WHAT FACTORS MAINTAIN THE MIXED MATING SYSTEM OF A LOCAL ANNUAL
PLANT, *TRIODANIS PERFOLIATA*?

by

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Abstract

A plant capable of reproducing by cross- and self-fertilization is known to have a mixed mating system. This system is an evolutionary puzzle to many researchers because the factors maintaining both outcrossing and selfing are not fully understood. Cleistogamy, a form of mixed mating, describes the production of both open (chasmogamous, CH) flowers that can be cross-pollinated and closed (cleistogamous, CL) flowers that are obligately self-fertilizing. Studies show that inbreeding depression, a negative consequence of selfing, is present in low quantities in CL offspring resulting in slightly reduced fitness relative to CH offspring. The minor fitness benefit of CH offspring cannot negate the advantage of producing low cost CL flowers that have guaranteed seed production. This prompts the question: why do plants continue to make costly, larger CH flowers? Heterosis, the increased fitness of offspring from crosses between different populations or species, may be a key factor in maintaining CH flowers. Heterosis can occur when populations are fixed for different mildly deleterious recessive alleles. The offspring of crosses between them will be heterozygous and show higher fitness. Since heterosis occurs through outcrossing, only CH flowers can benefit from this effect. We test the hypothesis that heterosis provides an advantage to CH flowers in *Triodanis perfoliata,* a weedy cleistogamous annual plant. In a growth room experiment, we hand-pollinated plants of three populations of *T. perfoliata* to generate offspring from self-fertilized CH flowers, within-population crosses, and between population-crosses with two populations. We also collected seeds from CL flowers to compare CL and selfed CH offspring to determine if the allocation of resources by the maternal plant was different for each flower type. Lifetime fitness was quantified by measuring seed germination, seedling survivorship, and plant biomass. On average across the three populations the CL offspring had 21.9% lower cumulative fitness than selfed CH offspring indicating a slight

flower effect. Cumulative fitness of selfed CH offspring were 23.7% lower than outcrossed CH

offspring due to inbreeding depression. Finally, no evidence of heterosis was found as the

between-population crossed offspring were 10% lower in fitness than the within-population

crossed offspring. These results indicate that heterosis cannot explain the maintenance of CH

flower in *Triodanis perfoliata*.

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DISCUSSION

Introduction

   Many hermaphroditic plants are capable of self-fertilization because they contain both the

male (stamen) and female (pistil) reproductive parts of a flower. One unusual form of this is

cleistogamy in which self-fertilization occurs within a closed flower (Culley & Klooster, 2007).

But most cleistogamous species also produce typical open flowers that can cross-fertilize.

Dimorphic cleistogamy refers to a plant that produces both closed cleistogamous (CL) flowers

and open chasmogamous (CH) flowers (Culley & Klooster,2007). Throughout the literature,

cleistogamy and dimorphic cleistogamy are considered interchangeable. Cleistogamy has been

reported in 693 species (50 families) with 536 of these producing both flower types (Culley &

Klooster, 2007). In general, the mixed mating system of hermaphroditic plants that are capable

of self- and cross-fertilization has been an evolutionary enigma for scientists. Theoretical studies

have shown that either self-fertilization or outcrossing should be stable, and mixed mating may

just be transitionary or unstable (Lande & Schemske, 1985; Schemske & Lande, 1985).

However, empirical studies have found mixed maing systems to be common (Goodwillie, Kalisz,

& Eckert, 2005).

   The transition from cross-fertilziation to self-fertilization has been recorded across numerous

flowering species (Stebbins, 1957). Self-fertilization provides multiple advantages for the plant.

First, plants capable of self- and cross-fertilization have a 3:2 (50%) benefit over those that only

outcross due to the transmission advantage (Fisher, 1941). These plants can self-fertilize their

own ovules and donate their pollen to pollinate flowers on other plants. Second, self-fertilization

occurs without the aid of a pollinator resulting in reproductive assurance (Darwin, 1876; Baker,

1955). When pollinators are rare or absent an autonomously selfing flower will fertilize its own

ovules and reproduce. Third, self-fertilization can occur in smaller flowers which are less expensive and require fewer resources allocated to them compared to their larger outcrossing counterparts (Oakley, Moriuchi, & Winn, 2007). One of the major forces opposing self-fertilization is inbreeding depression, which is the reduction in fitness of selfed offspring compared to outcrossed offspring (Charlesworth & Charlesworth, 1979; Lande & Schemske, 1985). Inbreeding depression occurs when deleterious recessive alleles are expressed in homozygotes thus lowering the fitness of offspring produced by self-fertilization (Charlesworth & Charlesworth, 1987). However, these deleterious recessive alleles are exposed to selection and can be removed from the population so inbreedin depression is reduced over time in historically self-fertilizing species (Lande & Schemske, 1985).

As suggested by the literature, selection should favor only one of the flower types not both in cleistogamous species. It seems that a shift towards CL flowers would be evolutionarily preferred. Although the transmission advantage is linked to flowers that can self- and cross-fertilize, CL flowers do not benefit from this as they are unable to pollinate other flowers. They are only capable of self-fertilizing within the flower itself. Even without this added benefit, CL flowers may be selected for as they are energetically less costly and inbreeding depression appears to often be purged leaving little disadvantage to selfing. Studies have shown that inbreeding depression is present in low quantities in CL offspring but may be balanced out by low seed and flower costs (Berg & Redbo-Torstensson, 1999). Data on the relative fertility, flower cost, and cost per seed across 15 species suggest CL flowers are cheaper to produce and have lower cost per seed than CH flowers (Oakley, Moriuchi, & Winn, 2007). So we are left with the question as to why are CH flowers are maintained in cleistogamous species. To

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counteract the high cost associated with CH flowers, there must be a big advantage to producing them.

One proposed hypothesis for the maintenance of CH flowers is heterosis, an increase in fitness of offspring from a cross between two populations (Lu, 2002; Oakley, Moriuchi, & Winn, 2007). Genetic drift can fix a population for mildly deleterious alleles by random chance and vary among different local populations (Kimura, Maruyama, & Crow, 1963; Whitelock, Ingvarsson, & Hatfield, 2000). When two populations are fixed for various mildly deleterious recessive alleles at different genes, the resulting crossed offspring show heterosis (Lu, 2002). The offspring will have an increase in heterozygosity thereby masking the deleterious alleles expressed in the parental populations resulting in an increase in fitness vigor (Crow, 1948; Whitelock, Ingvarsson, & Hatfield, 2000). Since heterosis occurs by outrcrossing, only CH flowers can benefit from this effect hence selecting for their maintenance.

In this study our goal was to quantify the cost and benefits of CL and CH flowers in *Triodanis perfoliata,* a weedy annual cleistogamous species. We measured the cost in terms of flower biomass and the benefits in terms of seed number produced and cumulative fitness of offspring. In measuring the relative benefits of each flower type, we quantified inbreeding depression, flower effect, and heterosis. The flower effect compares CL and selfed CH offspring biomass to determine whether the maternal plant allocates its resources differently to each flower type thereby affecting the offsprings ability to germinate and/or grow. While a few studies have been done on cleistogamous species not many have quantified heterosis or inbreeding depression in this context. For example, study on *Triodanis perfoliata* compared a few fitness parameters of CH and CL seeds; however, by doing so the genetic effect (inbreeding depression) and the

flower effect were not separated (Gara & Muenchow, 1990). By doing rigorous crossing study, I was able to separate these two by comparing selfed CH vs. outcrossed CH offspring in addition to CL and selfed CH offspring. I was also able to get measurements of heterosis to ultimatetely estimate the cost and benefits of each flower type in *Triodanis perfoliatia*.

Materials and Methods

Study Species and Study Site

Triodanis perfoliata is an annual herb/forb native to North America. It is commonly found in roadside ditches, woodlands, and disturbed habitats. *T. perfoliata* produces two types of flowers: chasmogamous (CH) and cleistogamous (CL). CH flowers have five blue/violet petals and range in diameter of 1 to 1.5 cm. They are frequently visited by small insects such as beetles, wasps, and bees (Gara & Muenchow, 1990). These flowers exhibit protandry, the appearance and maturation of male anthers before the female stigma becomes receptive. Primary CL flowers are petal-less and are typically located closer to the base of the plant. Smaller secondary Cl flowers generally develop after the formation of the first CH or primary CL flower on the same bract. An array of CH and CL flowers can be found everywhere else on the plant. When the capsule (fruit) matures, a pore in the middle forms and opens to release the seeds. In May 2016, maternal families of seeds were collected from *T. perfoliata* plants at three locations (populations) in Pitt County, NC. These locations were land near Otter Creek (OC), B's BBQ (BB) and the Pitt County Arboretum (AR).

Growth Room Study

In December 2017, seeds from eight families within each population were spread into 4.5 in square pots filled with a 1:1 mixture of Miracle Grow Potting Mix and a standard potting

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medium. The pots were placed in a growth room at East Carolina University where they received natural and artificial light. After emergence of the cotyledons, six seedlings from each family were transferred into individual Ray Leach "Cone-tainers" (Stuewe & Sons, Inc). Seedlings were arranged onto racks which were rotated once a week to minimize the effects of variable light conditions until February. The plants were watered using sub-irrigation and fertilized every week with a 12-55-6 fertilizer solution.

In February 2018, hand pollinations began. The experimental design included five treatment types: "cleistogamous" (CL flowers left unmanipulated); "chasmogamous selfed" (CH flower emasculated and pollinated by another CH on the plant); "within-population outcross" (CH flower emasculated and pollinated by a different plant from the same population); two "between-population outcrosses" (emasculated CH flower pollinated by plant from one of the other two populations). The healthiest looking plant from six families in each population served as the maternal plant. Each pollination treatment was replicated four or five times on the maternal plant. For within and between-population outcrosses, each replicate used pollen donors from a different family. Pollination treatments were indicated by placing a mark on the bract below the flower. Once the capsules were near or at dehiscence, seeds were collected and stored in small manila pouches until the fall of 2018.

Progeny from five maternal plants of each population were planted. Plant cultivation and growth conditions followed protocols described above. Once germination began in early September, sixteen seedlings of each cross-type were transferred to eight "cone-tainers" (two plants per pot) for a total of 600 pots (three populations × five maternal plants × five treatment types × eight seedling pairs). The pots were randomized onto 35 racks with each holding 17

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pots. Racks were rotated in groups of five around the growth room twice a week. Seedling survivorship was documented in early October, at approximately five weeks' post-germination, and the plants were then thinned to one per pot. Six weeks after germination, plant height was measured, and the number of plants of each cross-type with height greater than 15 cm were recorded. Once the plants reached senescence, they were cut at soil level and placed into individual bags. Two criteria were used for identifying senescence: the basal leaves and lower portion of the stem were yellowing, and the main stem had concluded its flowering. The bagged plants were stored in the growth room until January 2019.

In addition to survivorship, seed germination rate and dry above-ground plant biomass were recorded as measures of fitness. For the germination trials, 20 seeds from each of the 75 population-family-treatment combinations were placed onto moistened filter paper in a covered petri dish. The dishes were then placed into sealed Ziploc bags and kept at room temperature under adequate light conditions. Every week for three weeks, the number of ungerminated seeds was obtained. To measure biomass, all the plants were dried for 24 hours in a drying oven and weighed on an electronic balance.

To determine the relative cost of CH and CL flowers, the biomass of each flower type was measured. Before weighing, seeds were removed. Five of each flower type (CH, CL 1°, and CL 2°) were weighed at a time and divided by five to determine the weight of a single flower. For CL flower biomass, we calculated a weighted average of CL 1° and CL 2° flower based on previous estimates of their relative numbers (0.67 primary, 0.33 secondary) (Stewart, 2013).

Data Analysis

An analysis of variance (ANOVA) was used to analyze the above-ground plant biomass data. The factors in this model were treatment, population, and family (nested within population). Interactions considered were treatment*population and treatment* family (population). An ANOVA was also used to analyze data on the proportion of seeds germinated and the proportion of seedlings surviving to reproduction. To meet the assumptions of the model, we transformed both sets of data using the square root of the arcsine. Sources of variation were treatment, population, and treatment*population. The post hoc test, Tukey HSD, tested for pair-wise significance in treatment types for plant biomass, survivorship, and seed germination.

Estimates of Inbreeding Depression, Heterosis, and Flower Effect

Cumulative fitness (W) was calculated by taking the mean proportion of seed germinating, the proportion of seedlings surviving to adult, and the dry above-ground biomass of the different treatment types in each population. Inbreeding depression was calculated as $1 - (W_S/W_W)$ where W_S is the cumulative fitness of self-fertilized CH offspring and W_W is the cumulative fitness of the within-population crossed (outcrossed) offspring. Heterosis was calculated as $(W_B/W_W) - 1$ where W_B and W_W are the cumulative fitness of the between and within-population crossed offspring, respectively. Positive heterosis values indicate that offspring of between-population crosses had higher fitness while negative values indicate a reduction in fitness compared to the within-population crossed offspring. The flower effect was calculated as $1 - (W_{CL}/W_S)$ where W_{CL} is the cumulative fitness of CL flower offspring and W_S is the cumulative fitness of self-fertilized CH offspring.

Results

Flower Biomass

The overall mean of individual biomass for the CH, CL 1°, and CL 2° flowers across the three populations were 2.973, 0.581, and 0.326 mg respectively (Table 1). The weighted mean of the CL flowers was 0.496 mg per flower. From population-specific flower biomass data, the relative cost of producing CH flowers was calculated for each population: AR= 6.150, BB= 6.325, and OC= 5.526. On average the cost of CH flowers is 6.001 times more than the CL flowers in *T. perfoliata*.

Seed Germination

The ANOVA (Table 2) showed that only the treatment influenced the seed germination rates. The population and its interaction with the treatment did not have significant effect on the germination rates as the effect of treatments did not vary among populations. The Tukey HSD post hoc test indicated that CL seeds had significantly lower germination rates than the selfed CH seeds and the between and within-population crossed seeds (Figure 1). These three were not significantly different from each other.

Early Plant Growth

When early growth rate was measured for 575 plants, the observed vs. expected number of plants that were taller than 15 cm was compared. There were more selfed CH (34 observed vs 20 expected) and CL (28 observed vs 20.4 expected) plants observed that were taller than 15 cm than expected. In contrast, there were fewer between-population crossed (27 observed vs 42 expected) and within-population crossed (14 observed vs 20 expected) plants than expected that

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were taller than 15 cm tall. The expected number was determined using a contingency test, a variant of the chi square test. This result showed that treatment had an effect on the early growth rate (P<0.0001).

Survival

The ANOVA on survival from seedling to adult showed treatment has no effect on survivorship (P=0.538). The maternal population from which the plant came from showed just barely significant variation in survival (P=0.046). The interaction between treatment type and the maternal population showed no significant effect (P=0.763) meaning the treatments did not affect survival in the populations differently.

Above-Ground Dry Biomass

The ANOVA showed significant effect of treatment type, family (nested within the population), and their interaction on the above-ground dry biomass of *T. perfoliata* (Table 3). The maternal families showed considerable variation in biomass from each other. The interaction between treatment type and families showed significance as the treatment affected each family's biomass differently. The population and its interaction with treatment had no effect on the biomass of the plants. The Tukey HSD post hoc test indicated that the CL and selfed CH offspring had significantly lower biomass than the between and within-population crossed offspring (Figure 2).

Flower Effect, Inbreeding Depression, and Heterosis

Cumulative fitness, which takes into account seed germination, survival, and above-ground biomass was used to quantify flower effect, inbreeding depression, and heterosis (Table 4). On average across the three populations the cumulative fitness of CL offspring was 21.9% lower

than that of selfed CH offspring, indicating a fairly small effect of flower type on fitness. Selfed CH offspring had a 23.7% reduction in fitness when compared to the outcrossed CH offspring, indicating a modest amount of inbreeding depression. Finally, we found no evidence of heterosis; the between-population crossed offspring had 10% lower fitness than the within-population crossed offspring.

Benefit of CH Flowers

The benefit per CH flower was determined by multiplying the mean seed number produced by each flower type (estimated in Stewart, 2013) by the cumulative fitness. For CL flowers, the weighted mean seed number from the CL 1° and Cl 2° flower types was used. From this the benefit CH/ benefit Cl was determined for each population: AR= 4.039, BB= 4.318, OC= 3.271. On average CH flowers had 3.876 times the benefit of CL flowers in *T. perfoliata.*

Table 1. Flower biomass (mg) and the relative cost and benefits in producing CH flowers in *Triodanis perfoliata*. Relative cost is based on flower biomass, and relative benefit takes into account seed number and cumulative fitness.

Population	Ave. CH	Ave. CL 1º	Ave. CL 2º	Weighted CL	Relative Cost	Relative Benefit
AR	2.850	0.557	0.277	0.463	6.150	4.039
BB	3.227	0.620	0.290	0.510	6.325	4.318
OC	2.843	0.567	0.410	0.514	5.526	3.271
Overall Mean	2.973	0.581	0.326	0.496	6.001	3.876

Table 2. Analysis of variance of seed germination of each treatment type in *Triodanis perfoliata*: cleistogamous, self-fertilized CH, within-population cross, and two between-population crosses. The data were transformed by using the square root of the arcsine.

Source of Variation	d.f.	Mean Square	F	P
Treatment	3	0.426	5.337	0.040
Population	2	0.106	1.315	0.329
Treatment X Population	6	0.080	0.791	0.580

Table 3. Analysis of variance of above-ground dry biomass of offspring from each treatment: cleistogamous, self-fertilized CH, within-population cross, and two between-population crosses in *Triodanis perfoliata.*

Source of Variation	d.f.	Mean Square	F	P
Treatment	3	7.667	14.754	0.004
Population	2	2.070	0.281	0.761
Family (population)	12	8.359	5.737	0.000
Treatment X Population	6	0.520	0.346	0.908
Treatment X Family (population)	36	1.515	2.921	0.000

Table 4. Flower Effect, Inbreeding Depression and Heterosis in *Triodanis perfoliata.*

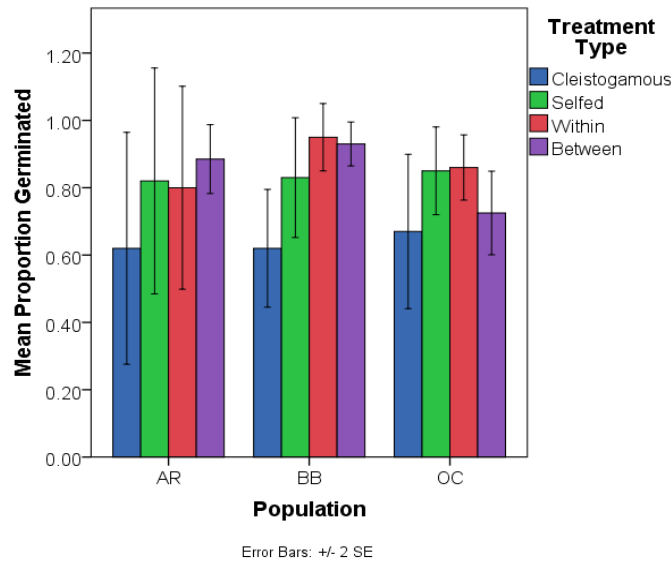| Maternal Population	Paternal Population	Flower Effect	Inbreeding Depression	Heterosis
AR	AR	0.236	0.329	
BB	BB	0.251	0.289	
OC	OC	0.168	0.092	
AR	BB			0.051
AR	OC			0.030
BB	AR			-0.082
BB	OC			-0.146
OC	BB			-0.238
OC	AR			-0.180
Overall Mean		0.219	0.237	-0.097

Figure 1. Mean proportion of seeds germinated from each treatment type in the maternal population in *Triodanis perfoliata* after three weeks. AR, Pitt County Arboretum; BB, B's BBQ; OC, Otter Creek.
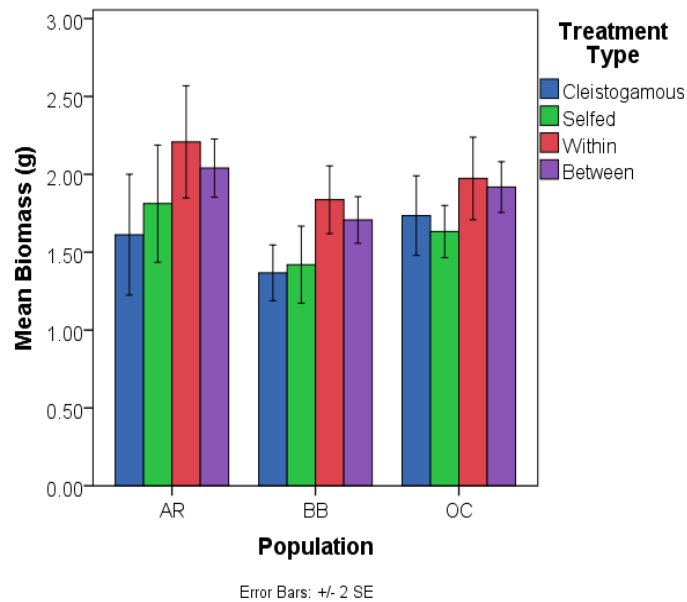

Figure 2. Mean above-ground dry biomass of offspring from each population-treatment combination in *Triodanis perfoliata.* AR, Pitt County Arboretum; BB, B's BBQ; OC, Otter Creek.
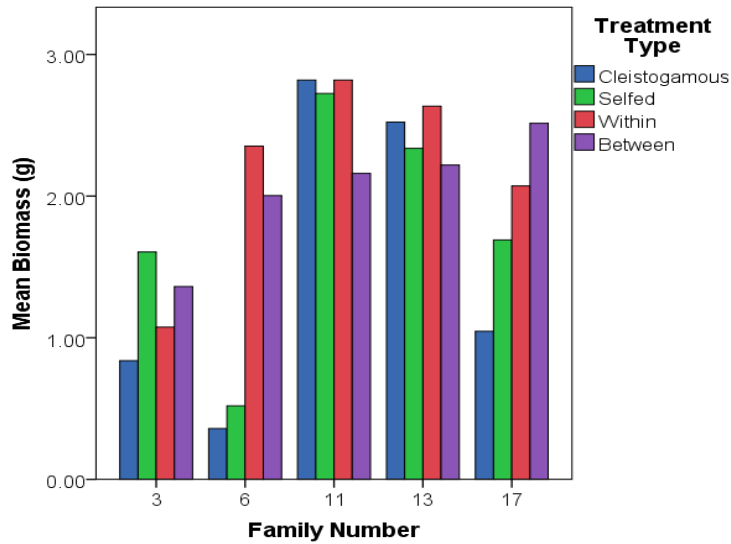
Figure 3. Family variation in above-ground dry biomass within the AR population. Each family-treatment combination is shown. AR, Pitt County Arboretum; BB, B's BBQ; OC, Otter Creek.

Discussion

Heterosis

We found no evidence to support the proposed hypothesis of heterosis in *Triodanis perfoliata*. Between and within-population crossed offspring did not significantly differ from each other in germination rates or biomass. However, a slight trend towards lower cumulative fitness in between-population crossed offspring was detected since heterosis was -10%. The negative trend was in the opposite direction of what we had hypothesized as the between-population crossed offspring should have increased in fitness.

The maternal populations (AR, BB, OC) used in this experiment were relatively close in distance. We chose to use populations at close distances to be representative of the spatial scale of potential gene flow caused by pollinators. In turn, populations in close proximity may have been genetically similar and have not diverged from each other enough to show heterosis. A study on *Leavenworthia alabamica* plant found that heterosis was highest (110.2% in

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germination and 73.6% in fitness) for the most secluded and genetically divergent population (Busch, 2006). This finding supports the idea that genetic loads fixing in populations is the basis for heterosis, the increase in fitness in offspring of a between-population cross (Whitelock, Ingvarsson, & Hatfield, 2000). Another reason why heterosis may not have been observed in our study is that *T. perfoliata* populations were not totally fixed for mildly deleterious recessive alleles. Family differences in biomass can be seen within the populations especially in the AR population (Figure 3). Each family responded differently to the treatment types indicating genetic variation across the population. If genetic drift had fixed the population for an allele at a gene, the offspring in all the families should have responded in the same manner in seed germination and above-ground dry biomass.

Inbreeding Depression

Depending upon the life stage, *T. perfoliata* exhibited non-significant or low amounts of inbreeding depression. Seed germination trials showed no significant inbreeding depression as selfed CH and (outcrossed) within-population crossed seeds did similarly well. However, measurements of above-ground dry biomass indicated significant evidence of inbreeding depression as the selfed CH offspring had consistently lower biomass than the outcrossed offspring. Based on cumulative fitness, the overall inbreeding depression across the three populations was 23.7%. The above-ground dry biomass of selfed CH offspring showed a 20.4% reduction due to inbreeding depression compared to the within-population crossed offspring. In comparison, a study on *Viola Canadensis,* another plant that exhibits cleistogamy, found that inbreeding depression for vegetative biomass was 14% (Culley, 2000).

The inbreeding depression of *T. perfoliata* (23.7%) is highly comparable to the average of primarily selfing species (23%) but not those that are predominantly outcrossing (53%) (Husband & Schemske, 1996). When inbreeding depression is lower than 50%, selfing is selected for but when it is over 50% outcrossing is selected for (Lande & Schemske, 1985). Low levels of inbreeding depression are expected in *Triodanis perfoliata* due to purging in historically self-fertilizing species. Deleterious recessive alleles that are expressed are selected against and removed from the population (Lande & Schemske, 1985). In the early life stages of *T. perfoliata*, inbreeding depression was negligible but in later life stages when the plant was mature there is a modest amount of inbreeding depression. One mechanism for how this occurs is during early life stages the lethal recessive alleles are purged out but at later stages the plant gained mildly deleterious recessive mutations either randomly or through outcrossing which are difficult to purge out (Husband & Schemske, 1996; Cully, 2000).

The pattern of low inbreeding depression early but higher later in life exhibited in *T. perfoliata* is consistent with other species that are predominantly self-fertilizing. In a comprehensive survey, 14 out of 18 self-fertilizing species exhibited most of their inbreeding depression at the growth and reproductive stages compared to seed production and germination (Husband & Schemske, 1996). In fact, on average only 2% inbreeding depression was found in predominantly selfing species at seed germination (Husband & Schemske, 1996). However, the level of inbreeding depression in *T. perfoliata* calculate in this experiment may be lower than the level in the environment as the expression of inbreeding depression is typically greater in more stressful environments (Armbruster & Reed, 2005).

Flower Effect

    We found significant evidence of the flower effect in the seed germination trials but no

evidence for it in above-ground dry biomass. CL seeds had significantly lower germination than

CH seeds produced by self-fertilization. Low germination success in CL seeds may indicate that

the maternal plant allocated fewer resources to the CL flower type and thereby affecting the

quality of certain parts of the seeds. For example, the seed coat (testa) is derived from the

maternal plant tissue rather than the embryo itself. The seed coat provides protection and support

for a viable embryo to grow (Fiqueiredo, Köhler, & Sveriges, 2014). However, a lack in

resources provided to the CL seeds by the maternal plant may impact the structure and condition

of the seed coat thus reducing its capability to protect the seed and ultimately affecting the seeds

germination success. Later on in life when the plant is mature, its success or fitness relies more

on its genetic makeup and not on how much mom had initially put into it.

    Our finding of lower CL seed germination rates in *Triodanis perfoliata* contradicts the results

of a few other studies. A literature survey compiled from 252 studies on 29 cleistogamous

species found that CL seeds either germinated better or equal to CH seeds in 67.9% of the studies

(Baskin & Baskin, 2017). In addition, a study on *Viola canadensis*, another species exhibiting CL

and CH flowers, found no significant fitness difference in the offspring of the CL and selfed CH

flowers during the early life stages. However, fitness difference due to flower type were found in

V. canadensis in later life stages such adult biomass (Culley, 2000) which is contrary to our

findings in *T. perfoliata.*

Fitness Measurements: Seed Germination, Survivorship, and Biomass

In many studies, seed production is seen as one of the best measures of overall plant fitness. However, measuring exact seed production is virtually impossible for *Triodanis perfoliata* because the fruits dehisce and drop their seeds. We estimated that one plant can produced as many as 90,000 seeds based on flower counts and mean seed numbers from previous work. We decided to use above-ground dry biomass as another good representative of fitness (Younginger, Sirova, Cruzan, & Ballhorn, 2017). In addition, preliminary analysis shows a strong correlation between flower number, an accurate measure of fitness, and biomass in *T. perfoliata* (unpublished data).

Variation among maternal populations in offspring survival was marginally significant mainly due to a slightly lower survival rate in the AR population. Survival across the BB and OC population was quite consistent. As the seedlings matured into adults, mortality was quite low. Additionally, the early growth rate of the plants showed variation due to different treatment types. It was quite interesting that CL and selfed CH offspring, which had consistently lower plant biomass, tended to grow more rapidly in the early stages than the within and between-population crossed offspring. CL and selfed CH offspring had non-significant amounts of inbreeding depression in seed germination which may be seen in early growth rate as well. However, there is significant inbreeding depression resulting in lower biomass in the selfed CH offspring in addition to CL offspring. Perhaps there may be an adaptive mechanism in *T. perfoliata* that speeds up the development and early growth of plants with inbreeding depression to ensure the production of some seeds.

Relative Cost and Benefit Analysis

The cost of producing CH flowers in *T. perfoliata* is about six times more than producing CL flowers, based on the dry biomass of each flower type. Since inbreeding depression is low or modest, little disadvantage to selfing is apparent. Considering the seed number and cumulative offspring fitness, the benefit provided by CH flowers is about four times the amount of CL flowers. However, the cost still outweighs the benefits. The cost of CH flowers calculated may be slightly conservative as neither nectar production nor metabolic costs in maintaining the flower was accounted for. Carbon loss via respiration and water loss via transpiration have been shown to be additional costs to large flowers. Large petals (larger surface area) of *Cistus ladanifer*) experience high transpiration rates and require more water compared to smaller flowers of *Cistus albidus* (Teixido & Valladares, 2014). Therefore, it is highly probably that the cost of CH flowers in *T. perfoliata* may in fact be more than six times of CL flowers.

A possible alternative to explain the maintenance of CH flowers in dimorphic cleistogamous species is adaptive phenotypic plasticity. The adaptive phenotypic plasticity model refers to the separate production of CH or CL flowers based on environmental factors throughout the flowering season (Schoen & Lloyd, 1984). A review of annual species exhibiting cleistogamy found that CL flower production has priority over CH production as the amount of CL flowers changed in response to variable environmental conditions in light, water, and disturbance (Oakley, Moriuchi, & Winn, 2007). They were also unable to demonstrate that CH flowers were able to contribute to parental fitness more than the CL flowers as suggested by Schoen & Lloyd's (1984) model of adaptive phenotypic plasticity.

Conclusion

The high cost in producing CH flowers, no evidence for heterosis, and the little disadvantage to selfing due to purging leaves us still with the unanswered question as to why *Triodanis perfoliata* maintains a mixed mating system. Heterosis has been proposed as a potential benefit of outcrossing, but it is not a factor in the maintenance of CH flowers in this species. The cost of CH flowers in terms of biomass is about six-fold while the benefit by seed production and cumulative offspring fitness is only four-fold. Other benefits to CH flowers to offset the cost are the production of outcrossed offspring with genetic variability and adaptive phenotypic plasticity as a response to environmental factors should be looked at further in depth. As of now, the mixed mating system of *Triodanis perfoliata* and other cleistogamous species still remains a mystery.

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