, gametophytic competition and style length: Does size affect paternity? *Evolutionary Ecology Research* 3: 729–745.

Vasek, F. C. 1965. Outcrossing in natural populations 2. *Clarkia unguiculata*. *Evolution* 19: 152–156.

Waser, N. M., M. V. Price, A. M. Montalvo, and R. N. Gray. 1987. Female mate choice in a perennial herbaceous wildflower *Delphinium nelsonii*. *Evolutionary Trends in Plants* 1: 29–33.

Wright, M. A. R., M. D. Ianni, and M. Costea. 2012. Diversity and evolution of pollen–ovule production in *Cuscuta* (dodders, Convolvulaceae) in relation to floral morphology. *Plant Systematics and Evolution* 298: 369–389.

Zhang, C., N. Tateishi, and K. Tanabe. 2010. Pollen density on the stigma affects endogenous gibberellin metabolism, seed and fruit set, and fruit quality in *Pyrus pyrifolia*. *Journal of Experimental Botany* 61: 4291–4302.