

THE ROLE OF BREEDING SYSTEM AND INBREEDING DEPRESSION IN THE MAINTENANCE OF AN OUTCROSSING MATING STRATEGY IN *SILENE VIRGINICA* (CARYOPHYLLACEAE)¹

MICHELE R. DUDASH² AND CHARLES B. FENSTER

Department of Biology, H. J. Patterson Hall, University of Maryland, College Park, Maryland 20742 USA; Mountain Lake Biological Station, Route 1, Pembroke, Virginia 24136 USA; and Department of Botany, Norwegian University of Science and Technology, N-7491, Trondheim, Norway

The goal of this study was to understand the interaction among breeding system, mating system, and expression of inbreeding depression in the hermaphroditic, primarily hummingbird-pollinated, iteroparous, short-lived perennial *Silene virginica*. We performed hand-selfed and hand-outcrossed pollinations in the field, conducted detailed floral observations within individual flowers and plants, and assayed adult tissue from flowering plants for a genetic estimate of population outcrossing rate. We quantified the opportunity for geitonogamy as the proportion of days each plant exhibited simultaneous male and female function, i.e., asynchronous expression of male- and female-phased flowers. Expression of cumulative inbreeding depression based on germination rate and total flower production in the glasshouse was ~40% and was congruent with the estimated high outcrossing rate of 0.89. Floral observations demonstrated strong temporal protandry within each flower (dichogamy) as well as complete spatial separation between male and female function within each flower (herkogamy). On average, 29% of the time there were both male- and female-phased flowers present on an individual plant. We conclude that our estimate of inbreeding depression is compatible with a largely outcrossing mating system and the amount of selfing observed, likely results from geitonogamy. This study illustrates the utility of examining both the causes and the consequences of inbreeding via selfing to provide additional insights into the evolution of plant mating systems.

Key words: breeding system; Caryophyllaceae; geitonogamy; hummingbird; inbreeding depression; mating system; perennial; *Silene virginica*.

Many theoretical models of mating system evolution recognize the importance of inbreeding depression as a selective agent preventing the evolution of selfing (e.g., Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Holsinger, 1991). Surveys of the inbreeding depression literature reveal that inbreeding depression increases throughout the life cycle of many plant species (reviewed by Husband and Schemske, 1996), which suggests that deleterious alleles contributing to inbreeding depression vary in their effects across the life cycle, with more weakly deleterious alleles expressed later in the life history. These results suggest that perennial species are more likely to harbor a higher genetic load than annual species because of the increased number of cell divisions and greater difficulty of purging weakly vs. strongly deleterious alleles from the population (Barrett and Eckert, 1990).

The evolution of selfing is also accompanied by the evolution of inbreeding depression with highly selfing species expressing less inbreeding depression than outcrossing species (e.g., Lande and Schemske, 1985; Schemske and Lande, 1985; Carr and Dudash, 1996; Husband and Schemske, 1996). The purging of inbreeding depression is commonly ascribed to the increased efficiency of selection to remove recessive deleterious alleles which are more frequently expressed in the ho-

mozygous state under various systems of inbreeding (e.g., Charlesworth and Charlesworth, 1987; Dudash and Carr, 1998). Because perennial species are more likely to harbor deleterious alleles, we expect that perennial species are also more likely to be highly outcrossing. This expectation is indeed observed, as the evolution of selfing is frequently associated with the annual life habit (e.g., Stebbins, 1950; Jain, 1976). A more recent analysis based on electrophoretic markers demonstrated a positive correlation between longevity and outcrossing rate as well (Barrett and Eckert, 1990). Furthermore, the reproductive assurance advantage associated with selfing (e.g., Stebbins, 1957; Schemske, 1978; Lloyd, 1979) is less likely to be a selective agent in the evolution of mating systems in perennials compared to annuals, because perennials have the opportunity for multiple bouts of reproduction (Morgan, Schoen, and Bataillon, 1997).

However, many perennial species often are not completely outcrossing, exhibiting some selfing (e.g., Schemske and Lande, 1985; Barrett and Eckert, 1990). Thus, a relevant question is what factors are responsible for any departure from complete outcrossing in perennials? One possible reason is that many plants are dependent on vectors for pollen removal and pollen receipt and thus may experience constraints in the expression of their mating system. For example, selection for an increase in floral display size may indirectly increase selfing through geitonogamy (e.g., Harder and Barrett, 1995). Mixed-mating systems may not be a direct product of selection, but rather an outcome of the abovementioned constraint. If we are to examine the relationship between inbreeding depression and mating system, it is necessary to not only document deviation from complete outcrossing but also to determine the causes of selfing.

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² Author for reprint requests (e-mail: md59@umail.umd.edu).

The opportunity for geitonogamous self-pollination in species that exhibit within-flower dichogamy and/or herkogamy has been of interest for some time (e.g., Darwin, 1876; Knuth, 1906). Many researchers have recently noted the increased probability of geitonogamy with increasing floral display size (e.g., Dudash, 1991; de Jong, Waser, and Klinkhamer, 1993; Harder and Barrett, 1995; reviewed in Snow et al., 1996; Brunet and Eckert, 1998; Vrieling et al., 1999; Eckert, 2000). Between-flower selfing does not provide the reproductive assurance of within-flower selfing because a vector is needed to transfer pollen from one flower to another, and it reduces the pollen available for outcrossing, i.e., pollen discounting (Harder and Barrett, 1995). Although dichogamy and herkogamy are thought to effectively prevent within-flower selfing by promoting outcross matings and/or preventing interference between pollen presentation and female receptivity (Lloyd and Yates, 1982; Lloyd and Webb, 1986; Webb and Lloyd, 1986), they do not necessarily decrease selfing via geitonogamy. Dichogamy will prevent geitonogamy if all flowers on an inflorescence or plant are in temporal synchrony, i.e., all in male or female phase. Dichogamy may also prevent between-flower selfing by combining protandry with acropetalous pollinator movement (e.g., Pyke, 1978, 1979; Waddington and Heinrich, 1979). Certain floral morphologies such as enantiostyly and heterostyly, in addition to self-incompatibility, may also limit geitonogamy (e.g., Fenster, 1995; Harder and Barrett, 1996). However, many species do not exhibit complete synchrony in gender function or the abovementioned inflorescence architecture or floral morphology, providing an opportunity for selfing to occur between flowers on a single plant. We need baseline data to quantify the relationship between the asynchronous expression of dichogamy and/or herkogamy and the potential for selfing via geitonogamous pollination.

We investigated whether inbreeding depression can be an important selective force in maintaining a primarily hummingbird-pollinated outcrossing mating system in the herbaceous perennial *Silene virginica*. The objectives of our study were to describe the breeding system of *S. virginica*, estimate its mating system with electrophoretic markers, examine the opportunity for selfing through geitonogamous self-pollination in the field, and quantify inbreeding depression among maternal families through one bout of reproduction from two populations in a glasshouse study. This approach allowed us to simultaneously assess both the causes and consequences of potential inbreeding via selfing in this system.

MATERIALS AND METHODS

Study system—*Silene virginica* (Caryophyllaceae) is a tetraploid (Kruckeberg, 1955) short-lived perennial (Dudash and Fenster, 1997) found in eastern North America. It flowers from late May through June at our study sites near Mountain Lake Biological Station (Allegheny Mountains, Giles County, Virginia, elevation ~1330 m). As demonstrated in this paper, the bright red flowers are protandrous and highly outcrossing. The primary pollen vector of *S. virginica* is the ruby-throated hummingbird, *Archilochus colubris*, with occasional visits by *Bombus* spp., syrphid flies, and small solitary bees (Fenster and Dudash, 2001). *Silene virginica* exhibits little evidence of clonal spread, and any additional leaf production occurs along the aboveground stem and is clearly interconnected upon inspection. This species is not pollen limited in terms of total seed production per individual for any given flowering season, and there is no future cost of reproduction reflected from current levels of reproduction in our study populations (Dudash and Fenster, 1997).

Study site—The breeding system, outcrossing rate, opportunity for geiton-

ogamy, and levels of inbreeding depression were quantified from plants located in a woodland site and/or an open meadow site. Plants in the woodland site occurred in the shaded understory of an oak-hickory and formerly chestnut forest, while plants in the meadow site were growing without a canopy cover along a power line cut. Approximately 2.5 km separated the two study areas, and the meadow site is ~50 m higher in elevation and begins flowering ~1 wk later than the woodland site.

Breeding system—The breeding system was documented by following 30 randomly chosen unmanipulated individuals in both field sites where each flower was labeled on each plant throughout the entire flowering season. The progression and the period of each flower's phenology was documented daily. To determine stigma receptivity, pollen supplementation was performed on another 30 randomly chosen individuals that were then monitored in the same manner. Hand-pollinations were performed each day the stigmas were receptive until the indicated darkening and wilting of the stigmas and petals occurred, signaling a successful pollination.

Quantifying outcrossing rates—We were unable to assay individuals directly from seed because of the small seed size or from the seedling stage owing to extreme variation in germination rates. Thus we estimated the outcrossing rate of *S. virginica* by assaying 60 adult plants from the woodland population. We assayed the individuals for six polymorphic loci. Proteins were extracted with phosphate grinding buffer (Soltis et al., 1983). The enzymes MDH (two alleles), SKDH (three alleles), and 6PGD (three alleles) were scored on morpholine citrate buffer system (Ritland and Gander, 1987), LAP (four alleles) was tested on a lithium borate buffer system, and PGI (five alleles) and PGM (three alleles) on histidine citrate buffer system (systems 7 and 11, respectively, of Soltis et al., 1983). Staining recipes followed Werth (1985). The outcrossing rate of the population was calculated by determining F statistics for each of the loci [$F = 1 - [(\text{observed heterozygosity})/(\text{expected heterozygosity if random mating})]$], and then using the relationship

$$\text{outcrossing rate} = (1 - F)/(1 + F)$$

to estimate population outcrossing rates. This procedure assumes equilibrium conditions (Crow and Kimura, 1970) and is an indirect measure of mating events, since inbreeding coefficients may decrease through the lifetime of a cohort as inbreeding depression reduces the frequency of inbred progeny (Ritland, 1996). The advantage of calculating outcrossing based on the inbreeding coefficient is that it is a cumulative measure across years and is, therefore, less sensitive to yearly variation in selfing rates as are direct measures of the selfing rate. The mean and standard error were calculated by jackknifing the calculated F statistics of each of the six loci (Sokal and Rohlf, 1995).

Quantifying the opportunity for geitonogamy—During 1992–1995 we quantified the number of flowers in female phase and male phase for each marked individual on a daily basis throughout the flowering season in the woodland site (sample sizes in Table 1). The meadow site experienced extensive large mammal herbivory, which prevented us from keeping such detailed data at that site. This census allowed us to quantify the opportunity for geitonogamy or the potential transfer of pollen from flowers in male phase to flowers in female phase on the same individual, effectively, selfing. For each flowering individual in the study we counted the number of flowers in female phase open simultaneously with flowers in male phase divided by the total number of flowers in female phase. This measure is integrated across the flowering phenology of each plant. Thus a single flower in female phase may have the opportunity to be self-pollinated one day and not another. We have never observed within-flower selfing fruit production, probably because of the expression of both dichogamy and herkogamy within individual flowers of *S. virginica* (personal observation).

Inbreeding depression study—In late May 1992, just prior to flowering, 17 and 11 plants from the meadow and woodland sites, respectively, were randomly chosen for hand-selfed and hand-outcrossed pollinations along 100-m transects at the two sites. To prevent pollination by natural vectors, pollinator enclosures, consisting of window screening supported on a wood and

TABLE 1. Mean proportional time flowers of *Silene virginica* in female phase are asynchronous with flowers in male phase on a plant in the woodland population across 4 yr. This measure is integrated across the flower phenology of each plant. Thus the mean values below represent the sample of flowers in female phase capable of receiving self-pollen from another flower opened on the same plant, or in other words, the opportunity for geitonogamous self-pollination on any given day in the flowering season. The value ranges from zero, corresponding to all females in female phase without any flowers in male phase, to 1.0, which corresponds to a female flower always having a male flower open simultaneously on the same plant. N = number of plants followed each year.

Year	N	Mean time in asynchrony \pm 2 SE
1992	201	0.221 \pm 0.036
1993	178	0.281 \pm 0.046
1994	145	0.295 \pm 0.048
1995	166	0.345 \pm 0.046

poultry-wire framework, were placed over each plant. Upon flowering, flowers on each plant were randomly chosen for self and outcross hand-pollination. To carry out self-pollinations, fresh pollen was transferred geitonogamously and applied by rubbing newly dehiscent anthers on the newly emergent receptive stigmas. Uniparental outcross-pollinations were conducted by transferring pollen in the same manner from plants between 1 and 10 m away. Hand-pollinations on each flower were repeated for both treatments over a 2–3 d period to help ensure that viable pollen was transferred to receptive stigmas. Because the hand-pollinations were repeated for several days it is likely that the fruit developing from outcross pollinations represent multiple sires. Eighteen days after the final pollination, developing fruit were covered with stocking material, so that all seed could be recovered when the fruit became ripe and began to dehisce. Fruit generally began to dehisce ~23–25 d after last pollination and fruits were individually collected and placed in envelopes.

Fruit set, seed set, and seed mass were quantified in the laboratory. Fruits were scored as seed-producing as long as they contained at least one developed seed without any evidence of herbivory. Fruits were scored as herbivorized if the entire fruit was absent or if there was any evidence of seed herbivory, including a lack of ovary or presence of frass or in some cases the herbivore itself. The noctuid moth larva *Hadena ectypa* is a common herbivore on both flowers and developing fruits of *S. virginica* at our study sites. If there was no evidence of herbivory and there was still no seed, then the fruit was scored as aborted. Under a 10 \times binocular dissecting scope, the number of aborted seed or unfertilized ovules and “normal” seed were counted. Normal seeds of *S. virginica* are fully round and weigh ~0.8 mg, while aborted seed or unfertilized ovules appear paper thin and are much smaller in size and mass.

In February 1993, from 20 to 150 seed from each hand-pollination treatment per plant were placed on water-saturated filter paper in petri dishes and stratified for 14 wk at 2°C, with a 14 h d and 10 h night. At the end of this period, the seed for each treatment and maternal parent were planted in potting soil in individual seed flats and placed on greenhouse benches where they were watered to maintain moist soil conditions. When virtually no seed germinated, the seed flats were transferred outdoors in June to cold frames where they were watered to prevent the soil from drying. The seed flats remained in the cold frames over winter and through spring 1994, when germination occurred. Germination was noted over a 2-wk period for each family and treatment in mid-April 1994.

In early May 1994, up to 10 seedlings per treatment (self or outcross) per original female plant (15 and 6 maternal families from the meadow and woodland site, respectively) were transplanted into 7-cm clay pots and one seedling per treatment per female was randomly placed into each of ten blocks on a greenhouse bench. Seedlings were only chosen from families where both self and outcross seeds germinated. On average, 9.4 seedlings (SD = 1.3) per female per treatment were planted. This unbalanced design represents a strategy to sample self and outcross progeny from as many maternal families as

possible, although less than ten seeds per treatment per family may have germinated. The seedlings were watered and fertilized ad libitum, and in September all leaves >1 cm in length were counted to quantify the effects of selfing and outcrossing on juvenile plant size. Plants that died were given a value of zero leaves.

Following the leaf census, all plants were transferred to the cold frame, maintaining their original position with respect to block. In late March 1995, all plants were transferred back to the greenhouse benches, again maintaining their position with respect to block. Plants commenced flowering in late April, and all flowers or buds within 2 d of opening were counted on each plant. In addition, tallest inflorescence height (a trait that is under positive directional selection in our populations; C. B. Fenster and M. R. Dudash, unpublished data) was measured for each plant. By late May, when almost all the plants had flowered, the experiment was terminated and aboveground biomass, excluding bolting reproductive stems, was harvested. This approach was taken to be able to compare vegetative growth independently from flower production as well as compare flowering and nonflowering individuals. Plants that died were given a value of zero biomass and zero flower production. Plants were dried in 50°C ovens for 2 mo before the effect of selfing and outcrossing on progeny aboveground biomass was quantified.

Statistical analyses of inbreeding depression—All data analyses were performed with SAS version 6.12 (SAS, 1989–1996). We quantified inbreeding depression in three ways. First, using ANOVA we examined the effect of selfing and outcrossing on percent fruit set, percent seed set, seed mass, percent germination, juvenile leaf number, adult flower number, adult biomass, and height of the tallest inflorescence. To meet the assumptions of ANOVA the above characters were transformed: percentage characters by arcsine square root, juvenile leaf and adult flower number by square root, and seed mass, adult biomass, and inflorescence height by log. All of the ANOVA models included cross, population, and maternal family identification nested within population except for the analyses examining the effect of selfing on percent fruit set and percent germination. For percent fruit set and percent germination we used the maternal family as the unit of replication, thus we could not quantify a maternal effect on these characters. For all of the analyses the main effects of population and maternal family nested within population (where maternal family was included in the model) and the interactions cross \times population and cross \times maternal family (populations) were considered as random effects, and variation between pollination treatments (i.e., cross) was analyzed as a fixed effect. Additionally, we used the SAS RANDOM statement with the TEST option to produce the error mean squares for hypothesis tests. Many of the interactions were found to be highly nonsignificant and were pooled into the error term for the analyses. For all analyses described above and below, the inclusion of zero values because of death had no effect on the significance values because so few plants died in the course of the experiment (survivorship after germination = 99%).

Second, we quantified the magnitude of inbreeding depression for each maternal family by calculating a relative performance measure (RP) where $RP = (\text{outcross-self})/\text{maximum}$. This measure of RP ranges from 1 to -1 and can be summed in an unbiased fashion across traits expressed during the life cycle (Ågren and Schemske, 1993). A value of zero corresponds to equivalent performance of inbred and outcross progeny, while positive or negative values correspond to outcross progeny outperforming self progeny and self progeny outperforming outcross progeny, respectively. To determine whether RP differed significantly from zero, t tests were used and were corrected with a sequential Bonferroni test (Rice, 1989). Using these maternal family level estimates, the independence of RP for the eight traits was tested using Pearson's correlation coefficient, corrected also with a sequential Bonferroni test. Only plants that survived were used in these correlation analyses in order to maintain independence among the traits, e.g., a plant that died, producing no leaves or flowers, was not included in the correlation analysis.

Finally, we calculated a multiplicative fitness function to estimate the amount of total inbreeding depression expressed due to selfing, through one bout of reproduction. We did not include percent fruit set and percent seed set because we felt that they were equivalent between the two treatments for the reasons given below. The multiplicative fitness function was constructed

by multiplying the ratio of the mean percent germination of self/outcross progeny by the ratio of the mean of total flower production of self/outcross progeny. Total inbreeding depression was equal to one minus the product of these two ratios and represents the effect of one generation of selfing on offspring survivorship and reproduction.

RESULTS

Breeding system—Each hermaphroditic flower opens first as a male with a whorl of normally five, but sometimes four, stamens emerging from the functionally tubular flower. Each flower normally spends an additional second day as a male with the emergence of the second whorl of stamens. Pollen is removed as soon as pollen dehiscence occurs, and the anther sacs often fall off once the pollen has been removed. At our study sites each flower often spends a day “neuter” with neither male or female function since the anther sacs are empty or have fallen off and the three stigmas have not yet emerged from the functionally tubular flower. The three styles must elongate and the stigmas must bend forming a “hook” at the end of each stigma before they are receptive. This observation has been repeatedly tested from hand-pollinations conducted by both researchers. Each flower then remains female for at least 1 d or until successfully pollinated (usually within 2 d). Extremely cold temperatures or heavy precipitation tends to slow the phenological progress, often “freezing” each flower’s state for a few days or until suitable environmental conditions occur allowing the resumption of the described phenology above. The phenology may result in flowers of various floral stages occurring on a given individual at any particular time, thus geitonogamous self-pollination is possible.

Outcrossing rate—For each of the six loci F statistics were the following: 0.05 (MDH), 0.19 (6PGD), 0.28 (SKDH), 0.19 (PGM), -0.07 (PGI), and -0.22 (LAP). The jackknifed mean and one SE of the population outcrossing rate are calculated as $t = 0.89 (\pm 0.13)$.

Opportunity for geitonogamy—The opportunity for geitonogamous pollinations to occur within an individual ranged from 22 to 35% with an average of 29% (Table 1) across the 4 yr of the study. The modal value was zero for each year indicating that the most frequent condition was the presence of a flower in female phase without any additional flowers in the male phase on a given individual.

Inbreeding depression—An ANOVA detected no significant population-level effects except for percent fruit set and inflorescence height. Thus in the presentation of the results we pooled the maternal family data from both populations and estimates of inbreeding depression represent the mean of the family values. There were no significant differences among the self and outcross progeny types performance in terms of percent fruit set, percent seed set per fruit, mean seed mass per fruit, percent germination, and inflorescence height (Table 2). We believe the nonsignificant higher seed and fruit set following selfing reflects the experimental artifact that pollen used for self-pollination came only from caged plants, whereas outcross pollen came from mostly uncaged plants that had already been naturally visited, reducing the available pollen for collection. Therefore, we hypothesize that the quantity of viable pollen placed on the stigmas may have differed between the two pollination treatments.

Significant inbreeding depression was detected in the self

TABLE 2. Average of family means for fitness-related traits of self and outcross progeny throughout the life history from two populations of *Silene virginica*. Means (and 2 SE) are presented, and significance was determined by ANOVA (df are in parentheses below the F ratio). Number of families from the meadow site = 15 and from the woodland site = 6.

Character	Type of cross		F	P
	Self	Outcross		
Percent fruit set	0.79 (0.12)	0.70 (0.14)	0.259 (1, 58)	0.618
Percent seed set	0.57 (0.08)	0.51 (0.06)	2.412 (1, 149)	0.123
Seed mass (mg)	0.87 (0.18)	0.86 (0.10)	0.86 (1, 149)	0.888
Percent germination	0.39 (0.09)	0.49 (0.08)	2.46 (1, 41)	0.125
Juvenile leaf no.	108.7 (10.2)	128.7 (10.0)	18.032 (1, 21)	<0.001
Adult biomass (gm)	9.0 (0.66)	11.1 (0.46)	24.980 (1, 15)	<0.001
Adult flower no.	63.6 (7.4)	84.5 (5.6)	31.463 (1, 15)	<0.001
Inflorescence height (cm)	31.2 (2.3)	32.3 (1.8)	0.162 (1, 197)	0.688

progeny for the traits juvenile leaf number, adult biomass, and flower production. We only detected significant interactions between maternal family with cross for two traits, mean seed mass ($F[20,128] = 1.642$, $P = 0.052$) and inflorescence height ($F[14,182] = 2.246$, $P < 0.001$). These significant interactions suggest that the amount of inbreeding depression varied among families, however, neither trait exhibited a main effect of cross. Additionally, all traits examined exhibited significant maternal effects suggesting a genetic basis to the observed variation (percent seed set, $F[27,25.7] = 1.983$, $P < 0.05$; seed mass, $F[27,23.8] = 3.323$, $P < 0.005$; juvenile leaf number, $F[16,374] = 3.257$, $P < 0.0001$; adult flower number, $F[15,303] = 3.774$, $P < 0.001$; adult aboveground biomass, $F[15,303] = 2.024$, $P = 0.014$; inflorescence height, $F[15,197] = 4.342$, $P < 0.0001$).

The analysis based on relative performance generally reflected the results of the ANOVA. The magnitude of inbreeding depression expressed was similar for percent germination, juvenile leaf number, adult biomass, and total flower production (Fig. 1). There was only a marginal effect of selfing on percent germination ($0.10 > P > 0.05$), but other traits measured later in the life cycle exhibited significant inbreeding depression ($P < 0.05$). After tablewise correction, significant correlations in relative performance were only observed for two correlations: juvenile leaf number with adult biomass ($r = 0.723$, $P < 0.05$, 21 df) and adult biomass with adult flower number ($r = 0.737$, $P < 0.05$, 21 df).

A multiplicative inbreeding depression estimate using fitness traits, percent germination, and total flower production demonstrated that self-progeny were only ~60% as fit as outcross progeny, resulting in an overall inbreeding depression level of ~0.4. We included percent germination in the multiplicative measure even though we observed only a marginal effect of cross on this trait (relative performance analysis above). The magnitude of inbreeding depression exhibited for percent germination was approximately the same in magnitude as those traits exhibiting significant inbreeding depression later in the life cycle (Fig. 1). It is likely that the lack of precision

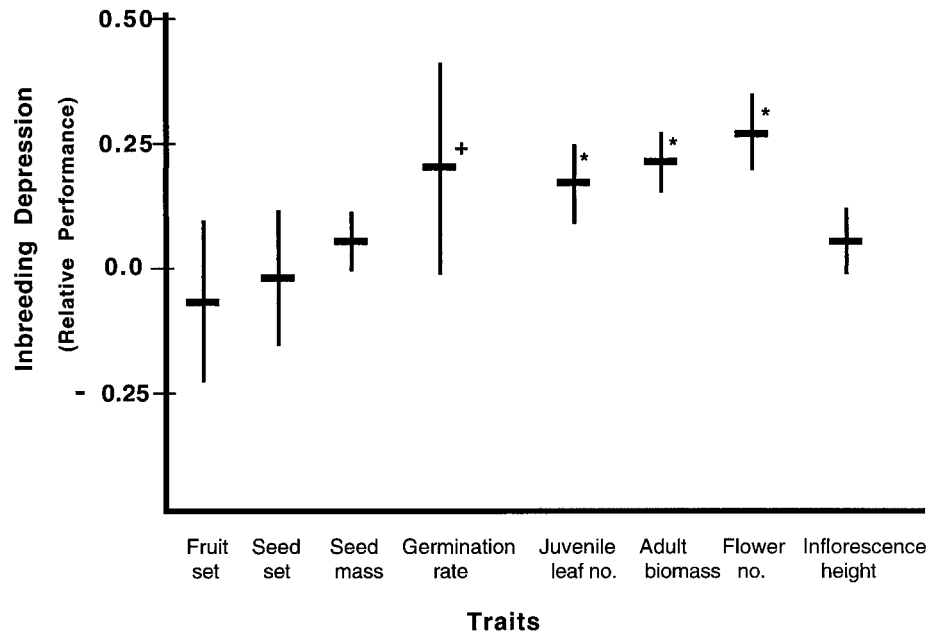


Fig. 1. Trait means of relative performance of selfed and outcrossed progeny (± 2 SE) averaged across families of *Silene virginica*. Values >0 indicate outcross-progeny outperform self-progeny within the same family, and values <0 demonstrate that self-progeny outperform outcross-progeny within the same family. The plus sign denotes $0.10 > P > 0.05$. The asterisk denotes $P < 0.05$ (t test value significantly different from zero after sequential Bonferroni correction).

of our estimate of inbreeding depression for germination reflects the absence of replication below the maternal family.

DISCUSSION

The breeding system observations, mating system estimate, and expression of inbreeding depression complement one another, indicating that *S. virginica* is a highly outcrossing perennial species. Significant inbreeding depression was detected in a number of traits measured throughout one bout of reproduction (Table 2; Fig. 1). There was no expression of inbreeding depression for traits early in the life cycle (percent seed set, percent fruit set), which is consistent with a recent review of the inbreeding depression literature (Husband and Schemske, 1996). In addition, we observed no inbreeding depression for inflorescence height. This trait is positively correlated to both male and female reproductive success in *S. virginica* (C. B. Fenster and M. R. Dudash, unpublished data), and we believe it is under strong natural selection. Thus we have some evidence for no significant expression of inbreeding depression for several traits closely associated with fitness in this species. Carr and Dudash (1996) have also observed a lack of inbreeding depression in two traits closely linked to individual fitness, pollen and ovule production in the primarily selfing *M. micranthus*.

We observed consistently similar levels of inbreeding depression from the percent germination stage onward (Fig. 1), which differs from the results reported by Husband and Schemske (1996) where the amount of inbreeding depression expressed increased as traits were measured later in the life cycle. The contrast between our results may be in part due to the benign greenhouse environment in which our inbreeding study was examined (e.g., Dudash, 1990). Additionally, *S. virginica* is a tetraploid (Kruckeberg, 1955), thus the expression of inbreeding depression may be reduced compared to a dip-

loid following only one generation of enforced selfing (e.g., Husband and Schemske, 1997), given that most evidence indicates inbreeding depression arises from the expression of recessive deleterious alleles (e.g., Charlesworth and Charlesworth, 1987; Dudash and Carr, 1998). We found no correlation between the earliest stage, which resulted in inbreeding depression, germination rate, with traits expressing significant inbreeding depression later in the life cycle. The observed significant correlations between juvenile size, adult aboveground biomass, and total flower production suggest a nonindependence in the expression of inbreeding depression for traits measured later in the life cycle. Overall it appears that our likely conservative estimate of inbreeding depression (40%) for *S. virginica* is congruent with an outcrossing mating strategy.

The quantification of inbreeding depression in herbaceous iteroparous perennials from germination until flowering is limited in the literature. Inbreeding depression is commonly quantified across the life cycle of a herbaceous perennial to some stage of early growth in a glasshouse environment (e.g., Schemske, 1983; Husband and Schemske, 1995; Ramsey and Vaughton, 1996). However, a few studies have followed individuals through at least one bout of reproduction in either the glasshouse (this study; Johnston, 1992; Culley et al., 1999; Kephart, Brown, and Hall, 1999) or field (Johnston, 1992; Ouborg and Van Treuren, 1994; Byers, 1998) and is in contrast to studies with annual and/or monocarpic plants where total reproductive effort can be readily measured (e.g., Dudash, 1990; Fenster, 1991; Husband and Schemske, 1996; Dudash, Carr, and Fenster, 1997; and many others). Thus, the estimates of inbreeding depression in the literature on iteroparous, herbaceous perennials are probably conservative because adult life stages are often omitted and we do not know how consistently inbreeding depression is expressed in subsequent bouts

of reproduction. We need more inbreeding depression studies conducted under field conditions throughout at least one, but preferably, multiple bouts of reproduction to gain additional insights on the magnitude of inbreeding depression expressed by iteroparous perennials.

Each flower of *S. virginica* exhibits both herkogamy and dichogamy totally separating within-flower male and female function, precluding within-flower selfing. Thus the opportunity for selfing arises from between-flower selfing, i.e., geitonogamy in this species. The high outcrossing rate of 0.89 was based on adult tissue and could be biased in the direction of increased outcrossing because inbreeding depression was documented to act from the germination stage onward in the life history. In *S. virginica*, naturally establishing first-year seedlings often take 4–5 yr before they reproduce (M. R. Dudash and C. B. Fenster, unpublished data) allowing ample time for juveniles to express inbreeding depression prior to flowering. Thus the selfing rate ($1 - \text{outcrossing rate}$) calculated from adult flowering plants may be inflated owing to selective pressures. If we use our conservative estimate of the relative fitness of self to outcross progeny of ~ 0.6 to factor out mortality subsequent to germination due to selfing, then the selfing rate at zygote formation is 0.18 [$(1.0/0.6) \times 0.11$], close to the estimate of potential selfing based on the proportion of time there are overlapping male and female phases on a plant (0.29). In addition, if the order in which hummingbirds visit flowers on an inflorescence is independent of the sexual phase of a flower, then hummingbirds will first visit female flowers as likely as male flowers. Taking this aspect of pollinator behavior into account halves the opportunity for selfing via geitonogamy (0.145) and places it intermediate to the outcrossing rate based on allozymes alone (0.11) and the allozyme outcrossing rate adjusted for inbreeding depression (0.18). Thus the selfing that does occur can likely be explained as either a by-product of geitonogamy (via asynchronous dichogamy and herkogamy of flowers on an individual) and/or biparental inbreeding and is less likely the result of selection to maintain a mixed mating system. In artificial array studies with *S. virginica* hummingbirds demonstrated a preference to first visit larger inflorescence displays over smaller floral displays. Secondly, hummingbirds readily visited multiple flowers on an individual potentially affecting geitonogamous self-pollination that was noted by human observation and verified by counting both pollen and fluorescent dye deposition on virgin stigmas with a compound microscope (M. R. Dudash and C. B. Fenster, unpublished data). Thus, the maintenance of a floral display size allowing geitonogamy may be an outcome of strong positive directional selection for floral display size on male function in hermaphroditic *S. virginica* (C. B. Fenster and M. R. Dudash, unpublished data). A decrease in progeny quality following selfing via geitonogamy may constrain the response to selection for increasing floral display size in self-compatible species (e.g., Dudash, 1991; Harder and Barrett, 1995; Snow et al., 1996).

The reproduction of iteroparous perennial species in any given season represents only a portion of its potential output. For example, in *S. virginica* average daily floral display size is two flowers (C. B. Fenster and M. R. Dudash, unpublished data), and seasonal average total flower production ranges from three to six flowers per individual (Dudash and Fenster, 1997). These modest numbers in any given season, however, reflect an effective outcrossing strategy when combined with both dichogamy and herkogamy within a flower but asynchro-

ny within an individual. Recent attention has been drawn to the relative importance of current vs. future reproduction and between-year seed discounting to better understand the evolution of selfing in perennials (Morgan, Schoen, and Bataillon, 1997). These models, however, assume there exists a cost between current reproduction and future reproduction, even though this assumption is not met in roughly one-half of the published studies (e.g., reviewed in Dudash and Fenster, 1997; Morgan, Schoen, and Bataillon, 1997). There is a need for more theoretical work that helps to explain the consequences of an iteroparous life history strategy without trade-offs between current and future reproduction on mating system evolution, as seen in this study system (Dudash and Fenster, 1997) and others (e.g., Whelan and Goldingay, 1989; Calvo, 1990; Jennersten, 1991). Finally, this study illustrates the importance of examining both the causes and consequences of inbreeding via selfing to provide additional insights into the evolution of plant mating systems.

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