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Low levels of inbreeding depression and enhanced fitness in cleistogamous progeny in the annual plant *Triodanis perfoliata*

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SHORT RUNNING TITLE: Low inbreeding depression in a cleistogamous plant

Abstract

The maintenance of outcrossing in cleistogamous plants that produce both open, facultatively outcrossing chasmogamous (CH) and closed, obligate selfing cleistogamous (CL) flowers is puzzling because CL reproduction is thought to be more reliable and less costly. A possible explanation for the maintenance of CH flowers is the avoidance of inbreeding depression. However, inbreeding depression for cleistogamous species has rarely been quantified. In this study, we estimate levels of inbreeding depression in plants from three populations of *Triodanis perfoliata* (L.) Nieuwl., a dimorphic cleistogamous annual, in greenhouse conditions. Estimates of inbreeding depression at multiple life stages in all three populations were low and often not different from zero. Inbreeding depression at specific life stages varied, with two populations showing later acting inbreeding depression, which is also found in other selfing species. In two of the study populations, selfed CL progeny outperformed selfed CH progeny, indicating a flower type effect. The low levels of inbreeding depression and the superior fitness of CL compared to selfed CH flowers we observed make the maintenance of CH flowers in this system surprising, and suggest that other advantages of outcrossing CH flowers are likely responsible for maintaining mixed mating in this species.

Key words: breeding system, Campanulaceae, cleistogamy, inbreeding depression, mixed mating system, plasticity, reproductive ecology

24 **Introduction**

25 Plant reproductive systems are remarkably diverse, but the major selective
26 pressures affecting the evolution of plant mating strategies can be distilled into a few key
27 ecological and genetic factors. For instance, inbreeding depression influences the relative
28 favorability of selfing versus outcrossing reproduction (Schoen and Lloyd 1984; Lande
29 and Schemske 1985; Goodwillie et al. 2005). Inbreeding depression describes decreased
30 fitness of progeny resulting from reproduction by two closely related parents or through
31 self-fertilization (Ritland 1984; Charlesworth and Charlesworth 1987; Brown et al. 1990).
32 Genetic explanations of inbreeding depression include increased homozygosity of
33 deleterious recessive alleles and decreased prevalence of advantageous heterozygotes
34 (Charlesworth and Charlesworth 1987; Roff 2002).

35 Selfing lineages are theorized to have low inbreeding depression due to purging of
36 deleterious recessive alleles as they are exposed to selection in homozygous genotypes
37 (Crow 1970; Lande and Schemske 1985; Husband and Schemske 1996; Byers and Waller
38 1999). Selfing species often display particularly low levels of inbreeding depression at
39 early life stages, which reflects strong selection against early-acting lethal recessives
40 (Charlesworth et al. 1990; Husband and Schemske 1996). However, inbreeding
41 depression expressed at later life stages is common in selfing populations (Charlesworth
42 and Charlesworth 1987; Hedrick 1994; Husband and Schemske 1996; Wang et al. 1999;
43 Willis 1999). These later-acting alleles are presumed to have a lesser impact on fitness
44 because older plants are expected to contribute less to reproduction than younger plants
45 (Charlesworth 1980; Kirkwood and Rose 1991).

A number of factors may select for the evolution of selfing. Because selfing genes are passed on through both the ovule and pollen in the maternal plant, selfing genes are expected to experience a 50% automatic transmission advantage over outcrossing genes (Fisher 1941; Holsinger 1992). Another factor that may select for selfing is indicated by the reproductive assurance hypothesis, which describes the advantage of selfing over outcrossing when pollination conditions or mate density limit outcrossing reproduction (Darwin 1877; Müller 1883; Baker 1955). The reproductive assurance hypothesis predicts that selfing will be favored in species in which individuals frequently colonize new areas; even when fitness of selfed offspring is quite low, producing any offspring is favorable over producing none. Finally, selfing may facilitate local adaptation by limiting the introduction of maladaptive genes through outcrossing (Antonovics 1968; Jain 1976).

The opposing evolutionary forces of inbreeding depression and the automatic transmission advantage are at the center of classical mating system theory, which describes mixed mating (reproduction through a mixture of self- and cross-fertilization) as a transitional state between predominant outcrossing and predominant selfing (Lande and Schemske 1985). Prior theoretical work (Lande and Schemske 1985) demonstrated that purging of deleterious alleles results in changes in the level of inbreeding depression, such that decreasing inbreeding depression corresponds with increasing selfing rates. The correlated evolution of selfing rate and inbreeding depression led to the prediction of the evolution of predominant outcrossing when inbreeding depression is high (selfed progeny are less than half as fit as outcrossed progeny), or predominant selfing when inbreeding depression is low (selfed progeny are more than half as fit as outcrossed progeny).

In contrast to this earlier view of mixed mating as a temporary, transitional state,

69 between selfing and outcrossing, current models of mating system evolution suggest that
70 mixed mating may be evolutionarily stable (Goodwillie et al. 2005). Explanations for the
71 potential stability of mixed mating incorporate other ecological factors beyond inbreeding
72 depression, namely pollination conditions (Holsinger et al. 1984; Schoen and Lloyd
73 1984) and resource availability (Oakley et al. 2007). However, the evolution of mixed
74 mating remains enigmatic to evolutionary plant biologists, and our understanding may be
75 limited by gaps in empirical data that adequately quantify inbreeding depression.

76 The current study focuses on inbreeding depression in a dimorphic cleistogamous
77 plant. Species with dimorphic cleistogamy (referred to hereafter simply as cleistogamy)
78 have a mixed mating system, producing both open, hermaphroditic, chasmogamous (CH)
79 flowers and closed, obligately selfing, cleistogamous (CL) flowers. Cleistogamy is
80 thought to be an adaptive reproductive strategy that allows for plasticity in reproduction
81 in response to pollinator and resource environments. Seeds from cleistogamous flowers
82 cost, on average, only one third as much as chasmogamous seeds (Culley and Klooster
83 2007), and thus closed flowers are expected to be favored when pollen and resources are
84 scarce. In contrast, open, potentially outcrossing flowers may have an adaptive advantage
85 when resources are abundant because selfed progeny may suffer from high inbreeding
86 depression.

87 Quantitative knowledge of the fitness consequences of cleistogamy will critically
88 inform our understanding of the factors influencing the evolution of cleistogamy. While
89 inbreeding depression has been studied extensively in a variety of systems (Husband and
90 Schemske 1996), it has been measured in relatively few mixed mating, cleistogamous
91 plant species. Furthermore, previous studies of inbreeding depression in cleistogamy have

largely equated CH flowers with outcrossing, and compared CH fitness to CL fitness for the purpose of quantifying inbreeding depression, which ignores the potential for selfing in hermaphroditic CH flowers (for exceptions see (Culley 2000; Oakley and Winn 2008)). In addition, comparison between outcrossed CH and selfed CL fitness does not account for non-genetic flower type effects on offspring fitness, driven by inherent differences in CL and CH flower types (Culley 2000). Flower type effects on progeny fitness can result from spatial and temporal differences in fruit maturation that affect resource allocation to fruits. For instance, position along the stem and timing of flowering often differs in CH and CL flowers and may influence resource availability for seed maturation (Ernst 1981; Cavers and Steel 1984).

A best but rarely used practice to determine the magnitude of inbreeding depression in cleistogamous species is to compare fitness of selfed CH progeny to that of outcrossed CH progeny (Culley 2000). Further, a comparison of fitness between CL and selfed CH progeny can be used to quantify the non-genetic effects of floral type (Culley 2000). A study that includes both comparisons can provide critical insight into both genetic and non-genetic factors that influence the evolution and maintenance of a cleistogamous mating system. Additionally, because the magnitude of inbreeding depression may vary across populations and throughout the plant life cycle, studies that quantify inbreeding depression at multiple stages and in more than one population are particularly useful.

This study quantifies inbreeding depression and fitness effects of different floral types in greenhouse-grown *Triodanis perfoliata* (L.) Niewl. (Campanulaceae) plants from three source populations. Specifically, we measured the level of inbreeding depression of

CH flowers at three stages across the entire life cycle of this annual species. We also compared fitness of experimentally selfed CH flowers with CL flowers, which are obligately selfing, to determine fitness effects associated with flower type. We used this information on inbreeding depression and flower type effects to test the hypothesis that inbreeding depression potentially favors more expensive CH flowers and helps to maintain the mixed mating system in this species.

Materials and methods

Study species

Triodanis perfoliata is a dimorphic cleistogamous annual, producing CH and CL flowers on the same plant. This herbaceous plant is native to and considered common throughout North America. Cleistogamous (CL) flowers of this species do not develop a corolla and are obligately self-pollinating, whereas chasmogamous (CH) flowers have a 5-petalled, bluish-violet corolla and are capable of both selfing and outcrossing (Trent 1942). Fruits resulting from the two flower types are also distinct in size and seed production, with smaller CL fruits producing fewer seeds (Trent 1942; Gara and Muenchow 1990). Chasmogamous flowers are protandrous; the male reproductive phase precedes the female reproductive phase, reducing the likelihood of self-fertilization (Goodwillie et al. 2018). However, some autonomous selfing could occur (Goodwillie et al. 2018). This species exhibits secondary pollen presentation, with relocation of pollen from the anthers to pollen-collecting hairs on the outside of the style prior to anthesis (Goodwillie et al. 2018).

Pollination experiment

Maternal plants were grown in a greenhouse from seeds collected from each of three natural populations in summer 2014. Source populations were located in Falkland, NC near Otter Creek (OC), Lynchburg, VA at the Claytor Nature Study Center (CLA), and Bronx, NY at the New York Botanical Garden (NYBG; GPS coordinates in **Table 1**). Rough estimates of population sizes were thousands of individuals at OC, 75-100 at CLA, and 25-50 at NYBG. Maternal source plants were collected at least 2 m apart to minimize relatedness. The pollination experimental design followed previous methods (Culley 2000) and resulted in selfed (from both CL and CH flowers) and outcrossed (from CH flowers) offspring for 15 maternal plants in each population. Outcross pollination entailed removing anthers from two donor plants, using them to scrape pollen off the style of the paternal plant and coating the receptive stigma surface of the maternal plant until visibly saturated with pollen. At least five flowers (range = 5 - 8) were outcross-pollinated per maternal plant, each with different pairs of donor plants. For selfed CH flowers, pollen used for hand pollination originated from flowers within the maternal plant. Fruits of selfed chasmogamous (CH-S), outcrossed chasmogamous (CH-OX) and selfed cleistogamous (CL) flowers were collected upon maturation. Seeds were stored at room temperature for three months prior to planting.

Seeds from controlled pollinations or CL flowers were pooled by treatment type for each maternal plant. Fifty seeds were assessed for germination for each of the three progeny types (CH-S, CH-OX, CL) in each maternal family ($n=6,750$ seeds total). Ten seeds per petri dish were placed on damp filter paper (5 petri dishes/family). Germination was analyzed as proportion seeds germinated per petri dish. Petri dishes were arranged in

a spatially randomized design on tables within the greenhouse and germinated at 22° C under a 14-hour light regime. Germination, determined by visual confirmation of cotyledons, was scored daily for 14 days, then every other day until no further germination was seen (a total of 24 days).

Several seeds of each type were potted in twelve individual cone-shaped pots (Cone-Tainers®, Stuewe and Sons, Inc., Tangent, OR, USA) filled with potting soil (Sunshine Mix #1, Sungro, Agawam, MA) and fertilized with Osmocote fertilizer (14-14-14). Within maternal lines, 10-12 (mean = 11.5) seedlings of each treatment category survived and were assessed for multiplicative fitness, with fitness multiplied across each measured life stage ($n=1,550$ offspring total). In instances when multiple seedlings emerged in the same pot, they were thinned before exceeding one cm in height to one plant per pot. Plants were grown in the greenhouse at ~22° C under a 14-hour light regime, watered daily and fertilized once weekly until senescence. Plants were arranged haphazardly, and all pots were rotated several times weekly to reduce the possibility of positional effects. Upon senescence, total number of flowers (inclusive of CH and CL types) was counted. Aboveground biomass was harvested, dried to constant weight at 80° C and weighed to the nearest mg.

Detecting inbreeding depression and flower type effects at different stages

To understand the effect of progeny type (CH-S, CH-OX, CL) on fitness at each stage, we used mixed models (Crawley 2012) to compare values for proportional germination, biomass, and flower production across progeny types with treatment category as a fixed effect and maternal plant as a random effect for each study

population. All statistical analysis was done separately for each population. Models were built using the lme4 package version 1.1-7 (Bates et al. 2015) in R (R Core Development Team 2013). General linear mixed models were used to determine the effect of treatment (i.e., progeny type) on flower number and biomass (glm function). We built models for proportional seed germination using generalized linear mixed models with a binomial error distribution and a logit link function (glmer function). Model significance was determined using the ANOVA function in the car package, version 2.0-25, in R (Fox and Weisberg 2011). When ANOVA results did not show a significant effect of progeny type on trait values, the results test statistic shown was the Wald χ^2 . When the ANOVA revealed a significant effect of progeny type on trait values, we used Tukey's post hoc tests to determine significant differences between CH-S and CH-OX progeny, and between CH-S and CL progeny, with the test statistic the Z-score (multcomp package, version 1.4-1, glht function; (Hothorn et al. 2008)). A finding of significantly ($P < 0.05$) lower performance in CH-S than in CH-OX progeny indicated inbreeding depression. A significant difference between CH-S and CL progeny indicated a significant flower type effect.

We assessed variation in fitness across maternal lines by comparing log-likelihood of models with and without the inclusion of maternal family as a random effect (likelihood ratio test). Models for comparison of glmer and corresponding glm models (lacking maternal effects) were made in glmmADMB Version 0.8.3.3, which can handle both glmer and glm objects (Skaug et al. 2012). The glmmADMB package provides the same estimates as the lme4 package, because both use Laplace approximation, but the glmmADMB allows for likelihood comparison of models lacking random effects,

whereas the lmer package does not (Bolker et al. 2009). Comparison between models was made using the ANOVA function in R. A significant *P*-value, paired with lower log-likelihood of the model including maternal effects, indicated that maternal effects were significant. Because glmmADMB cannot handle linear or linear mixed models, RLRsim version 3.1-3 was used for comparison of the lmer models and corresponding linear model (excluding maternal plant as a random effect; (Scheipl et al. 2008)).

Cumulative inbreeding depression (δ) and flower type effects

The inbreeding depression coefficient (δ) is quantified at the population-level as: one minus the relative fitness of selfed to outcrossed progeny (W_{CH-S} / W_{CH-OX}) at each life stage (Equation 1). In species with both CH and CL flowers, the relative fitness of CL flowers compared to selfed CH flowers quantifies the flower type effect on fitness (Equation 2; Culley 2000). The cumulative inbreeding depression coefficient quantifies relative fitness across the entire life cycle and is calculated by multiplying relative fitness of individual life stages together and subtracting that product from one. Because two of the measured traits, biomass and flower number, are both representative of the adult life stage, we included only flower number (the more direct measure of fitness potential) in the calculation of cumulative relative fitness.

Equation 1 $\delta = 1 - \left(\frac{W_{CH-S}}{W_{CH-OX}} \right)$

Equation 2 $W_{rel-CL} = 1 - \frac{W_{CL}}{W_{CH-S}}$

While significant inbreeding depression is detected as greater fitness of outcrossed than selfed progeny for each individual life stage (see section above), the

inbreeding depression coefficient provides information about the *magnitude* of inbreeding depression. To test whether cumulative population-level inbreeding depression coefficients and flower effect coefficients were significantly different from zero, 95% confidence intervals (CI) were generated by bootstrapping with resampling in R. Population means were considered significant when the confidence interval did not include zero.

Results

Inbreeding depression at different stages

The magnitude of inbreeding depression varied across life stages, and patterns differed by population, but in general, inbreeding depression coefficients were low and often not significantly different from zero. Differences in CH-OX and CH-S germination provided evidence for inbreeding depression in the CLA and OC populations ($\delta = 0.09$, $Z = -4.32$, $P < 0.0001$; and $\delta = 0.05$, $Z = -4.56$, $P < 0.0001$, respectively; **Fig 1**). In the NYBG population, mean germination was similar for CH-OX and CH-S plants ($\delta = -0.03$, $\text{Wald } \chi^2 = 4.39$, $P = 0.11$; **Fig 1**), indicating a lack of inbreeding depression for this population at this life stage.

In the OC population, inbreeding depression was significant for biomass: CH-S progeny weighed 16% less than CH-OX progeny ($\delta = 0.16$, $Z = -4.03$, $P < 0.001$; **Fig 1**). Inbreeding depression was not significant for biomass in the NYBG or CLA populations ($\delta = 0.22$, $Z = -2.18$, $P = 0.074$; and $\delta = 0.06$, $\text{Wald } \chi^2 = 4.45$, $P = 0.11$, respectively; **Fig 1**). Inbreeding depression in flower production was found in OC and NYBG populations, with 17% and 26% lower flower production in CH-S plants, compared to CH-OX plants,

respectively ($\delta = 0.17$, $Z = -3.85$, $P < 0.001$; and $\delta = 0.26$, $Z = -2.65$, $P = 0.022$, respectively; **Fig 1**). Inbreeding depression was not significant at flower production for the CLA population ($\delta = 0.02$, $Wald \chi^2 = 1.11$, $P = 0.575$, **Fig 1**). Data for germination, biomass, and flower production for all populations can be found in the supplementary information (**Supplementary Table S1**).

Comparison of log-likelihood of models with and without maternal family as a random effect detected significant variation in fitness across maternal lines for all life stages in all study population (all P -values < 0.0001 ; **Supplementary Table S2**). The magnitude of inbreeding depression varied across maternal families and across life stages (**Fig 2**).

Cumulative inbreeding depression (δ)

We observed no significant population-level lifetime inbreeding depression coefficients in any of the study populations, as indicated by bootstrapped 95% confidence intervals that overlapped with zero (**Table 1**, **Fig 3**). Thus, despite some evidence for low but significant inbreeding depression at some individual life stages for some populations (see above), there was no evidence for cumulative inbreeding depression across the life stages for any of the populations.

Flower type effects between CH selfed and CL progeny

The effect of flower type on progeny fitness, evaluated by comparison of selfed CH and CL progeny, varied across life stages and across populations (**Fig 1**).

The CLA population exhibited no significant flower type effects at any life stage and had a low cumulative flower effect coefficient (Equation 2) that was not significantly different from zero (-0.12 ($CI = -0.43 - 0.20$); **Figs. 1, 3; Table 1**). The OC population exhibited flower type effects at germination; seeds produced by CH-S flowers had an average germination rate that was 20% lower than those from CL flowers ($Z = 2.95$, $P = 0.009$; **Fig. 1**). Further, the cumulative flower effect coefficient was significantly different from zero for OC (-0.43 ($CI = -0.77 - -0.08$); **Table 1, Fig. 3**). In the NYBG population, CH-S progeny produced 23% fewer flowers than CL progeny ($Z = -2.6$, $P = 0.027$; **Fig 1**). The overall flower effect coefficient for NYBG plants was also significantly different from zero (-0.37 ($CI = -0.70 - -0.03$); **Table 1, Fig. 3**). Negative cumulative flower effect coefficients in OC and NYBG indicates higher fitness of progeny produced by CL relative to CH-S flowers.

Discussion

Low inbreeding depression

Inbreeding depression is thought to be a major reason for the maintenance of more energetically costly outcrossing flowers in species exhibiting dimorphic cleistogamy (Culley and Klooster 2007). However, we did not find substantial levels of inbreeding depression in the mixed cleistogamous species *T. perfoliata*. Our estimates of cumulative inbreeding depression across the life cycle were not significantly different from zero. While some estimates of inbreeding depression at individual life stages in some populations were significantly different from zero, all were very low, indicating a lack of evidence for strong inbreeding depression. Therefore, it does not appear that

inbreeding depression alone can explain the maintenance of the more showy, facultative outcrossing CH flowers in *T. perfoliata*.

Simple models of mating system evolution predict that predominantly outcrossing should occur when inbreeding depression coefficients are above 0.5 (Lande and Schemske 1985), which is well above what we observed. However, pollen discounting, which is the reduction in outcross fitness as a result of selfing (Holsinger and Thomson 1994) could reduce this critical threshold (de Jong and Klinkhamer 2005). Because CL flowers only self, they experience 100% pollen discounting. Thus production of outcrossing, chasmogamous flowers could potentially be selected for at inbreeding depression values below 0.5 in cleistogamous species, although differences in the costs and seed production of the two flower types are also expected to modify the threshold level (Schoen and Lloyd 1984).

Our estimates of inbreeding depression levels and those of the two other studies that quantify inbreeding depression without conflating CH flowers as outcrossing in cleistogamous species were all below 0.5 (Culley 2000; Oakley and Winn 2008). One study (Culley 2000) reported a lifetime inbreeding depression estimate of 0.10 for greenhouse grown, perennial, *Viola canadensis* L. plants, and another study (Oakley and Winn 2008) found a lifetime inbreeding depression value of 0.43 in the cleistogamous perennial, *Viola septemloba* Leconte.

The apparent lack of inbreeding depression we found leads to the question of what other factors can account for the maintenance of CH flowers. Across all cleistogamous species for which the benefits and costs have been quantified, CH reproduction is one-third as reliable and twice as costly as CL reproduction (Oakley et al.

2007). But the extent to which CL flower and fruit production are relatively less costly, as well as the value of CL flowers in providing reproductive assurance will vary with species. In *T. perfoliata*, CH reproduction is not pollen limited, and CL fruits produce only 10-50% the seeds of CH fruits, depending on pollination conditions, so reproductive assurance via CL flowers may not be as important in this species (Ansaldi et al. 2018). Maintenance of cleistogamous mixed mating also involves the adaptive advantage of plastic responses to environmental heterogeneity via production of the flower type that best suits the environmental conditions (Schoen and Lloyd 1984). High pollination and resource availability are frequently invoked as conditions that favor the production of CH, outcrossed flowers. However, the advantage of outcrossing in these models is the avoidance of inbreeding depression, which does not appear to be an important factor in *T. perfoliata*. One alternative explanation for the maintenance of CH flowers suggests that CH flowers may be advantageous as a means to procure new, potentially adaptive alleles, while CL flowers provide a means for uninterrupted selfing, and therefore proliferation of favorable alleles introduced by CH reproduction (Lu 2002). However, empirical evidence explaining the concurrent production of CH and CL flowers remains inconclusive.

Our results are consistent with historically high selfing rates causing the purging of deleterious recessive alleles in *T. perfoliata*. Average estimates of inbreeding depression coefficients for predominantly selfing species are 0.23, which is lower than the estimate of 0.53 for primarily outcrossing species (Crow 1970; Barrett and Charlesworth 1991; Husband and Schemske 1996; Byers and Waller 1999). Overall selfing rates are unknown in *T. perfoliata*, but are likely to be high due to a high proportional production of CL flowers combined with the capacity for selfing in CH

344 flowers; (average % chasmogamy $\pm$ 1 SE= $23 \pm 15\%$ (Ansaldi et al. 2018)). With this
345 reduced genetic load, CL flowers could be maintained because selfing would not result in
346 the loss of fitness, and would produce the low estimates of inbreeding depression that we
347 found in this study.

348 Estimates of inbreeding depression can be influenced by whether the study is
349 conducted in the greenhouse or the field, but the effects of the conditions on estimates of
350 inbreeding depression is controversial. Greenhouse experiments, like the present study,
351 have the advantage of minimizing micro-environmental variation, which is present to a
352 greater extent in the field, and which can cause errors in estimates of inbreeding
353 depression because differences among progeny may be artifacts of relatively favorable or
354 unfavorable micro-environmental conditions (Clay and Antonovics 1985). However,
355 estimates of inbreeding depression from greenhouse experiments could potentially be
356 artificially low due to lack of stress and competition in the greenhouse. Some previous
357 studies found that measures of inbreeding depression in greenhouse conditions were
358 lower than that found in the field (Dudash 1990; Montalvo 1994; Roles and Conner
359 2008). But a meta-analysis failed to detect differences in inbreeding depression estimates
360 between greenhouse compared to field studies (Angeloni et al. 2011). Furthermore,
361 stress, which is generally lower in the greenhouse than in the field, could potentially
362 influence estimates of inbreeding depression, but there is no clear relationship between
363 stress and inbreeding depression (Armbruster and Reed 2005). One experiment on *Silene*
364 *vulgaris* (Moench) Garcke found that inbreeding depression was actually lower in
365 stressful conditions (Sandner and Matthies 2016), in contrast to predictions, and other
366 work suggests that severe stress may not lead to greater inbreeding depression for

populations that have generally low inbreeding depression values (Rehling et al. 2019). A study investigating inbreeding depression in the field in this species would be complementary to this work, but would not be free of potential bias nor would it necessarily provide more accurate estimates. It is nevertheless important to recognize that our estimates of inbreeding depression could potentially be underestimates. Furthermore, it is also possible that our estimates of inbreeding depression could have been altered if other fitness components, such as seed production of self versus outcrossed flowers, had been included, but it was unfortunately not feasible to include other components in this study.

Because plants in the present study were not emasculated, we cannot eliminate the possibility that some self-pollination occurred in CH-OX flowers. CH flowers in *T. perfoliata* display protrandry, partial separation of the male and female reproductive phase, which inhibits self-pollination (Goodwillie et al. 2018). However, some selfing does occur in CH flowers of *T. perfoliata*. Experimental floral manipulations showed that some of the selfing is delayed, and may occur as the stigma curls back towards the style in the later stages of anthesis to make contact with self-pollen present on the style (Goodwillie et al. 2018). However, pollination studies in both field and greenhouse indicate that autonomous selfing results in only about 60% as many seeds as flowers that have been supplemented with self-pollen (Ansaldi et al. 2018; Goodwillie et al. 2018). Therefore, we are reasonably confident that our CH-OX progeny were largely the result of our controlled hand-outcross pollinations.

Magnitude of inbreeding depression (δ) across the life stages

Variation in the expression of inbreeding depression across life stages is typically the result of different deleterious alleles acting in each life stage. Lethal recessive alleles acting at early life stages should be quickly purged from populations because of their severe negative effect on fitness in the homozygous state (Husband and Schemske 1996). Inbreeding depression in the later life stages is thought to have less of an impact on plant fitness because the relative contribution to reproduction decreases as a plant ages (Charlesworth et al. 1990; Kirkwood and Rose 1991). Therefore, deleterious alleles may accumulate when their effect is mild, resulting in larger inbreeding depression in the later life stages (Charlesworth and Charlesworth 1987; Hedrick 1994; Husband and Schemske 1996; Wang et al. 1999; Willis 1999). The pattern observed in the NYBG population is consistent with that of late acting inbreeding depression in predominantly selfing species (Husband and Schemske 1996). Similarly, inbreeding depression in the cleistogamous plant *Viola canadensis* was low for seed production, flower production and survival, but outcrossed plants produced 14% more vegetative biomass than selfed plants (Culley 2000). And in *Viola septemloba*, inbreeding depression at the individual life stages was not significant but tended to increase at later life stages (Oakley and Winn 2008). This mechanism of effective purging at early life stages does not explain the patterns observed in CLA or OC plants, where inbreeding depression was expressed either at early life stages or consistently across all life stages, respectively.

Variation among maternal lines

Maternal family had a significant impact on plant fitness, and patterns of the magnitude of stage-specific inbreeding varied. In some cases, differences across maternal family were quite pronounced, as is seen in the germination stages of OC and NYBG

populations. In both of these populations, there were two families that exhibited large, negative inbreeding depression coefficients, which indicates that CH-S plants were more vigorous than CH-OX plants within these lines. Within these maternal families, the negative inbreeding depression coefficients generally resulted from a combination of particularly low CH-OX germination (relative to the population mean) and high CH-S germination. An exception was a maternal family in the OC population where CH-OX germination was average, but CH-S germination was relatively high. The observed low fitness associated with outcrossed progeny is not likely a result of biparental inbreeding, because in a highly selfing population, purging of deleterious alleles is expected to mitigate biparental inbreeding depression (Ritland 1984; Uyenoyama 1986; Brown et al. 1990). A more likely explanation is that outcrossing in these families led to unfavorable allelic combinations, resulting in the low germination in outcrossed progeny.

The observed variation in inbreeding depression among maternal lines may reflect different genetic loads (i.e. the number of deleterious recessive alleles) present in each family or differences in the scale of the effect of the present alleles (Charlesworth and Charlesworth 1987; Husband and Schemske 1996). It is not unusual to observe differences in inbreeding depression across maternal lines within a population (Dudash et al. 1997; Culley 2000; González-Varo and Traveset 2010). However, maternal variation cannot necessarily be ascribed to inbreeding depression as maternal effects, and other genetic mechanisms can contribute to differences in the relative fitness of outbred and selfed progeny across families (Johnston and Schoen 1994; Fox 2005; Kelly 2005; Moorad and Wade 2005). For instance, outcrossing can introduce novel genetic combinations, while selfed progeny are restricted to inheriting alleles present in the

selfing parent (Fox 2005). And, while selfing should lead to low variance in fitness, outcrossing can lead to higher fitness variance due to the possibility of an unfavorable allelic combination following outcrossing (Waller 1980).

Flower type effect: CH-S vs CL progeny

CH flowers in dimorphic cleistogamous species such as *T. perfoliata* can either outcross or self-fertilize. If there is a difference in fitness between offspring produced by selfing CH flowers and by CL flowers, which are obligately selfing, this would be a flower type effect. Historically, multiple authors have largely assumed that progeny from CH flowers are the result of outcrossing, thus ignoring potential flower type effects (Wilken 1982; Clay 1983; Waller 1984; Antlfinger 1986; Gara and Muenchow 1990; Lu 2002; Winn and Moriuchi 2009). Few prior studies of cleistogamous species experimentally address the potential for non-genetic flower type effects on fitness of progeny (Culley 2000; Oakley and Winn 2008).

In this study, we demonstrated differences in fitness between selfed progeny from CH and CL flowers in *T. perfoliata*, showing a flower type effect. In two populations, NYBG and OC, CL progeny were more vigorous than CH-S progeny. Our finding contrasts with another study that failed to find a flower type effect in *Viola canadensis* (Culley 2000). Our result indicates that CL flowers may have several advantages over CH-S flowers, including greater fitness of offspring, being less energetically expensive to produce, and offering reproductive assurance, which can help to explain the maintenance of CL flowers.

Flower type effects may arise from inherent differences in CL and CH fruit

structure, positioning, and timing of maturation. In many species, including *T. perfoliata*, flowering times for CL and CH flowers differ slightly, and it is possible that these differences could lead to differential resource allocation to flower types or other environmental effects during seed maturation. In many species, position along the stem differs between flower types, with primary CL flowers beginning low on the stem and CH flowers occurring along the upper half of the stem. Positional effects are well documented in the literature, and may result from temporal effects in maturation timing (Ernst 1981; Cavers and Steel 1984) and structural heterogeneity in the maternal plant that influences intra-plant resource allocation (Cheplick 1996; Cheplick and Sung 1998). Differences in CH and CL flower environment may be even more pronounced in perennial species that produce the flower types in separate seasons and in species with subterranean CL flowers.

It is important to note that our estimates of flower type effects are from a greenhouse study, and results could potentially be different in the field. For example, if resource such as nutrients are lower in the field than greenhouse, and nutrient availability influences the relative fitness of CL compared to CH flowers, flower type effects could differ in magnitude or be absent or even reversed in the field compared to the greenhouse. However, our study populations occur in areas that do not appear to be substantially limiting in nutrients. Furthermore, if nutrients were lower in the field than the greenhouse, we might expect that the relative fitness of CL flowers, which are cheaper to produce, might be even greater rather than less in the field. We expect that our experiments provide reasonable estimates of flower type effects. However, it is possible that field experiments would provide different information on flower type effects. Future

work on flower type effects under different conditions would be useful.

Conclusions

The low levels of inbreeding depression we observed in *T. perfoliata* suggests the potential for a selective advantage of self-fertilization. Selfing via cleistogamy appears to be particularly advantageous given the flower type effect that we found, with greater fitness from CL than CH-S flowers, as well as the lower cost of CL compared to CH-S flowers. Likely, ongoing high levels of selfing have purged many deleterious recessive alleles in these populations. However, levels of inbreeding depression varied substantially across life stages, indicating that the frequency and effects of deleterious recessive alleles likely varied across life stages. Inbreeding depression also varied widely by maternal family, indicating that individual genetic loads probably vary among genotypes. Further, significant flower effects in these populations indicate that the effects of self-fertilization vary depending on whether it occurs in obligately selfing CL flowers or in facultatively selfing CH flowers. Specifically, finding that CL progeny are more fit than those from CH-S flowers suggests the need for further study of non-genetic effects that underlie differences in selfed progeny from the two flower types in cleistogamous breeding systems. Overall, low levels of inbreeding depression demonstrate that this factor alone cannot explain the maintenance of CH flowers, and that other ecological and evolutionary factors should be considered.

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Table 1. Population locations, and population-level inbreeding depression (IBD)

coefficient (δ) and flower effect (1-cumulative relative fitness) averaged across maternal lines ($N=12$ for each population). Bootstrapped 95% confidence intervals are indicated in parentheses. Subscripts denote statistical significance levels as determined by 95% confidence intervals that do not overlap with zero. Negative flower effects indicate that CL progeny outperformed CH-S progeny.

Population	GPS Coordinates	Cumulative IBD mean	Cumulative flower effect mean
OC	35.43141 N, -77.34327W	0.233 (-0.142 – 0.609)	-0.426 (-0.773 – -0.0795)*
NYBG	40.85984 N, -73.87558W	0.226 (-0.0335 – 0.485)	-0.365 (-0.696 – -0.0336)*
CLA	37.38731 N, -79.53914W	0.0972 (-0.083 – 0.277)	-0.115 (-0.433 – 0.203)

* $P < 0.05$, ** $P < 0.01$

Figure 1. Population trait means for germination, flower number, and biomass for each progeny type: CH-OX (progeny from outcrossed, chasmogamous flowers), CH-S (progeny from selfed, chasmogamous flowers), and CL (progeny from cleistogamous flowers). Bars represent mean (± 1 SE). Bars with different letters were judged to be significantly different at $P < 0.05$ as determined by means of Tukey's *post-hoc* comparison of trait values between offspring types following GLMMs. Marginally significant differences are not annotated here.

Figure 2. Average inbreeding depression coefficient ($1 - (W_{CH-S}/W_{CH-OX})$) by trait for maternal line ($N=15$) within each of three study populations. Points represent average relative fitness within a maternal plant, with lines connecting life stages within a maternal line.

Figure 3. Cumulative coefficients of inbreeding depression ($1 - (W_{CH-S}/W_{CH-OX})$) and flower effects ($1 - (W_{CL}/W_{CH-S})$), averaged across maternal lines within each study population. Asterisks (*) indicate values that are significantly different than zero, as determined by bootstrapped 95% confidence intervals that do not overlap with zero. The dashed red line indicates a critical threshold inbreeding depression coefficient ($\delta = 0.5$) below which, the Lande and Schemske (1985) mating system model predicts evolution of predominant selfing.

CLA

Botany
NYBG

OC