

RELATIVE ENERGETIC ECONOMY OF CLEISTOGAMOUS SELFING IN THREE POPULATIONS OF THE PERENNIAL *RUELLIA HUMILIS*

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Editor: Jennifer J. Weber

Premise of research. The maintenance of mixed selfing and outcrossing is an enduring mystery in evolutionary biology. Cleistogamy, in which individuals produce both potentially outcrossing chasmogamous (CH) and obligately selfing cleistogamous (CL) flowers, provides an ideal framework in which to study the evolutionary forces maintaining mixed mating. Despite their promise for providing insight into mating system evolution, few studies of cleistogamous species have comprehensively investigated the relative costs and benefits of CL selfing.

Methodology. We quantified the necessary components to calculate the relative energetic cost of reproduction of each flower type—CH and CL—in a greenhouse study using three populations of the perennial *Ruellia humilis* Nutt (Acanthaceae). These components included flower dry mass, fertility (i.e., fruit set), seed mass per fruit, and number of seeds per fruit. We also measured pollen-ovule ratios for both flower types as another measure of relative energetic investment. Last, we tracked phenology of the two flower types and used the proportion of CH flowers to estimate maximum potential outcrossing rates.

Pivotal results. We found that the energetic cost of reproduction via CL flowers was four to 10 times lower than that of reproduction via CH flowers. This energetic economy derived both from the reduced mass and from the increased fertility of CL flowers. Pollen-ovule ratios in CL flowers were one-third to one-half those of CH flowers, providing additional support for their energetic economy. Maximum potential outcrossing rates in these populations based on CH flower production were between 43% and 61%, but CH flowers can autogamously self at rates between 33% and 75%.

Conclusions. These results suggest that selection should favor only CL flowers due to their greater energetic economy. Additional work in this species demonstrates that neither inbreeding depression nor heterosis can provide an advantage strong enough to overcome the energetic benefits of CL reproduction. Further research on the developmental genetics of the two flower types and potential genetic constraints on the loss of CH flowers is needed.

Keywords: cleistogamy, flowering phenology, mating system, pollen-ovule ratio, *Ruellia*, selfing.

Online enhancement: appendix table.

Introduction

The persistence of mixed mating—that is, reproduction by a combination of selfing and outcrossing—is an old and unresolved mystery in plant evolutionary biology. The evolution of mating systems depends on the balance between the costs and benefits of self-fertilization. Inbreeding depression—that is, the reduction in fitness of inbred progeny relative to outbred progeny—plays a central role as a cost of selfing (Lande

and Schemske 1985; Charlesworth and Charlesworth 1987; Goodwillie et al. 2005; Winn et al. 2011). However, by fertilizing both its own ovules and the ovules of other individuals, a facultative-selfing plant can pass on three gamete copies for every two passed via obligate outcrossing. This 50% increase in fitness of facultative selfers is known as the automatic transmission advantage (Fisher 1941) and leads to the expectation that, if outcrossing is to be maintained, inbreeding depression must exceed the fitness benefit of the automatic transmission advantage (Lande and Schemske 1985). Self-fertilization is also beneficial because it can provide reproductive assurance in cases with limited availability of pollinators or mates (Baker 1955; Schemske 1978; Lloyd 1992; Kalisz et al. 2004). Additionally, reduced flower size in selfing species can provide a benefit to

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Manuscript received May 2023; revised manuscript received August 2023; electronically published December 14, 2023.

selfing via greater energetic economy (Randle et al. 2009; Grosenbacher and Whittall 2011).

One additional consideration in understanding the adaptive significance of mixed mating is that some self-fertilization may simply be accidental. For example, large floral display sizes may attract more pollinators but also lead to greater selfing among different flowers on the same plant (de Jong et al. 1993; Harder and Barrett 1995). An ideal system for investigating the benefits of selfing would be one with autonomous self-fertilization and for which the relative energetic economy of selfing and outcrossing could be quantified. Floral heteromorphism in cleistogamous species provides such an opportunity, but these species have nonetheless received less attention in the plant mating system literature (Goodwillie et al. 2005; Oakley et al. 2007; Winn et al. 2011).

Individuals of cleistogamous species produce both potentially outcrossing chasmogamous (CH) flowers and closed, obligately selfing cleistogamous (CL) flowers. Following historical precedent (Lord 1981), we define cleistogamy in plants or populations as the syndrome of producing both CL and CH flower types, but when speaking of flowers, cleistogamous refers only to the selfing flower type. Mixed production of CH and CL flowers occurs in over 50 families and has evolved independently multiple times from an ancestral state with only CH flowers, and there are only three documented evolutionary losses of CH flowers (Culley and Klooster 2007). Several observed facts—the repeated independent origins, broad taxonomic occurrence, and persistence of both obligately selfing and potentially outcrossing flowers on the same individual—provide strong evidence that cleistogamy is an adaptive strategy for mixed mating (Culley and Klooster 2007; Oakley et al. 2007).

The conclusion that the production of both flower types is an adaptive strategy of mixed mating, however, is at odds with the results from studies on the costs and benefits of CL selfing. Taken together, this body of work indicates that it is difficult to explain the maintenance of CH outcrossing (Oakley et al. 2007). Empirical estimates of the costs of CL selfing suggests that these costs are low. Relative components of fitness of progeny derived from CL selfing are similar on average (across about 20 studies in 14 species) to those of progeny derived from CH flowers (reviewed in Oakley et al. 2007), and estimates of inbreeding depression in cleistogamous species fall below the 50% threshold expected to prevent the evolution of complete selfing (Culley 2000; Oakley et al. 2007; Oakley and Winn 2008; Ansaldi et al. 2019). Reduced inbreeding depression with recurrent selfing is expected because strongly deleterious, partially recessive alleles may be purged by selection or drift (Lande and Schemske 1985; Glémin 2003) and mildly deleterious alleles may be fixed by drift (Whitlock et al. 2000). It is important to note that the automatic transmission advantage will also be lower in cleistogamous species because the CL flowers are unable to donate pollen to outcrossing—that is, they experience complete pollen discounting (Harder and Wilson 1998).

The factors that are likely to provide the greatest benefit to CL flowers are therefore reproductive assurance and energetic economy. Higher rates of fertility (i.e., fruit set) in CL flowers compared to CH flowers may assure reproduction in the absence of pollinators (Culley 2002; reviewed in Oakley et al. 2007; Ansaldi et al. 2019). Additionally, energetic economy may favor CL flowers over CH flowers because they are much

smaller (Schemske 1978; Waller 1979; Oakley et al. 2007) and typically lack pigmentation and nectar (Lord 1981).

To quantify the relative energetic costs of reproduction of the two flower types, estimates of flower mass, fertility, seed number per fruit, and seed weight for both CH and CL flowers are needed. Dry flower mass has been used as a proxy for resource investment to reproduction, and CL flowers can be one- to 100-fold lighter than CH flowers (Schemske 1978; Waller 1979; Schoen 1984; Sun 1999; Oakley et al. 2007; Seguí et al. 2021). Fertility of CL flowers is typically high, whereas fertility of open-pollinated CH flowers can range from 25% to 96%, depending on the species (Oakley et al. 2007; Abdala-Roberts et al. 2014; Goodwillie et al. 2018). Higher fertility of CL flowers decreases the cost of reproduction because less energy is invested into flowers that do not produce seeds. Seed mass is directly related to the energetic cost of reproduction and is important because there can be dramatic differences in the mass of seeds produced by CH and CL fruits (Sun 1999; Berg and Redbo-Torstensson 2000; Albert et al. 2011; Cheplick 2023). Finally, standardizing the cost of reproduction of each flower type by seed number per fruit allows direct comparison of the two flower types (Oakley et al. 2007), they produce different numbers of seeds (Culley 2000; Albert et al. 2011; Ansaldi et al. 2019).

Another way to quantify relative energetic investment in reproduction of the two flower types is the ratio of pollen to ovules in a flower. Pollen is lightweight, and may thus not contribute detectably to differences in flower mass, but producing male gametophytes is nonetheless expected to be costly (Schemske 1978; Schoen and Lloyd 1984). Lower pollen-ovule ratios are expected in CL flowers because of the efficiency of self-fertilization in the closed flowers, adding to their energetic benefit (Cruden 1977). This is one explanation for why cleistogamy is common in wind-pollinated grasses, which must invest heavily in male gametophytes relative to female but can limit this energetic cost by producing CL flowers (Campbell et al. 1983). The only two studies that we are aware of that have measured pollen-ovule ratios in both flower types of cleistogamous species have documented up to a fivefold greater pollen-ovule ratio in CH flowers than in CL flowers (Lord 1980; Sun 1999).

Relative phenology and degree of chasmogamy (i.e., proportion of all flowers that are CH) are two additional traits that can provide insight into the selective forces that may be involved in maintaining CH flowers. For example, one model (Schoen and Lloyd 1984) frames the floral heteromorphism as a type of adaptive plasticity, in which the plant matches the flower type to seasonal variation in order to maximize fitness. Thus, a pattern of producing different flower types in distinct seasons would be a necessary but insufficient condition for this hypothesis (Winn and Moriuchi 2009; Stojanova et al. 2016). Information on the degree of chasmogamy likewise provides an upper limit on the population-level outcrossing rate, although the realized outcrossing rate may be much lower depending on rates of self-fertilization in CH flowers (Oakley et al. 2007).

There are estimates of the individual traits discussed above in cleistogamous species (reviewed in Oakley et al. 2007). However, our current knowledge of the relative benefit of CL flowers is mostly pieced together from estimates of isolated components of these costs and benefits across many different species (but see Schemske 1978; Waller 1979; Winn and Moriuchi

2009; Seguí et al. 2021). The specific selective forces that determine the balance between the costs and benefits of CH flowers may differ among species because of differences in life history, demographic history, pollination syndrome, or other factors. Therefore, measuring a complete suite of traits related to the costs and benefits of each flower type in the same system will allow for a better estimate of how strong selection for outcrossing must be to maintain production of CH flowers. While logistically challenging, collection of these data for multiple populations of the same species may permit more general conclusions. Alternately, dramatic variation among populations in mating system–related traits (see Whitehead et al. 2018) may provide an opportunity to explore the selective forces that determine that variation.

Here we quantify the necessary components of the energetic cost of reproduction—flower dry mass, fertility, seed weight, and seed number—in a greenhouse common garden, using three natural populations of *Ruellia humilis* Nutt (Acanthaceae). In addition to these components, we measured pollen-ovule ratios of both flower types to provide another proxy for energetic investment in reproduction. Last, we tracked the phenology of CH and CL flower production and calculated the degree of chasmogamy in all three populations.

Material and Methods

Study System

Ruellia humilis is a short-lived perennial native to rich, sandy soils of prairies in the midwestern United States. Like many species in this genus (Tripp 2007), *R. humilis* is a cleistogamous species, and individuals produce both CH and CL flowers. This species is primarily pollinated by hawkmoths, but recent declines in visitation to *R. humilis* have been documented (Heywood et al. 2017, 2022). The anthers of both CH and CL flowers dehisce around dusk, and flowers of both types last for less than a day. Selfing of CH flowers has been observed; the anthers brush against the stigma when the corolla tube abscises in the afternoon (Long and Uttal 1962).

The number of native populations of *R. humilis* are limited because of widespread conversion of prairie habitat for agricultural land use. Remnant populations do occasionally persist along roadsides and cemeteries established on the sandy soils that serve as the habitat for this and other prairie species. We identified three native populations in Indiana and Illinois, from which we collected the source material for this work: two cemetery prairie remnant populations that we designate Sand Ridge (lat. 40.4085°N, long. 86.9665°W; approximately 400 individuals) and St. Mary's (lat. 40.3952°N, long. 86.9172°W; approximately 50 individuals) and a roadside population that we designate Wilmington (lat. 41.3124°N, long. 88.1321°W; approximately 250 individuals). All sites are subject to mowing, with the greatest frequency at St. Mary's. We observed intense seed predation at Sand Ridge and less seed predation at the other two sites. Seed predation is common in *Ruellia* (Munguía-Rosas et al. 2013), and disproportionately high rates of predispersal seed predation on CH fruits compared to CL fruits have been reported in *R. humilis* (Heywood and Smith 2021). Inbreeding depression is lower than 27% in both Sand Ridge and Wilmington and is negative (26% fitness advantage of selfing) in St. Mary's (Soto et al. 2023).

Germination of Seeds for Floral Trait Measurements

We collected seed from fruits of 20–23 maternal lines from these natural populations (unknown flower type). Seeds were germinated and plants were grown for a generation in the greenhouse to minimize maternal environmental effects. The next generation of seeds was collected from eight to 15 (mean = 10.33) maternal lines per population from CL fruits that developed in the greenhouse.

To grow plants for trait measurements, seeds were cold stratified at 4°C on moist paper towels in the dark for 8 wk to break dormancy. After stratification, seeds were individually sown across six 285-cell, 7-cm³ flat inserts (Greenhouse Megastore) filled with a 2:1 mixture of potting soil (BM2, Berger Horticulture Products) and sand. After sowing, flats were placed in an incubator (Percival Scientific) set at a constant temperature of 28°C with 13-h days at 65 μmol/m²/s light intensity, which is the maximum for this incubator. Once seedlings had two sets of true leaves, we transplanted them into 9-cm, 245-cm³ pots (Greenhouse Megastore) filled with the same sand and soil mixture and placed the pots in a heated greenhouse (minimum temperature: 20°C). Four weeks after the initial transplant, we transplanted the plants again into larger pots (10.79 cm × 10.79 cm × 12.38 cm; Greenhouse Megastore), filled with the same sand and soil mixture, and kept them in the greenhouse for the remainder of the experiment. Individuals began flowering 9 wk after sowing, in April 2022.

Traits for Calculating the Energetic Costs of Reproduction

For dry flower mass, we sampled one to three flowers of each type (CH and CL) on replicate plants from eight to 11 maternal lines. Entire flowers (minus two anthers used to produce pollen counts for pollen-ovule ratios; see “Pollen-Ovule Ratio”) were harvested, placed in a coin envelope, and set in a drying oven at 60°C for 10 d. The flowers were then weighed to the nearest 0.01 mg using a microbalance (ML104, Mettler Toledo). We estimated mean fertility of CL and CH flowers as autogamous fruit set by marking buds with uniquely colored thread. Autogamous fruit set in a pollinator-free environment is an estimate of minimum fertility because outcrossing in the field may increase CH fertility. After 2 wk, fruit set was visually scored as 1, for flowers that produced fruit, or 0, for those that did not. In autumn 2022, once they had turned brown, these fruits were collected and placed in coin envelopes, where they were allowed to dehisce prior to weighing and counting seed number per fruit. Estimates of mean seed weight per fruit were then obtained by dividing total seed mass per fruit by the number of seeds per fruit.

To calculate the relative cost of reproduction by CL flowers compared to CH flowers (Oakley et al. 2007), we first produced average values over replicate flowers on an individual plant separately by flower type for each of four measurements: dry mass, fertility, seed number per fruit, and seed weight. We then averaged over all replicate individuals for each trait and flower type to obtain population-level means (table S1 [available online]). Using these population averages, we calculated a population-level cost of reproduction of each flower type using the following equation:

$$\frac{\left(\frac{\text{dry flower mass}}{\text{fertility}} \right) + \text{total seed mass per fruit}}{\text{seed number per fruit}}$$

Fertility in the denominator of the first term accounts for the investment in flowers that do not yield seeds, and seed number per fruit standardizes the average cost of reproduction per fruit (above the bar) if CH and CL fruits produce different numbers of seeds. To get a relative cost of reproduction of the two flower types (CL/CH), we divided the cost of reproduction we calculated for CL flowers by that calculated for CH flowers.

Pollen-Ovule Ratio

While the production of pollen and ovules does not contribute greatly to total flower mass, the production of these structures is nonetheless expected to be costly. We therefore also quantified the pollen-ovule ratio as an alternative approach to quantifying relative costs of reproduction. For each of the same flowers harvested for dry mass (see “Traits for Calculating the Energetic Costs of Reproduction”), we counted the number of ovules by dissection under a stereomicroscope. The total number of pollen grains was then divided by the number of ovules to get pollen-ovule ratio per flower.

For pollen counts, we collected two of the four anthers from a given flower bud approximately 2 h before anther dehiscence. The two anthers were placed in a microcentrifuge tube at room temperature for 12 h. Once the anthers had dehisced, we added to each centrifuge tube 500 μL of a mixture consisting of 450 μL of a glycerol-ethanol-water solution and 50 μL of 1% aniline blue, to aid in visualizing pollen grains. We vortexed the tubes for 1 min to release pollen grains from the anther sacs. After this initial vortex, five replicate 20- μL aliquots of the suspended solution were pipetted onto five different ruled microscope slides with an etched grid of 64 2-mm squares (Flinn Scientific), vortexing the microcentrifuge tube for an additional 30 s between pipettings. We counted pollen grains on the entire grid of each slide at $\times 10$ magnification under a light microscope and averaged the five separate counts for each flower. This metric represents the average number of pollen grains in a 20- μL sample from two of the four anthers from each flower, suspended in a total of 500 μL . Total pollen grains per flower were then estimated by multiplying this average by 25 (20 $\mu\text{L} \times 25 \mu\text{L} = 500 \mu\text{L}$) to obtain the total number of pollen grains in the 500- μL solution, which was then multiplied by 2 to yield an estimate of the total pollen production from all four anthers on a flower.

Flowering Phenology and Degree of Chasmogamy

We quantified the timing of production of each flower type in *R. humilis* by counting the number of each of the CH and CL flowers on all flowering individuals every day for 22 wk starting in April 2022. Because daily flower production was sparse, we summed weekly CH and CL production for each population over all replicate plants. Average number of CH and CL flowers produced per individual per population per week was then calculated for each population by dividing weekly totals by the number of flowering individuals. We also quantified the proportion of total flowers produced that were CH per individual by summing the total number of CH and CL flowers produced over the 22-wk period. The resulting ratio, $\text{CH}/(\text{CH} + \text{CL})$, gives the maximum potential outcrossing rate per population, although realized outcrossing rates may be lower due to CH selfing.

Statistical Analysis

For each trait, we tested for the effects of maternal population, flower type, and their interaction using ANOVA. All terms were treated as fixed effects because of the small number of maternal populations. Individual plant means by flower type were used for all traits, including flower dry mass, fertility, seed number per fruit, average seed weight per fruit, and pollen-ovule ratio. Although the residuals were not perfectly normally distributed with equal variance for all traits, we used normal error distributions for all traits because qualitatively similar results were obtained for both models with alternative error distributions and nonparametric models, indicating that our results are robust to moderate violations of the assumptions of ANOVA.

In an ANOVA for any given trait, a significant effect of flower type indicates a difference between CH and CL flowers averaged over the effect of population. A significant interaction between these two effects indicates that differences between flower type depend on the maternal population. A significant effect of maternal population would indicate genetic differences among populations averaged over the effect of flower type, although this is not of central interest to our objectives. For CH/total we used a single-factor ANOVA to test for an effect of maternal population, because this trait is a ratio of both flower types. All analyses were performed in JMP version 16.1.

Results

Traits Measured on Individual Flower Types

We found highly significant effects of both maternal population and flower type for flower dry mass, fertility, and pollen-ovule ratios and a significant interaction between maternal population and flower type for flower dry mass (table 1). Dry mass of CL flowers was consistent across the three populations (fig. 1A; table S1), so the significant interaction effect for this trait was driven by modest variation among populations in CH flower dry mass. Flower mass of CL was only 27% that of CH for the Sand Ridge population, and this proportional decrease was more pronounced for the St. Mary’s (22%) and Wilmington (20%) populations (fig. 1A; table S1). Over both flower types, St. Mary’s had significantly greater flower mass than the other two populations. Fertility of CL flowers was approximately 50% greater than CH flowers overall, and although the interaction was not significant, this proportional difference was smaller in Sand Ridge than in the other two populations at 19%, compared to 94% for St. Mary’s and 83% for Wilmington (fig. 1B; table S1). Sand Ridge had greater fertility than the other two populations when considering both flower types combined. The pollen-ovule ratio of CL flowers was 42% that of CH flowers overall (fig. 2; table S1). Although there was no significant population \times type interaction for pollen-ovule ratio, there was some variation among populations (range: 33%–50%) in the pollen-ovule ratio of CL relative to CH (fig. 2; table S1). Over both flower types, Sand Ridge had significantly greater pollen-ovule ratio than the other two populations.

If there are large differences in seed size and/or weight between CH and CL fruits, seed traits can be important components of the relative cost of reproduction in the two flower types. However, results for average seed number per fruit and average seed weight per fruit confirm initial observations

Table 1
ANOVA Results for the Effects of Maternal Population, Flower Type, and Population \times Type Interactions on Components of Relative Energetic Cost of Reproduction and the Degree of Chasmogamy

Component	Population		Flower type (chasmogamous vs. cleistogamous)		Population \times type		Error	
	Mean square	<i>F</i> ratio	Mean square	<i>F</i> ratio	Mean square	<i>F</i> ratio	Mean square	Denominator df
	Flower dry mass (mg)	73.99	8.00***	6112.64	661.08***	74.03	8.01***	9.25
Fertility	1.22	9.94***	1.92	15.64***	.08	.68	.12	121
Mean seed number per fruit	.51	.44	.00	.00	1.67	1.44	1.16	127
Mean seed weight per fruit (μg)	34.47	1.61	12.66	.59	16.69	.78	21.44	127
Pollen-ovule ratio ^a	13.48	9.51***	242.68	171.13***	1.23	.87	1.42	111
Chasmogamous flowers-to-total flowers ratio	.30	8.11***	na	na	na	na	.03	119

^a Pollen-ovule ratios are divided by 100 to keep to keep mean square values in scale with other traits.

*** $P < 0.001$. *F* ratios not marked with asterisks had $P > 0.05$.

that there were no differences with respect to flower type. In fact, there were no significant differences for these traits for any of the model terms (fig. 1C, 1D; table 1). Average seed number per fruit ranged between 2.8 and 3.4 across popula-

tions and flower types (fig. 1C; table S1). Average seed weight per fruit was 9.2–9.9 μg across populations and flower types, with the exception of CL seeds in Sand Ridge, which were somewhat larger at 12.1 μg .

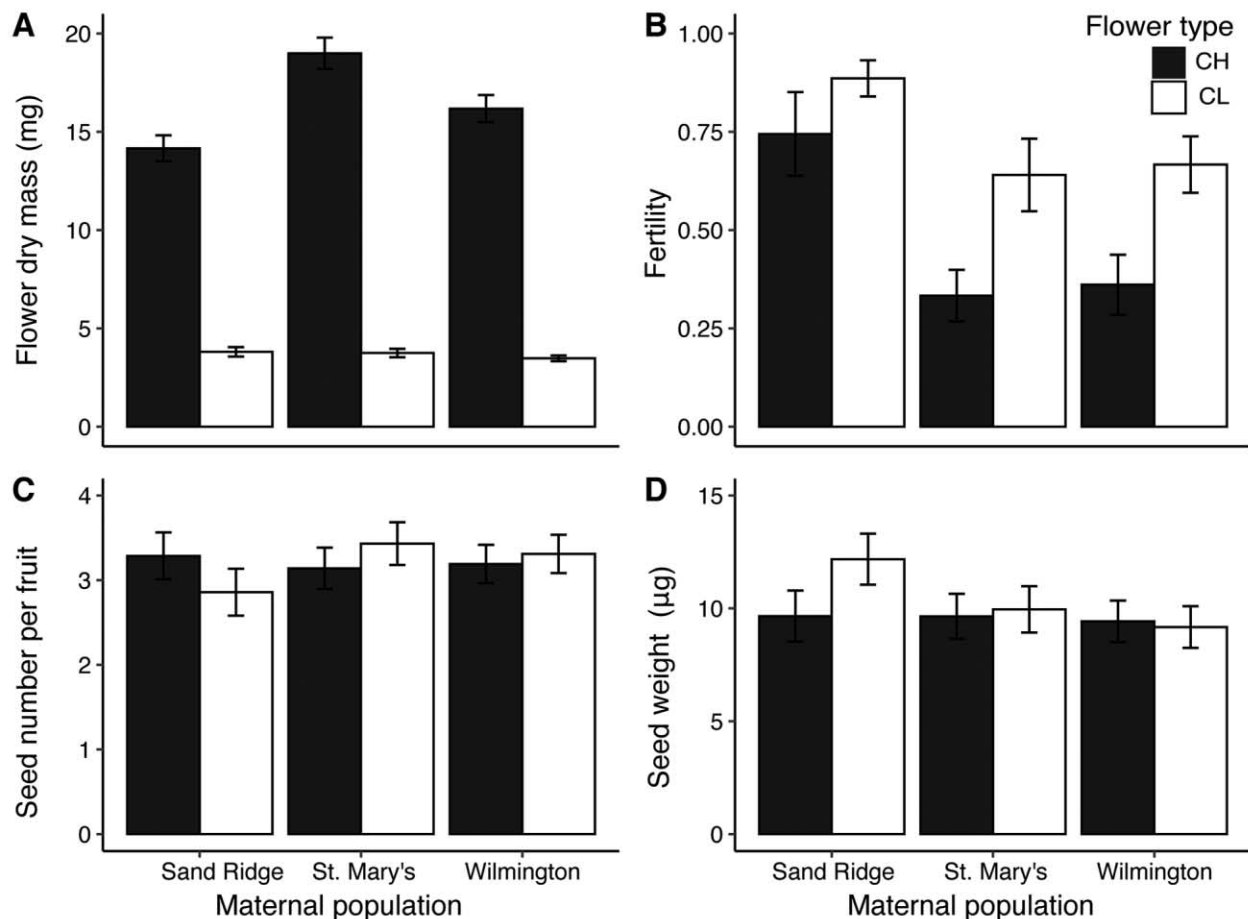


Fig. 1 Mean (\pm SE) trait values of chasmogamous (CH; filled bars) and cleistogamous (CL; open bars) flowers by maternal population. A, Flower dry mass (mg). B, Fertility (probability of fruit set). C, Seed number per fruit. D, Seed weight (μg).

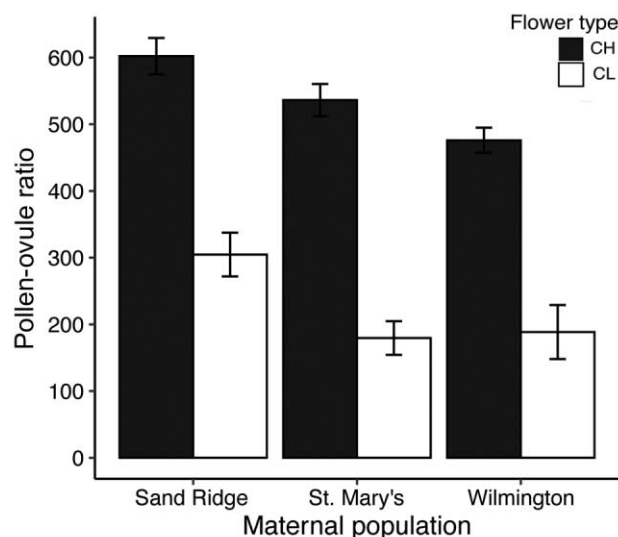


Fig. 2 Mean (\pm SE) pollen-ovule ratio of chasmogamous (CH; filled bars) and cleistogamous (CL; open bars) flowers by maternal population.

Energetic Costs of Reproduction

Means for each flower type for dry mass, fertility, seed number per fruit, and the mass of those seeds are all components of the energetic costs of reproduction. Costs of reproduction via CL flowers were consistent across populations and ranged from 1.5 to 1.7 mg/seed (table S1). Cost of reproduction via CH flowers was more variable among populations: 5.8 mg/seed in Sand Ridge, 14.1 mg/seed in Wilmington, and 18.2 mg/seed in St. Mary's (table S1). Reduced overall cost of CH reproduction in Sand Ridge appears to be driven both by greater relative fertility (i.e., greater autogamous selfing) of CH flowers (fig. 1B) and by reduced relative mass of CH flowers (fig. 1A). The relative (CL/CH) cost of reproduction in Sand Ridge (0.26) was more than two times greater than that of St. Mary's (0.09) or Wilmington (0.11). Because these derived variables are calculated at the population level, we were not able to perform a formal statistical analysis. However, results for individual components of the cost of reproduction (fig. 1) suggest that the difference between Sand Ridge and the other two populations is meaningful.

Flowering Phenology and Degree of Chasmogamy

Patterns of relative phenology of CH and CL flowers were qualitatively different among populations. In Sand Ridge, CH flower production started early and peaked by the third week of flowering, with CH flower production about nine times greater than CL flower production during the third week. This was followed by mixed production of both CH and CL flowers at a rate of about two flowers per individual per week per flower type during weeks 4–14 (fig. 3A). After week 14, CH flower production declined while CL production remained constant or increased slightly. In St. Mary's, individuals started flowering 2 wk later than the other populations (fig. 3). In this population, CH flowers were almost exclusively produced for the first 6 wk (fig. 3B), peaking around week 7, at which point CH flower pro-

duction was about eight times greater than CL flower production. Production of CL flowers in this population began increasing approximately 8 wk after first flowering, with mixed production of both flower types until week 16 followed by a shift toward almost exclusive CL production afterward. In Wilmington, both CH and CL flower production began in the first week, and overall flower production was consistently lower across the 22 wk compared to the other populations. Peak CH flower production occurred in week 1, and maximal CH production was about one-half that of the other two populations (fig. 3). Production of both CH and CL flowers in a ratio of about 3:1 continued until week 17, when CH production decreased and CL production increased slightly. For the average ratio of CH flower number to total flower number over the entire flowering period, which represents the maximum potential outcrossing rate for each population, we found values of 0.43 for Sand Ridge (SE = 0.03), 0.61 for

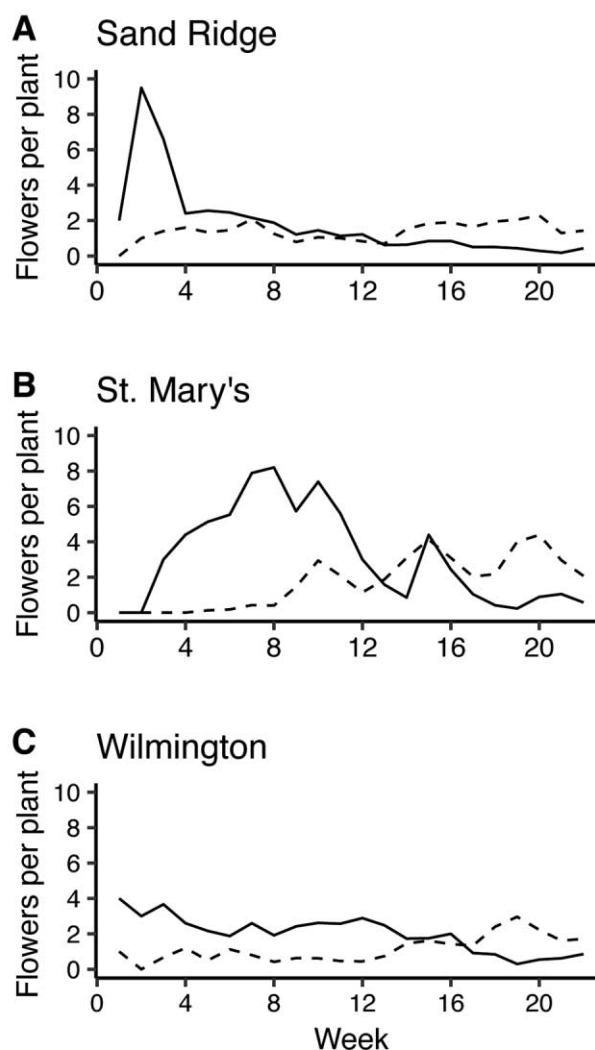


Fig. 3 Average number of chasmogamous (solid line) and cleistogamous (dashed line) flowers produced per individual per week over a 22-wk period for the three maternal populations, Sand Ridge (A), St. Mary's (B), and Wilmington (C).

St. Mary's (SE = 0.03), and 0.52 for Wilmington (SE = 0.03). The lesser value for Sand Ridge is responsible for the significant effect of population on the degree of chasmogamy (table 1).

Discussion

The continued maintenance of CH flowers in cleistogamous species is a long-standing evolutionary puzzle. However, only a few studies have collected all the information needed to understand the magnitude of selection required to maintain CH flowers. Overall, we found that the energetic cost of reproduction via CL flowers was between four and 10 times lower than the cost via CH reproduction and that this energetic economy stems from the lower flower mass and higher fertility of CL flowers. Pollen-ovule ratios in CL flowers were one-third to one-half those of CH flowers, providing additional support for the energetic economy of CL flowers. Finally, we found that about one-half of the total flowers produced by these populations are CH, and these flowers are capable of autogamously selfing at rates of 33%–75%.

Traits for Calculating the Energetic Costs of Reproduction

We found that flower dry mass was lower for CL than for CH flowers. The dry mass of CL flowers relative to CH flowers observed here is somewhat lower than the average but within the range previously reported for cleistogamous species (Oakley et al. 2007; Seguí et al. 2021). This suggests that there is a greater relative energetic economy to producing CL flowers in *Ruellia humilis* when compared to other species, with exceptions in *Impatiens capensis*, *Impatiens pallida* (Schemske 1978; Waller 1979), and *Scutellaria indica* (Sun 1999). We observed significant variation among populations of *R. humilis* for relative dry mass of the two flower types (i.e., a population \times type interaction) that appears to be entirely due to variation in CH flower size. It is possible that larger CH flowers are necessary in St. Mary's and, to a lesser extent, Wilmington to attract pollinators because these are relatively smaller populations.

For fertility, we found that CL flowers had a greater proportional fruit set than CH flowers, although this difference was less pronounced in Sand Ridge. The values of relative fertility of CL flowers we observed here are close to the mean of 1.8 reported for 13 species (Oakley et al. 2007), after excluding the extreme value of 19.2 for *S. indica* (Sun 1999). A recent estimate of 14.3 for *Viola jaubertiana* is likewise extreme (Seguí et al. 2021) but suggests that considerable variation in the relative fertility of CL flowers among species is possible. In *R. humilis*, moderate values of relative fertility appear to be due to both CL flowers' overall fertility of only 72% and the greater fertility of CH flowers derived from their ability for delayed selfing (Long and Uttal 1962; Heywood et al. 2017). While we did not observe a significant interaction between population and flower type for fertility, the high fertility of both flower types for Sand Ridge is noteworthy. In particular, the high absolute fertility of CH flowers in this population indicates a greater potential for autogamous selfing of CH flowers. While our estimates of CH fertility are conservative because outcrossing is not possible in the greenhouse, they are similar to field estimates of fertility of CH flowers from other species (25%–95%; Oakley et al. 2007; Abdala-Roberts et al. 2014; Goodwillie et al. 2018).

Relative investment in seeds of the two flower types can be an important component of overall relative energetic costs of re-

production. In some species, such as *Amphicarpaea bracteata* (Schnee and Waller 1986) and several grasses (Cheplick and Clay 1989; Cheplick 2023; Jones et al. 2023), there is a seed size heteromorphism, with at least some of the CL-derived seeds being much larger than CH-derived seeds. Additionally, there can be variation in seed number between CH and CL fruits even with similar seed mass (Seguí et al. 2021). However, in *R. humilis*, we found that neither seed number per fruit nor average seed weight per fruit differed significantly between CH and CL. Additionally, we have not observed any difference in germination rates between CH and CL seeds (T. Soto, unpublished data). We can thus rule out greater seed set and larger seeds as potential selective forces favoring CH flowers. This lack of flower type effect on seed weight has been documented in other cleistogamous species such as *Lamium amplexicaule* and *Viola pubescens* (Culley 2002; Stojanova et al. 2016). Similar seed numbers per fruit between flower types is also not uncommon in other cleistogamous species (Culley 2002; Albert et al. 2011).

Total Energetic Costs of Reproduction via the Two Flower Types

The energetic cost of reproduction via CL flowers relative to CH reproduction (CL/CH) is of central interest in understanding the magnitude of advantage needed to maintain continued production of CH flowers. We found that this relative cost of CL reproduction varied considerably by population and was between 10% and 25% of that of reproduction via CH flowers. Only four prior studies have collected all the necessary data in the same system to calculate the relative cost of reproduction of the two flower types (Schemske 1978; Waller 1979; Winn and Moriuchi 2009; Seguí et al. 2021), with values ranging from 0.06 to 0.67 (mean = 0.42). The values we estimated for Sand Ridge are comparable to those reported for *I. pallida* (Schemske 1978). Similar values between *Ruellia* and *Impatiens* make for an interesting comparison, because CH dry mass poses a much greater relative cost in *Impatiens* than it does in *Ruellia*, but this is counterbalanced by reduced seed number in CL fruits compared to CH fruits in *Impatiens*. The values for Wilmington and St. Mary's are nearer to the value of 0.06 that we calculated using data from *V. jaubertiana* (Seguí et al. 2021). These two populations of *R. humilis* and three populations of *V. jaubertiana* are the clearest examples of the energetic economy of CL flowers to date. However, other studies with large values of relative fertility of CL flowers may yield even lower values of relative cost per seed, but we do not have all of the requisite data to make these calculations. Our novel finding of among-population variation in the relative cost per seed suggests that, while technically challenging, estimates of these parameters for multiple populations per species are necessary for more general conclusions about a species' mating system. With the extent of intrapopulation variation observed here, even more populations may be needed to tease apart any confounding factors that may underlie such variation.

Pollen-Ovule Ratio

We measured pollen-ovule ratios to provide another estimate of the energetic costs of the two flower types. Although

gametophytes do not contribute much to overall flower mass, there is reason to expect considerable energetic investment in producing pollen and ovules (Schemske 1978; Schoen and Lloyd 1984). We found that pollen-ovule ratios of CL flowers in *R. humilis* were about twofold lower than those of CH flowers overall. Lord (1980) found a similar proportional decrease in pollen-ovule ratio of CL flowers of *L. amplexicaule*. These findings are consistent with the expectation that CL flowers reduce investment in producing pollen because selfing mechanisms are effective (Cruden 1977; Queller 1984). Although there was no significant population \times cross-type interaction for this trait, Sand Ridge had greater pollen-ovule ratios for both CH and CL flowers. The combination of CH flowers in Sand Ridge that were smaller and more autogamously selfing and that had greater pollen-ovule ratios could indicate greater selection for CH selfing in this population and an increased investment in pollen to overcome an inefficient delayed-selfing mechanism. The greater pollen-ovule ratio in CL flowers in Sand Ridge is puzzling, as CL flowers should be very efficient at self-fertilization, but we note that CL fertility in Sand Ridge is greater than that of the other two populations.

Patterns of Relative Phenology and Degree of Chasmogamy

We observed considerable variation among populations in the relative phenology of the two flower types. Wilmington had a pattern of simultaneous low-level production, whereas Sand Ridge and St. Mary's had partially overlapping distributions (CH before CL) of the two flower types, with an earlier and narrower peak of CH flower production in Sand Ridge. It is unclear what might be driving these genetic differences in relative phenology among populations. It is possible that an early and compressed peak of CH flower production in Sand Ridge could be a strategy to increase the chances that some seeds escape predation (Honek and Martinkova 2005), although seed predation may be constant across the CH flowering period in this species (Heywood and Smith 2021). Different patterns of relative phenology in St. Mary's and Wilmington are more challenging to explain but likely involve forces such as pollinator attraction (particularly in the very small St. Mary's population), seed predation avoidance, and possibly even the frequency of mowing. An in-depth field study in these populations over the course of the season might reveal greater insight about the forces driving these patterns, especially in St. Mary's and Wilmington, but such a study is difficult or even impossible to undertake at sites that are frequently mowed.

One thing in common among all three populations is that they do not produce different flower types in distinct seasons, as is common in perennial cleistogamous species (Oakley et al. 2007; Winn and Moriuchi 2009; but see Seguí et al. 2021; Austin et al. 2022). The lack of distinct seasons for the different flower types suggests that the maintenance of CH flowers is not an adaptive plastic response to producing CH flowers in the season in which they are more favorable (Schoen and Lloyd 1984; Winn and Moriuchi 2009). Additionally, in all three populations, CH flowers were produced either before or simultaneously with CL flowers, which appears to be un-

common in species with overlapping distributions of the two flower types (Oakley et al. 2007). These populations therefore do not exhibit a “pessimistic strategy” (Cheplick and Quinn 1982) in investing in CL flower production first but instead begin investing in the riskier CH flowers immediately. It has also been suggested that CH production is a bet-hedging strategy (Waller 1980), although this hypothesis is difficult to test. The proportion of total flowers that were CH (i.e., the degree of chasmogamy) suggests that these populations are about 50% outcrossing at most, but autofertility of CH flowers, particularly in Sand Ridge, means that realized outcrossing rates may be much lower. An important caveat is that all estimated trait values from our greenhouse common-garden experiment may vary if measured under field conditions.

Conclusions

Our results provide a clearer picture of the mating system and the selective advantage required to explain the continued maintenance of CH flower production in this species. We estimate that the maximum possible population-level outcrossing rates are between 43% for Sand Ridge and 61% for St. Mary's; considering estimated rates of autogamous selfing in CH flowers reduces these maxima to between 11% and 33%. Our biomass-based measures of the energetic economy of CL flowers suggest that progeny from CH flowers would have to be four to 10 times fitter to explain their evolutionary maintenance in the face of short-term selection. Reduced relative pollen-ovule ratios in CL compared to CH flowers suggests that the selective advantage of CH flowers would need to be even greater. Recent work in this system (Soto et al. 2023) has further shown that neither inbreeding depression within populations nor heterosis in crosses between populations can provide an advantage to CH flowers sufficient to explain their maintenance in any of these three populations. Thus, we have exhausted the potential short-term explanations for the maintenance of CH outcrossing. To our knowledge, this is the most comprehensive collection of data on the costs and benefits of CH/CL in multiple populations, but our results are in agreement with a body of literature on the overwhelming benefits of CL flowers. We suggest that new lines of investigation are needed to understand the persistence of CH flowers, which may include genetic constraints on the loss of CH flowers. We additionally suggest more research to understand the genetic mechanisms underlying the developmental switch that occurs to determine whether a CH or CL flower is produced at a given floral meristem.

Acknowledgments

We thank Bob Easter from NICHES Land Trust for allowing us to collect seed from Sand Ridge and David Rebmann at St. Mary's for pausing mowing to facilitate seed collection. We also would like to thank Gordon McNickle for helpful comments on an earlier version of the manuscript and Juan Diego Rojas-Gutierrez for assistance with the greenhouse experiment. This work was funded in part by USDA Hatch grant 7000281 to C. G. Oakley via Purdue College of Agriculture and a Purdue ARGE assistantship to T. Y. Soto.

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