

BRIEF COMMUNICATION

# Heteranthery in *Clarkia*: pollen performance of dimorphic anthers contradicts expectations

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**PREMISE OF THE STUDY:** Wild plant species that require the services of pollen-feeding insects for reliable pollination may evolve features that attract and reward their mutualistic partners. Heterantherous species have been proposed to exhibit a “division of labor” whereby “feeding anthers” (which produce pollen that may be consumed by an insect) are distinguished from “reproductive anthers” (which produce pollen more likely to contribute to reproduction). In some heterantherous species, including *Clarkia unguiculata* (Onagraceae), these two anther types differ with respect to stamen length, anther size, pollen production, and pollen color.

**METHODS:** The primary goal of this study was to test one component of the “division of labor” hypothesis by comparing the performance of the pollen produced by each type of anther in *C. unguiculata*. To achieve this goal, under greenhouse conditions, we hand pollinated and assessed pollen performance (using epifluorescence microscopy) within ~228 flowers.

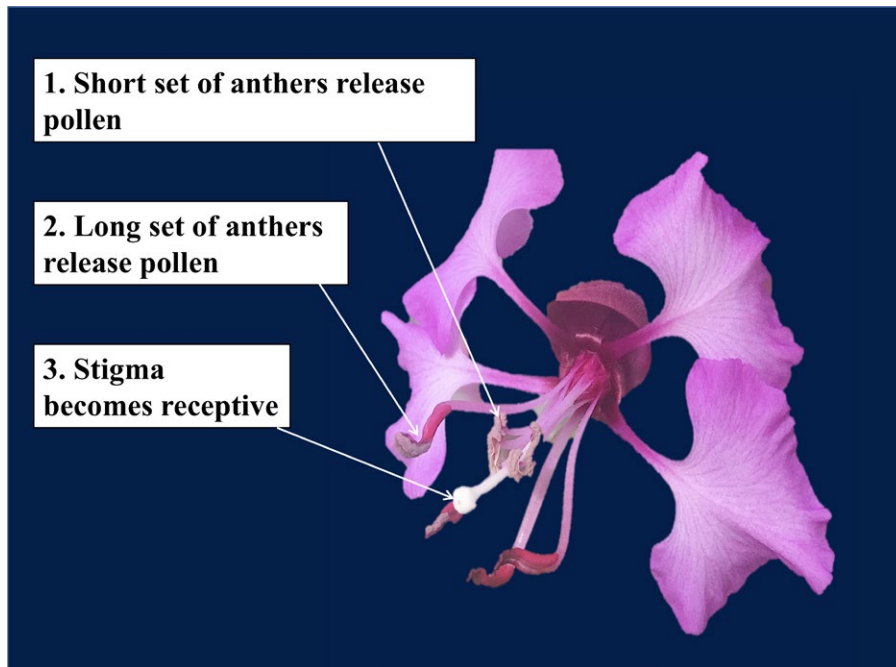
**KEY RESULTS:** The pollen produced by the two anther types differed significantly with respect to both stigma and style penetration. The inner series of anthers produce pollen with higher performance than the outer series of longer, dark red anthers.

**CONCLUSIONS:** These findings contradict previous descriptions of the genus, reporting that the inner diminutive series of anthers in *Clarkia* produce “abortive and nonfunctional” pollen. We outline the future research required to demonstrate the ecological function of heteranthery in this iconic wildflower group.

**KEY WORDS** *Clarkia unguiculata*; division of labor; floral evolution; heteranthery; Onagraceae; pollen performance; plant–pollinator interactions; trade-offs.

The transfer of fertile pollen grains between conspecifics is crucial for successful reproduction in many wildflower species. However, there are many pollinating insects that consume fertile pollen grains as food before they successfully reach a receptive stigma. The evolution of heteranthery, the presence of two dimorphic sets of anthers in the same flower, may mediate the evolutionary dilemma by which plants must both protect their gametes and provide food for pollinators. Fritz Müller and Charles Darwin both described a hypothesis characterized by a “division of labor” to explain the presence of multiple and morphologically distinct sets of anthers in many

outcrossing species (Darwin, 1862; H. Müller, 1881, 1882; F. Müller, 1883). Several studies have shown that heterantherous species that rely on pollen-feeding insects for outcrossing often have two specialized sets of anthers, which “divide the labor” between producing food as a reward for pollinators and producing pollen more likely to contribute to sexual reproduction (Luo et al., 2008; Vallejo-Marín et al., 2009; Li et al., 2015). Anthers that provide food for pollinators (“feeding anthers”) are often characterized by distinct coloration that contrasts with the petals (Jesson and Barrett, 2003). The feeding anthers are also often smaller than the anthers that produce



**FIGURE 1.** *Clarkia unguiculata* has two whorls of stamens with four stamens per whorl. (1) The anthers of the inner set of stamens mature first and are relatively short, (2) the second set of anthers mature several days later and are relatively long, and (3) the stigma becomes receptive 5–7 d after the outer set of anthers has split open, releasing its pollen.

pollen that is more likely to be transferred to conspecific stigmas (“reproductive anthers”; Jesson and Barrett, 2003; Vallejo-Marín et al., 2009). In addition, the reproductive anthers are not typically of a color that contrasts with the petal or sepal, and they produce pollen that is carried, but not consumed, by pollinators (Luo et al., 2008; Vallejo-Marín et al., 2009; Li et al., 2015). This study aims to help clarify the role of heteranthy in the annual wildflower *Clarkia unguiculata* (Onagraceae), flowers of which contain two sets of dimorphic anthers with unknown reproductive potential.

*Clarkia unguiculata* is a hermaphroditic, protandrous annual herb native to California. This species is relatively widespread in California and has been the focus of studies regarding geographic variation (Jonas and Geber, 1999), pollen performance (Németh and Smith-Huerta, 2003; Smith-Huerta et al., 2007; Mazer et al., 2018), and mating system (Ivey et al., 2016; Emms et al., 2018). This species has two sets of dimorphic stamens (Fig. 1). The first set is smaller and usually white; the second set is several millimeters longer, matures later, and bears anthers that are typically dark red to purple (Lewis and Lewis, 1955). However, there is some variation within and between populations in the color of each set of anthers. The most common combination is shown in Figure 1 (dark red/purple long anthers, white short anthers). Lewis and Lewis (1955) grew field-collected seeds of *C. unguiculata* in a greenhouse and conducted a series of crossbreeding experiments in which they examined a series of reproductive features including differences in viability between the two anther types. They described the pollen produced by the inner, shorter whorl of anthers as being completely inviable in all *Clarkia* taxa. The primary goal of the present study was to test the “division of labor” hypothesis by comparing the performance of pollen produced by each of the two anther types in *C. unguiculata*. To achieve this goal, under greenhouse conditions,

we hand pollinated and assessed pollen performance (using epifluorescence microscopy) within ~228 flowers.

## METHODS

### Study species

*Clarkia unguiculata* (Onagraceae) is a bee-pollinated, predominantly outcrossing, protandrous (anthers mature and dehisce prior to stigma receptivity), and dichogamous (having spatial separation of mature anthers and stigmas) winter annual native to California. This species occupies oak woodland, grazed or disturbed hillsides, and road cuts in the Coastal Ranges and the Sierra Nevada foothills south to the Peninsular Ranges. Outcrossing rates in field populations in the southern Sierra Nevada range from 64% to 98% (Vasek, 1965; Hove, 2012; Ivey et al., 2016). *Clarkia unguiculata* is pollinated primarily by a set of solitary bee species (the “*Clarkia*” bees) that use the genus as their exclusive pollen source, although they are also visited by generalists such as *Bombus* spp. (MacSwain et al., 1981; Moeller, 2005).

### Greenhouse study

Seeds from eight wild populations of *C. unguiculata* were collected and cultivated for this study (for location information, see Table 3). In 2015–2016, we sampled seeds from 35 maternal families per population. Seeds were placed in coin envelopes (one maternal family per envelope) that were stored in plastic zip-lock bags with silica desiccant in a dark refrigerator until use. In fall of 2016, ten seeds per maternal family (35 maternal families × 8 populations) were germinated in agar in 2-inch Petri dishes. Petri dishes holding dormant seeds were placed in a dark refrigerator for one week to promote germination. After germination, two seedlings per maternal family were each planted in individual Cone-tainers (SC10, width 1.5 inches, depth 8 inches; <http://www.stueweandsons.com>) filled with a custom soil mixture (5:1:1:1 Sunshine Grow no. 5, sand, worm castings, Island Seed and Feed fertilizer; <http://islandseed.com>). Cone-tainers were placed in racks in the greenhouse and bottom watered for the duration of the study. Plants were grown under controlled temperature (50–65°F nighttime temperature range and 55–85°F daytime temperature range), and the temperature was logged hourly during all hand pollinations so that the statistical models used here (described below) could include this variable. Trays of plants were rotated every week to disrupt any potential effects on pollen performance of location in the greenhouse.

### Experimental design and hand pollination

Each field-collected maternal family was represented by two siblings. One sibling per family was designated as a “pollen recipient” and the other as a “pollen donor.” As soon as the first flower opened on each pollen recipient, we removed both sets of anthers to prevent

self-fertilization. Pollen recipients were physically separated from pollen donor plants to prevent accidental cross-pollination. Pollen recipients and pollen donors were grown under Lumigrow Pro LED lights (<https://www.lumigrow.com>) for 10 h d<sup>-1</sup> for the duration of the study.

We harvested pollen from both anther types (Fig. 1) from each pollen donor plant. From each pollen donor flower, we harvested pollen from the short set of anthers and, on a different day, from the long set of anthers, to perform one to three hand pollinations (per set of anthers) on separate pollen recipient flowers (on separate pollen recipient plants). We conducted 137 hand pollinations using long-anther pollen and 91 pollinations using short-anther pollen; each recipient flower received pollen from only one donor. Many of the short anthers produced only enough pollen to sufficiently cover the surface of one stigma, so one set of short anthers was typically used only in one hand pollination. In all cases, the pollen recipients were from a different maternal family than the pollen donor plant. All hand pollinations were conducted using donors and recipients from the same population. We performed a total of 228 hand pollinations for which pollen performance was unambiguously scored (137 long-anther pollinations and 91 short-anther pollinations).

To perform each pollination, we removed either the long or short anthers from one pollen donor flower and placed them in a microcentrifuge tube. Pollen grains were easily dislodged from the anther surface by agitating the closed microcentrifuge tube. We used a dissecting spatula to cover the stigmatic surface of an emasculated pollen recipient flower's stigma with pollen from a single set of anthers. Four hours after each hand pollination, we collected the stigma and style by severing the style at its base with a scalpel. We placed each stigma and style in a microcentrifuge tube filled with formalin acetic acid to arrest pollen tube growth. Microcentrifuge tubes were labeled with an identification number linked to the maternity and paternity of the associated sample. We also recorded the floral sequence of each pollinated flower on the primary stem of each pollen recipient. The floral sequence refers to the number of flowers produced before the focal flower (e.g., the first flower produced by a plant has a floral sequence of 1).

### Stigma penetration and pollen tube growth

We used the methods described by Martin (1959) to soften and clear the styles and to stain them with aniline blue. This process allows the visualization and counting of pollen grains and pollen tubes using a fluorescence microscope. A negative effect of pollen load on pollen performance due to gametophyte competition for either space or maternal resources has been demonstrated in *C. unguiculata* (Mazer et al., 2016). Therefore, to control for the potential effects of variation in the intensity of early-stage pollen competition on pollen performance, we counted the number of pollen grains adhering to each stigmatic surface and included this variable in the models described below. Given that some of the pollen deposited on the stigma may have been dislodged when placed in solution in the microcentrifuge tube after they were harvested, the number of pollen grains adhering to the surface provides a measure of the number of grains that became anchored to the stigma within 4 h of pollination (likely due to features of the pollen wall or germination) and are competing for access to the stigma. We quantified pollen performance by determining the proportion of these pollen grains that penetrated the stigma surface and the proportion whose tubes successfully grew to a distance of 4.5 mm down the style (from the

base of the stigma where it meets the style) within the 4 h post-pollination period. We selected 4.5 mm because it represents ~25% of the mean style length (16.7 mm) of the styles examined in this study. We were able to determine how many pollen grains reached the base of the stigma and whose tubes reached 4.5 mm down the style by examining the aniline-blue-stained stigma and style under a fluorescence microscope (with a DAPI filter) and counting the number of callose plugs deposited in those regions (<https://www.olympus-lifescience.com>; Mazer and Hove, 2013; Mazer et al., 2016).

### Statistical analyses

**Proportion of pollen tubes penetrating the stigma: Effects of anther type, population, pollen load, and floral sequence**—We conducted a four-way fixed-effect analysis of variance (ANOVA, using Type III sum of squares) to detect the independent effects of anther type (short or long anther), population, pollen load (number of pollen grains on the stigma), floral sequence of the sampled flower (on the pollen recipient plant), and their two-way interactions on the proportion of pollen grains to penetrate the stigma. No interactions had a significant effect on pollen performance and all were excluded from the model.

**Proportion of pollen tubes to reach 4.5 mm down the style: Effects of anther type, population, pollen load, and floral sequence**—We conducted a four-way fixed-effect ANOVA (using Type III sum of squares) to detect the independent effects of anther type, population, pollen load (number of pollen grains on the stigma), floral sequence (on the pollen recipient plant), and their interactions on the proportion of pollen grains to reach 4.5 mm (from the base of the stigma). No interactions were significant and all were excluded from the model. Both analyses were conducted using the program JMP Pro version 13.0.0 (SAS Institute, Cary, North Carolina, USA).

### Results

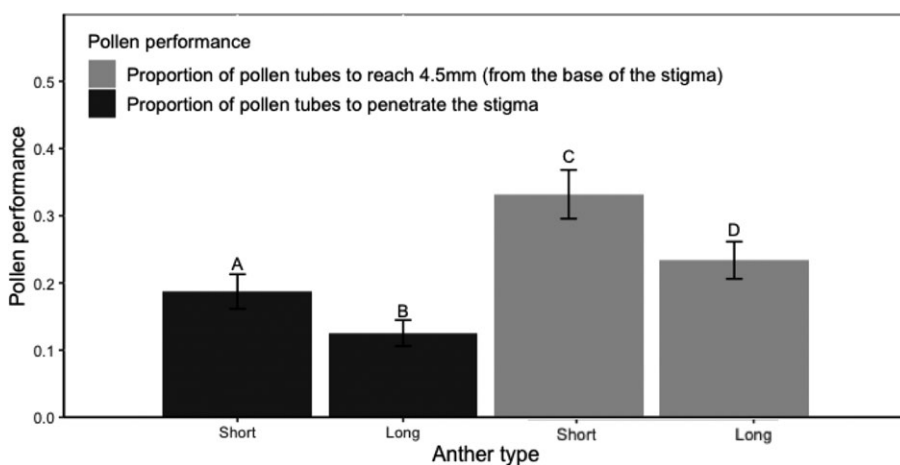
We used two metrics of pollen progression to estimate pollen performance: penetration of the stigma and entry into the style, and the growth of pollen tubes to a distance of 4.5 mm down the style. For the first trait, stigma penetrance, performance was estimated as the proportion of pollen grains on the stigmatic surface that reached the base of the stigma. For the second performance threshold, we estimated the proportion of all pollen tubes observed at the base of the stigma that successfully penetrated the style to a distance of 4.5 mm from the stigma base.

The models that included anther type, population, pollen load, and floral sequence explained a significant proportion of the variance in the proportion of pollen grains to penetrate the stigma (adj.  $R^2 = 0.21$ ,  $F = 7.13$ ,  $P < 0.0001$ ; Table 1) and the proportion of pollen tubes to reach 4.5 mm down the style from the base of the stigma (adj.  $R^2 = 0.12$ ,  $F = 3.96$ ,  $P < 0.0001$ ; Table 1).

**Performance of pollen from dimorphic sets of anthers in *C. unguiculata***—Anther type had a significant effect on pollen penetration of the stigma surface ( $P = 0.049$ ) and on the proportion of pollen grains to reach 4.5 mm down the style ( $P = 0.028$ ; Fig. 2 and Table 1), independent of the other factors in the model. Pollen harvested from the “short” anthers had a higher mean stigma penetrance rate (least square mean:  $0.18 \pm 0.03$  SE) than pollen harvested from the “long”

**TABLE 1.** Summary of multivariate models to detect the independent effects of anther type, population, pollen load, and the floral sequence of pollination on pollen performance in *Clarkia unguiculata*. Pollen color and greenhouse temperature did not significantly affect pollen performance and were excluded from the model. Significant values are in bold.

Proportion of pollen tubes to penetrate the stigma				Proportion of pollen tubes to reach 4.5 mm down the style (from the base of the stigma)			
df	Sum of squares	F ratio	Prob. > F	df	Sum of squares	F ratio	Prob. > F
1	0.171	3.897	<b>0.049</b>	1	0.426	4.850	<b>0.0287</b>
1	0.881	20.131	<b>&lt;0.0001</b>	1	0.006	0.072	0.789
7	0.415	1.356	0.226	7	2.346	3.819	<b>0.0006</b>
1	0.405	9.252	<b>0.003</b>	1	0.385	4.384	<b>0.037</b>
10	3.120	7.133	<b>&lt;0.0001</b>	10	3.477	3.962	<b>&lt;0.0001</b>
217	9.493			216	18.954		
227	12.614			226	22.431		
Adj. $R^2$ : 0.21				Adj. $R^2$ : 0.12			



**FIGURE 2.** Performance of pollen from dimorphic anthers in *Clarkia unguiculata* ( $n = 228$ ). Anther type had a significant independent effect on pollen penetration of the stigma surface and the proportion of pollen grains to reach 4.5 mm down the style (from the base of the stigma). Different letters indicate significant differences between least square means within pollen performance categories. Bars = standard error.

anthers ( $0.12 \pm 0.02$  SE). Following pollinations using short-anther pollen, 33% ( $\pm 0.03$  SE) of pollen grains that developed pollen tubes that successfully penetrated the stigma continued to grow to a distance of  $\geq 4.5$  mm down the style. By contrast, 23% ( $\pm 0.03$  SE) of the pollen grains produced by the long anthers developed pollen tubes that reached the same benchmark.

We recognized that the proportion of pollen grains whose tubes grew a distance of 4.5 mm might simply reflect their speed of germination; that is, rapidly germinating pollen grains would have an intrinsic temporal advantage that would enable them to grow longer distances during the first 4 h following pollination than slow-germinating pollen grains. If so, then our two measures of pollen performance (stigma penetration and pollen tube growth) would necessarily be correlated. One observation indicates that the effect of anther type on pollen tube growth is not due to a strong correlation between the two performance metrics in combination with the effect of anther type on stigma penetration. At the population level, a high proportion of pollen tubes penetrating the stigma (4 h after pollination) did not always result in a large proportion of pollen tubes (callose plugs) reaching a distance of 4.5 mm down the style (from the style base) (Table 2). For example, the population sampled from “Emerson Oaks” exhibited a low proportion

of stigma penetration compared to other populations (parameter estimate =  $-0.007 \pm 0.03$  SE) but a high proportion of pollen tubes that grew to a distance of 4.5 mm (parameter estimate =  $0.171 \pm 0.04$  SE). This indicates that our metrics of pollen performance are independent of each other at the population level.

#### **Independent effects of floral sequence, pollen load, and population on pollen performance**

**Floral sequence**—Flowers pollinated at relatively distal positions on the primary stem exhibited higher stigma penetration rates than those located at more basal positions. The floral sequence of the pollinated flower on the maternal plant had a significant and positive effect on the proportion of pollen grains to penetrate the stigma ( $F_1 = 20.12$ ,  $P < 0.0001$ ). However, there was no effect of floral sequence on the proportion of pollen grains to reach 4.5 mm down the style ( $F_1 = 0.07$ ,  $P = 0.789$ ).

**Population**—Populations differed significantly in mean pollen performance (Table 3). The ANOVAs detected a significant effect of population (the wild population from which the seeds were collected to grow the greenhouse plants) on the proportion of pollen grains to reach 4.5 mm down the style from the base of the stigma ( $F_7 = 3.82$ ,  $P = 0.0006$ ) but not on the proportion of pollen grains to penetrate the stigma ( $F_7 = 1.36$ ,  $P = 0.226$ ).

**Pollen load**—Our results corroborate those of previous work showing that pollen load has a significant negative effect on the proportion of pollen grains to successfully enter the style in *C. unguiculata* (Mazer et al., 2016). The number of pollen grains deposited on a stigma (the “pollen load”) negatively affects the proportion of pollen tubes to penetrate the stigma ( $P < 0.003$ ) and the proportion of pollen tubes to make it to 4.5 mm from the stigma base ( $P = 0.037$ ) (Table 1).

#### **Discussion**

Heteranthry is widespread among the angiosperms (Buchmann, 1983). The division of labor hypothesis was introduced more than a



**TABLE 2.** Parameter estimates and standard error (SE) of all fixed effects included in both multivariate models to detect the independent effects of anther type on pollen performance in *Clarkia unguiculata*.

Term	Proportion of pollen tubes to penetrate the stigma		Proportion of pollen tubes to reach 4.5 mm down the style from the base of the stigma	
	Estimate	SE	Estimate	SE
Population				
Auburn Road	0.037	0.038	0.018	0.054
Bear Creek	0.075	0.044	-0.109	0.062
Dark Canyon	-0.035	0.045	0.034	0.064
Evey Canyon	0.023	0.035	0.071	0.050
Emerson Oaks	-0.007	0.029	0.171	0.041
Garrapata Park	-0.041	0.045	-0.072	0.064
Matilija Creek	0.057	0.041	-0.114	0.059
Short anther type	0.031	0.016	0.049	0.022
Pollen load	-0.0002	8.246e-5	0.0002	0.0001
Floral sequence	0.008	0.002	-0.0006	0.002

hundred years ago (Darwin, 1862; H. Müller, 1881, 1882; F. Müller, 1883) to explain the adaptive significance of dimorphic sets of anthers in wildflowers. This hypothesis has been partially corroborated in several species (Bowers, 1975; Vallejo-Marín et al., 2009; Li et al., 2015), but thorough empirical tests remain uncommon (but see Luo et al., 2009). In one of the first experimental studies of this hypothesis, Mori et al. (1980) found that pollen grains produced by different sets of stamens of *Lecythis pisonis* (Lecythidaceae) and *Couroupita guianensis* (Lecythidaceae) differed with respect to the rate of in vitro germination, and they proposed that this differentiation had evolved in association with alternative functions of the distinct sets of stamens. Similarly, a study of *Lagerstroemia indica* (Lythraceae) found that only “reproductive” pollen produced pollen tubes capable of penetrating the stigma. “Feeding” anthers (which were shorter than “reproductive” anthers) released pollen that produced pollen tubes shorter than the diameter of the pollen grain itself (Nepi et al., 2003). Several studies comparing the viability of pollen produced by dimorphic sets of anthers have found that the longer set produced more viable pollen than the shorter set (Nepi et al., 2003; Luo et al., 2009).

Many *Clarkia* species rely on pollen-feeding insect pollinators for the majority of their reproduction and have two sets of four stamens. Lewis and Lewis (1955) describe the stamens of *Clarkia* in detail, stating that “the inner series is abortive and nonfunctional” (Fig. 1). This inner whorl of anthers is comparatively small in size, and their color contrasts with that of the petals; they conform to the physical description of “feeding anthers.” Additionally, the species meets the ecological requirement of relying on the services of pollen-feeding insects for outcrossing. The primary goal of the present study was to test the reproductive potential of the inner series of stamens in *C. unguiculata*. We found that the pollen produced by this set of “nonfunctional” anthers is viable and in many cases outperforms the pollen produced by the longer, dark red set of anthers (Fig. 2). Differences in pollen viability have been assessed differently across studies and species, which makes comparison challenging. Additionally, some studies of wild species have found no significant difference in viability between the pollen produced by morphologically distinct anther types (Bowers, 1975; Dulberger, 1981). However, to date, no study of a heterantherous taxon has found that the smaller (or relatively

**TABLE 3.** Locations of field populations of *Clarkia unguiculata*.

Population	Latitude	Longitude	Elevation (m)
Garrapata State Park	36.45499	121.91792	475
Bear Creek Road	37.16563	122.01796	651
Dark Canyon Road	39.68256	121.39094	811
Auburn Road	38.93301	121.01162	364
Evey Canyon	34.16367	117.68258	704
Emerson Oaks Reserve	33.46826	117.07271	445
Matilija Creek	34.51474	119.38087	506
Wishon Drive	36.18794	118.67141	1157

vestigial) set of stamens produces pollen that penetrates the stigma and style at a higher rate than the longer set.

While we were able to test the reproductive potential of these pollen/anther types, we did not quantify their relative contribution to reproduction in the field. Further studies are required to examine the proportion of pollen from each anther type that reaches a conspecific stigma in the field and to determine whether the realized contribution to reproduction of the two anther types differs. Future studies should also compare the nutritional content of different pollen types as well as their palatability to *Clarkia* bees. If the pollen produced by one anther type is preferentially consumed by pollinating insects, then the attributes of one set of anthers may have evolved to meet the food requirements of the species’ pollinators. Additionally, some studies have found increased granular ornamentation to be a feature of “reproductive” pollen while “nutritive” or feeding pollen is often characterized by a smoother exine (Pacini and Bellani, 1986; Luo et al., 2009). Examining the morphology, size, and ornamentation of pollen produced by each anther type may also improve our assessment of their contribution to reproduction.

## CONCLUSIONS

In conclusion, we found that anther “type” explains only a small percentage of observed variation in pollen performance in the heterantherous *C. unguiculata*. For example, population of origin significantly affects the proportion of pollen tubes to penetrate the stigma, which suggests that population or a correlated feature of geography or climate may be an additional source of variation in pollen performance. Additionally, we found that individuals exposed to elevated levels of ultraviolet light produce flowers with pollen that performs worse (according to the same performance metrics reported here) than flowers grown under LED lights that do not emit ultraviolet light (K. Peace and S. J. Mazer, unpublished data).

Our results demonstrate the need for continued empirical study of the ecological function of heteranthery in wild plant species. Continued research is required to understand the evolution of heteranthery in *Clarkia* and its implications for male fitness.

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