



Resources and pollinators contribute to population sex-ratio bias and pollen limitation in *Fragaria virginiana* (Rosaceae)

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Populations of gynodioecious species vary in the ratio of female versus hermaphroditic individuals they contain, and many exhibit higher frequencies of females under poor resource conditions. One important factor limiting female frequencies within populations is predicted to be pollen limitation of seed production, caused by either low abundance of pollen donors or insufficient pollen transfer. However, empirical studies measuring variation in pollen limitation with population sex ratios or resource gradients in gynodioecious plants are inconsistent. Part of this inconsistency may be that pollen limitation and its causes are context-dependent. Another possibility is that sex-specific daily flower production and/or sex-biased visitation are more relevant to the likelihood of pollen limitation than sex ratio based on counting individual plants. In this study, we examined context-dependent pollen limitation in gynodioecious/subdioecious *Fragaria virginiana*. We specifically examined the potential for resource availability to influence sex-specific daily flower production, sex-biased pollinator visitation, and their relationships with pollen limitation in experimental populations that contained either high or low frequencies of female plants. High resource availability reduced apparent female frequency by increasing daily flower production by hermaphrodites relative to females. This is important because pollinators increasingly discriminated against female flowers as floral sex ratios became more female-biased. Contrary to expectation, females in high-female populations were not consistently more pollen limited than those in low-female populations. The level of pollen limitation of females was better explained by sex-biased pollinator foraging and visitation frequency than by the plant sex ratio or floral sex ratio. Thus, negative frequency dependence of female pollen limitation was evident only considering sex ratio bias mediated by pollinator visitation.

Populations of many separate-sexed species vary in sex ratio. Understanding the causes of this variation provides important insights into the proximate and ultimate mechanisms generating and maintaining separate sexes (reviewed by Geber et al. 1999). In plant species that contain both female and hermaphroditic individuals (gynodioecy), females tend to increase in frequency and as resource availability declines (reviewed by Ashman 2006). This pattern suggests an important role for resource availability in generating and maintaining gender dimorphism in flowering plants, but the mechanism behind this association between resources and sex ratio is poorly understood.

Several hypotheses have been proposed to explain why female plants become more common with lower resources (reviewed by Ashman 2006). All of these assume that females produce either more or better quality seed offspring than hermaphrodites (i.e. achieve higher relative maternal fitness) under relatively stressful abiotic conditions. However, empirical evidence that relative maternal fitness can predict population sex ratios in gynodioecious plants is mixed (Kesseli and Jain 1984, Kohn and Biardi 1995, Wolfe and Shmida 1997, Weller and Sakai 2005, Ramula et al. 2007), possibly indicating that additional factors affect

the association between resources and sex ratio. The process of pollination and mating is a likely candidate, because females must receive sufficient pollen in order for outcrossing and relative fertility advantages to be manifested in nature. In general, seed production by females is predicted to be increasingly pollen-limited as they become more common (Lewis 1941, Lloyd 1974, Maurice and Fleming 1995, McCauley and Brock 1998). This is because high female frequency automatically reduces the population pollen:ovule ratio, increasing the potential for insufficient pollen receipt even with sufficient pollinator visitation. Empirical evidence for increased pollen limitation of females under high female frequencies or low resource availability is inconsistent (reviewed by Shykoff et al. 2003, Knight et al. 2005, Ashman 2006, Cuevas et al. 2008). This could be because most studies relate pollen limitation to population sex ratios estimated by counting plants; they do not incorporate the potential for sex-specific daily flower production, which directly determines pollen:ovule ratios at any given time during the flowering season as well as the population sex ratio that is perceived by floral visitors. We need detailed studies on the effects of both resources and sex ratio on the process of pollination to fully understand how

these ecological factors interact to promote females under low resource conditions.

This paper describes an experiment to measure how resource availability and population sex ratio influence pollination in the gynodioecious Virginian wild strawberry *Fragaria virginiana* (Rosaceae). We manipulated both factors in experimental arrays containing the same total number of plants, and documented variation in reproductive allocation, pollinator visitation, seed fecundity and pollen limitation for both sex morphs. We were particularly interested in whether manipulating resource availability would directly affect the sex ratio that is apparent to floral visitors through sex-specific patterns of daily flower production in response to resources, and the consequences of variation in daily sex ratios for patterns of insect visitation (Table 1). We also examined the potential for flowering sex ratios and visitation bias feed back to population sex ratio through patterns of relative fertility and pollen limitation of seed production under varying resource conditions.

We made several predictions about how each combination of resources and sex ratio could influence flowering sex ratios, visitation by pollinators and the potential for pollen limitation. Our expectations for pollen limitation are based on the general assumption that it should be negative frequency dependent, but vary slightly depending on whether pollen limitation of females is most sensitive to the plant sex ratio, flowering sex ratio, or sex-bias in the visitation patterns of pollinators. In our study, these expectations apply only to females because hermaphrodites of *F. virginiana* readily self-pollinate and show little inbreeding depression at the seed production stage (Ashman unpubl.), thus we do not expect to see pollen limitation of seed production by hermaphrodites in any context.

First, we predicted that all plants growing under high-resource conditions could produce more and/or larger flowers offering greater reward, and consequently receive higher insect visitation than plants in low resources (Eckhart 1991, 1999, Delph and Lively 1992, Delph et al. 1996, Vaughton and Ramsey 1998). Increased total visitation should generally reduce the likelihood of pollen limitation at high resources compared to low resources for both sex ratio treatments. Second, we expected high-resource conditions to bias floral sex ratios in favor of hermaphrodites, and consequently result in lower likelihood of pollen limitation compared to low-resource gardens. We base this expectation on the observation that high resource availability increases total flower production by hermaphrodites of *F. virginiana* significantly more than by females (Case and Ashman

2007). Third, we expected higher visitation to hermaphrodites than females, and a higher number of total visits to gardens with fewer female flowers. Insect visitors strongly prefer hermaphroditic flowers in many species (Bell 1985, Ashman and Stanton 1991, Eckhart 1991, Delph and Lively 1992, Ashman et al. 2000), particularly where insects seek pollen as a reward and where hermaphrodites produce much larger flowers than females (reviewed by Eckhart 1999).

Methods

Study system

Fragaria virginiana (Rosaceae), the Virginian wild strawberry, is a creeping perennial herb native to eastern North America (Staudt 1989), and commonly grows in meadows, old fields and along road or forest edges. Current evidence suggests that gynodioecy in *F. virginiana* is under nuclear control with at least two linked loci (or regions) of major effect, and male sterility (femaleness) dominant to male fertility (Spigler et al. 2008). Plants reproduce sexually via seed and asexually via plantlets produced on stolons, although seed production far exceeds clonal reproduction even under stressful conditions (unpubl.). Plants perennate as rosettes; flowering and fruiting occurs from late April to early June and stolons are produced throughout the summer in western Pennsylvania. Floral visitors include small solitary bees, flies, butterflies, and ants (Ashman and Diefenderfer 2001, Ashman and King 2005). Plants produce either pistillate (females) or perfect (hermaphrodites) flowers on pleiochasial inflorescences. Although pollinators prefer perfect flowers, which offer larger visual, olfactory cues and rewards than pistillate flowers (Ashman 2000, Ashman et al. 2000, 2005), female plants have very high fruit set (~90%) while hermaphrodites fruit set is generally low (<20%) even under optimal conditions (Staudt 1989, Case and Ashman 2007).

Source population for genotypes

The genotypes used for this experiment originated from a single natural population of wild strawberry in western Pennsylvania (population P in Ashman 1999; ~25% females). All plants used in this study were cloned from plantlets under greenhouse conditions in the summer and

Table 1. Two types of sex-ratio bias predicted to affect frequency-dependent pollen limitation in gynodioecious flowering plants. Unlike sex ratios based on counting plants of each sex (female, F, or hermaphrodite, H), both flowering sex ratios and sex-biased visitation may vary within the season, can be manifested at the level of individual plants or at the level of a patch, and can be affected by ecological factors, such as resource availability or plant sex-ratio, which may vary among patches. We expect plant sex ratio to directly affect patch-level bias (A.2. and B.2.) and plant-level visitation bias (B.1.), while resources should only have direct effects on plant-level sex ratio bias (A.1. and B.1.).

Type of sex ratio bias	Level at which bias is manifested	How the bias is measured	Directly affected by plant sex ratio?	Directly affected by resources?
A. Flowering sex-ratio	1. Plant-level	number of flowers produced per day by F vs H plants	N	Y
	2. Patch-level	proportion of F vs H flowers available per day	Y	N
B. Sex-biased visitation	1. Plant-level	number of flowers visited per day on F vs H plants	Y	Y
	2. Patch-level	proportion of visits to F vs H flowers per day	Y	N

fall of 2000 and 2001, and over-wintered at the Pymatuning Laboratory of Ecology (PLE, Crawford Co., PA, 41°34'N, 80°27'W) until the start of the experiment (30 April in both 2001 and 2002).

Imposition of resource and sex-ratio treatments

Full details of the array construction and experimental design can be found in Case and Ashman (2007). Briefly, we created four experimental treatments in each of two years. Two treatments were designated high-resource (HR) and two low-resource (LR). We maintained a three-fold difference in resource availability to plants in HR versus LR by manipulating pot size, soil nutrients, and water. One of each HR and LR treatment was assigned as a high female (HF, 60% female) and a low female (LF, 15% female) sex-ratio treatment, resulting in four resource \times sex ratio combinations – HRHF, HRLF, LRHF and LRLF. Each array consisted of 80 plants. The HF arrays were assigned 48 female and 32 hermaphrodite genotypes while the LF arrays were assigned 12 female and 68 hermaphrodite genotypes. The arrangement of plants within each array was random with respect to genotype but completely stratified by sex, such that each quadrant had the same sex ratio as the entire array.

Each resource-by-sex ratio treatment was represented by two clonally replicated arrays in order to estimate levels of pollen limitation in each treatment. One replicate in each treatment was assigned to be open-pollinated and the other hand-pollinated. Open-pollinated arrays were covered with deer netting to limit predation but allow insect visitation; hand-pollinated arrays were covered with grey nylon window screening to exclude all floral visitors. Each replicate contained one vegetative clone of each genotype. Individual clones were treated and arranged in exactly the same way in each replicate array.

Growth and maintenance of experimental gardens in the field

Each resource-by-sex ratio treatment consisted of a pair 1.2 m² sunken plots placed at the four corners of an old-field at PLE. Each corner hosted one open-pollinated and its clonal hand-pollinated array, and treatments were rotated within the field between study years. The four corners were at least 500 m apart to minimize gene flow between open-pollinated arrays (Ashman unpubl.). Each plant in its pot was placed inside a larger pot filled with clay chip, then the larger pots sunk into the plots in a 9 \times 9 grid on top of a layer of weed cloth. This nested pot design was used to: 1) minimize site effects by preventing roots from extending into the native soil, 2) space the plants evenly at a natural interplant density (11–13 cm apart, Ashman unpubl.), and 3) standardize height so that crowns of plants in both small and large pots would be at ground level.

Flower production and pollinator observation

We recorded daily flower production for each plant, by censusing every three days in 2001, every two days in 2002, and again at the start of each pollinator observation period.

We recorded pollinator visits to individual flowers, the identity of the insect and of each plant visited (based on its location within the garden), and the total number of flowers open on each plant. Pollinator visits were recorded during 20-min observation periods during mid-late flowering (Supplementary material Appendix 1), during which every plant in the experiment was observed at least twice on two different days. In 2001, we recorded two observation periods in each of five days (10 observation periods per open-pollinated garden; Supplementary material Appendix 1A) and in 2002, we recorded 1–2 observation periods in each of nine days (16 observation periods per open-pollinated garden; Supplementary material Appendix 1B). Two observers recorded all visits to one quarter of an array during each observation period, and classified insects into three functional groups: bees (largely solitary and halictid bees), flies (typically Syrphidae and Bombyliidae), and other (butterflies and ants). Visitation rates were calculated as the number of visits per flower per hour during each observation period. If a plant had no open flowers during an observation period, it was recorded as 'missing' rather than receiving zero visits.

Additionally, in 2002, we recorded visitation bouts by individual insects with the aid of an event recorder (<http://bioweb.biology.kent.edu/facultypages/lorch/software.html>). We set up the event recorder on a handheld computer with a 9 \times 9 grid identical to the layout of our experimental arrays. We recorded the location of all visits during each bout in sequence by tapping cells within the grid (representing individual plant genotypes). The program recorded the time and grid-location of each visit, the total number of flowers visited, and the identity of the visitor as either solitary bee or small fly. We then linked each visit to the sex of an individual plant based on its grid-location. In total, we observed 87 individual bouts, of which 59 were by solitary bees and 28 by small flies.

Assessment of pollen limitation

We estimated pollen limitation as the difference in seed production between open-pollinated and hand-saturated plants. All flowers on all plants in each hand-pollinated garden received supplemental outcross pollen to saturation every other day. Pollen for hand-pollinations was collected from additional clones of each hermaphrodite genotype in the greenhouse prior to the start of the experiment. We assumed that the pollen we collected for hand-supplementation was comparable in quality to pollen available in the field. Thus, our measure of pollen limitation represents insufficient pollen receipt. Pollen donors were assigned randomly within treatments, meaning that the potential pool of sires was the same for both open- and hand-pollinated gardens for each treatment. Each flower was pollinated with a single hermaphrodite pollen donor on each day, and pollen donors were randomly chosen from the sire pool with replacement each day.

Data analyses

We analyzed each dataset based on our expectations for resource and sex ratio effects at the plant level versus the patch level (i.e. arrays; Table 1).

Daily flower production

To determine whether resource availability altered daily flower production at the plant level (Table 1A.1.), we assessed the effects of resource treatment, sex-ratio treatment, and sex on mean daily flower production using split-plot ANOVA (Porvin 2001). Because the sex-ratio treatment could not be applied to individuals, resource and sex effects were tested at the level of each array, while differences between sexes were tested among individuals (see Case and Ashman 2007 for detailed justification).

Flowering sex ratio

To determine if patch-level flowering sex ratios deviated from plant sex ratios (Table 1A.2.), we tallied all open flowers of each sex in each array on each census day, and compared the flowering sex ratio to plant sex ratios using replicated goodness-of-fit G-tests (Sokal and Rolff 1995). When we detected significant heterogeneity among observation days, we did not pool, and explored the pattern of deviation in flowering versus plant sex ratios among census days using correlation analysis. To compare flowering sex ratios among treatments, we used a repeated-measures ANOVA in JMP ver. 7 (SAS Inst. 2007), including resources, sex ratio, and resources \times sex ratio as main effects, with census day as the repeated factor. Contrasts between least squares means were planned to test for differences between resource \times sex ratio treatments. We could not assess between-year effects in this model, but a separate analysis indicated no significant difference in floral sex ratios between years (DF = 1,5, $F = 0.0407$, $p = 0.67$, and compare grey boxes between years in Fig. 1). We only

analyzed flowering sex ratios on census days for which at least 10 total flowers were open in each array (Supplementary material Appendix 1).

Visitation rates

We used split-plot ANCOVA to assess differences between sexes and treatments in mean visitation rate per flower at the plant level. Main effects of resources, sex ratio and sex were included as per the analysis of daily flower production per plant. We could not use repeated-measures to analyze visitation sex ratios because we did not observe all plants each day. Therefore we included observation day as a random covariate to account for variation among days in conditions other those we measured (e.g. ambient temperature or precipitation).

Sex bias in pollinator foraging

Visitation bias at the plant level was calculated as the mean visitation rate per female relative to the mean visitation rate per hermaphrodite (Table 1B.1.).

At the garden level, we calculated the mean proportion of open female flowers visited during each observation period (Table 1B.2.). We used replicated goodness-of-fit G-tests to assess whether patch-level foraging differed from flowering sex ratios among observation days. Expected proportion of female flowers visited was determined by multiplying the observed total number of insect visits by the proportion of female flowers open in each garden for each observation period. In addition, we used generalized linear models (GLMs) in JMP to assess relations between floral sex

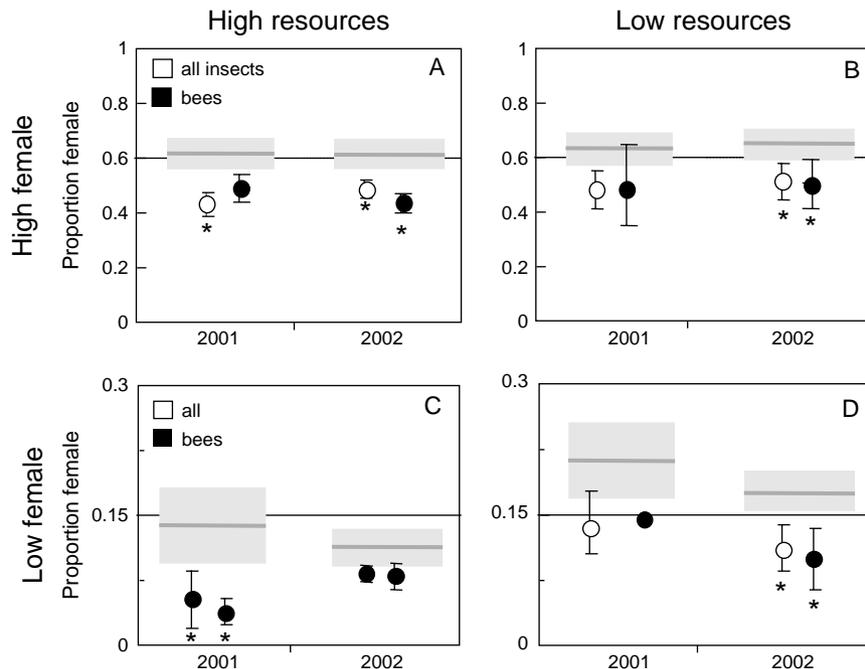


Figure 1. Sex bias in daily flower production and insect visitation in experimental arrays with varying resources (high vs low) and sex ratios (60% female plants vs 15% female plants). Solid black horizontal lines indicate the proportion of female plants in each array. The grey boxes indicate the mean and 95% confidence intervals of the mean proportion of female flowers open per day. Circles indicate the mean (± 1 SE) proportion of female flowers visited per observation period by all insects (open circles) and bees only (closed circles). Asterisks indicate sex-biased visitation that was significantly different from flowering sex ratios based on G-tests (Table 2). Note different y-axis scales for each sex-ratio treatment.

ratio and visitation bias among observation periods in each garden. Each point in the regressions represented data on a given observation day for the observed proportion of female flowers open versus the observed proportion of visits to female flowers. Based on Pearson goodness-of-fit tests, we used a binomial distribution and a logit link function. We included observation day as a covariate in both GLMs, and removed non-significant interactions using sequential backwards elimination ($\alpha = 0.10$). We used F-tests to determine significance of each regression from a slope of 0, and calculated 95% confidence intervals around the slope of each regression line to determine whether relations between floral sex ratios and visitation bias were proportional (i.e. not significantly different from a slope of 1), or if the degree of visitation bias varied disproportionately with the floral sex ratio.

In 2002, to assess whether our estimates of visitation bias were relevant at the level of single insects, we recorded the sequence of visits to each sex within individual bouts on four consecutive observation days during peak flowering. This is important because in gender-dimorphic species, pollen can only be transferred if both polleniferous and ovuliferous flowers are visited by individual insects. We used GLMs to assess differences between insect functional groups (bees vs flies), resource, and sex-ratio treatments in total number of visits per bout and proportion of female flowers visited per bout. Based on Pearson goodness-of-fit tests, we used an exponential distribution and a reciprocal link function for bout length and a normal error distribution with identity link function for proportion of female flowers visited. We included observation day as a covariate in both GLMs, and removed non-significant interactions using sequential backwards elimination ($\alpha = 0.10$).

Pollen limitation

In our study, we estimated pollen limitation for each individual genotype by comparing fecundity of each plant in the open-pollinated arrays with its clone in the fully hand-pollinated gardens (Ashman et al. 2004). Pollen limitation was measured as proportion seed set (seeds produced per ovule produced) of the hand-pollinated clone minus seed set of the open-pollinated clone. We used direct counts of seeds and ovules on a single fruit per plant that was standardized by its position in the inflorescence (i.e. the secondary position), which is known to accurately estimate the average values within an inflorescence for multiple floral traits (Ashman and Hitchens 2000, Case and Ashman

unpubl.). Conclusions based on this single-fruit estimate of pollen limitation of seed set were identical to analyses based on direct counts of total seeds per plant and estimates of total ovule production per plant (data not shown). Because we pollinated all flowers on each plant in the hand-pollinated arrays, our estimates of pollen limitation are not confounded by the potential effects of resource reallocation on seed production per fruit (Knight et al. 2006).

We used a two-way split-plot ANOVA assessing the effects of resources and sex ratio on pollen limitation; two separate analyses were run, one for each sex. For each garden, we first tested whether pollen limitation index differed significantly from 0 using paired one-tailed t-tests. We then used linear regressions to compare pollen limitation index to floral sex ratios and visitation bias as well as total visitation rate in each garden; regressions were conducted using garden means in each case.

Results

Daily flower production and flowering sex ratios

Overall, resources increased daily flower production by individual plants almost two-fold (least squares means: HR 2.8 ± 0.1 flowers per plant per day, LR 1.5 ± 0.1 flowers per plant per day; Table 2A); HR contained twice as many total open flowers per day as LR in both years (resources: $p < 0.0001$, Supplementary material Appendix 2). Female plants had 10% more flowers open per day than hermaphrodites (2.26 ± 0.12 vs 2.07 ± 0.1 flowers per day; Table 2A), a difference that was statistically significant. Sex-ratio treatments (HF vs LF) did not affect daily flower production by individual plants, and we found no significant resource \times sex interactions. Although the sexes did not differ significantly from each other within any of the gardens, we detected a resource \times sex ratio \times sex interaction for mean daily flowers (Table 2A); high resources did not significantly increase flower production by females in the LF plots, but did increase mean daily flowers for all other plants.

Based on replicated G-tests, all arrays in both years showed significant deviation of flowering sex ratio from plant sex ratio on at least one census day during the season (Table 3A–F). G-tests for heterogeneity indicated significant variation among census days in three of four gardens in 2001 and in one (HRHF) of the four gardens in 2002 (Table 3A, 3D). In all cases, this seasonal heterogeneity reflected a significant linear decline in the proportion of female flowers

Table 2. Split-plot ANCOVAs on mean daily flower production (A), mean visitation rate to each plant (per flower per hour) by all insects (B) and bees only (C). Datapoints represent means for each plant of all individual census or observation periods. Resource and sex ratio effects were tested over garden (DF = 1,4), sex effect was tested over individual plants (DF = 1,620). Observation day was included as a covariate in analyses of visitation (B and C). F-values are shown in the table. Statistically significant effects ($\alpha = 0.05$) are indicated in boldface; asterisks indicate p-values (*0.05, **0.01, ***0.001, ****0.0001).

Source	(A) Mean daily flowers	(B) Mean insect visits per flower per hour	(C) Mean bee visits per flower per hour
Resources	38.8**	1.49	1.6
Sex ratio	0.001	0.02	0.16
Res \times SR	0.07	0.01	0.001
Sex	4.12*	21.7****	17.8****
Sex \times Res	0.99	5.23*	3.9*
Sex \times SR	1.9	0.12	0.36
Sex \times Res \times SR	4.1*	0.11	0.01

Table 3. Deviation of flowering sex ratios (A–F) and sex-biased pollinator visitation (G–L) from expected values in four experimental gardens of *Fragaria virginiana* in 2001 and 2002. Treatments included two resource levels (high =HR; low =LR) at two plant sex ratios (60% female = HF; 15% female =LF). We compared percent female flowers to percent female plants among census days for which at least 10 total flowers were open. We compared percent female flowers visited to flowering sex ratios among observation periods for which at least 10 total visits were observed. Boldface values indicate statistically significant deviation from expected based on replicated G-tests; degrees of freedom for each test are indicated in parentheses.

		HRHF	LRHF	HRLF	LRLF
Floral sex ratio					
2001	(A) G_{HET}	61.7 (11)	22.2 (10)	38.1 (11)	14.4 (9)
	(B) G_{POOLED}	–	–	–	15.1 (1)
	(C) G_{TOTAL}	65.7 (12)	23.6 (11)	45.0 (12)	29.5 (10)
2002	(D) G_{HET}	64.2 (16)	18.2 (14)	21.2 (18)	11.7 (14)
	(E) G_{POOLED}	–	11.4 (1)	33.3 (1)	8.3 (1)
	(F) G_{TOTAL}	64.7 (17)	29.6 (15)	54.5 (19)	20.0 (15)
Sex-biased insect visitation					
2001	(G) G_{HET}	2.4 (6)	3.2 (4)	10.0 (6)	1.7 (3)
	(H) G_{POOLED}	10.0 (1)	2.5 (1)	9.0 (1)	0.001 (1)
	(I) G_{TOTAL}	12.4 (7)	5.7 (5)	19.0 (7)	1.7 (4)
2002	(J) G_{HET}	29.8 (14)	22.2 (10)	18.5 (13)	9.8 (8)
	(K) G_{POOLED}	–	–	3.0 (1)	7.5 (1)
	(L) G_{TOTAL}	60.1 (15)	29.9 (11)	21.5 (14)	17.3 (9)

open per day (Pearson's $r = -0.41$ to -0.84 ; all $p < 0.05$). Despite this heterogeneity, the mean flowering sex ratio did not differ significantly from plant sex ratios in any of the arrays (grey boxes overlapping solid black lines in Fig. 1A–C). However, flowering sex ratios were significantly biased in LRLF in both years, and in LRHF and HRLF in 2002 (Table 3B, 3E), but the direction of bias differed between resource treatments (Fig. 1C–D). HRLF was significantly more hermaphrodite-biased than expected in 2002, and tended the same direction in 2001 (Fig. 1C). In contrast, LRLF (both years) and LRHF (2002 only) were more female-biased than expected (Fig. 1B, 1D). When we contrasted flowering sex-ratios at the patch level, female flowers were significantly more abundant in LR versus HR (resource effect in MANOVA: $DF = 1, 4$, $F = 7.43$, $p = 0.053$). HF arrays were statistically indistinguishable from each other in terms of floral sex ratio (least squares means contrast: ($DF = 1, 4$, $F = 0.45$, $p > 0.5$, Fig. 1A–B), while increasing resources decreased the apparent proportion female in LF (least squares means contrast: $DF = 1, 4$, $F = 10.1$, $p = 0.033$; Fig. 1C–D).

Insect visitation

We observed a total of 271 insect visits in 2001, and 1596 visits in 2002. Patch-level visitation was almost four-fold greater in 2002 than in 2001 (78 ± 6.0 vs 21 ± 10.3 visits per array per hour, respectively). In all 2002 arrays and both HR gardens in 2001, 55–71% of all insect visits were made by bees. The two 2001 LR gardens received the fewest total visits overall (8 ± 2.8 visits per hour), with 17% (LRLF) and 30% (LRHF) of their total visits made by bees. Therefore we analyzed all insect visitors first, and then only bee visitors.

Neither resources nor sex-ratio significantly affected plant-level visitation rates (non-significant resource and sex-ratio effects in Table 2B). Although hermaphrodites received visits almost twice as often as females overall (sex effect in Table 2B; least squares means ± 1 SE for visits by all insects: 0.6 ± 0.23 visits per female flower per hour, 1.1 ± 0.22 per hermaphrodite flower per hour), this sex

preference was only significant in HR (resource \times sex effect, Table 2B). These results were identical when considering only visits by bees (Table 2C).

Sex-biased pollinator foraging

Scaling up to the patch level, we observed significant sex-bias in pollinator foraging in all four arrays in at least one of the two study years (Table 3G–L). In both years, the mean proportion of female flowers visited (by all insects and bees alone) was lower than expected (circles in Fig. 1). The bias against female flowers was significant only in HR in 2001 (Fig. 1A, 1C, Table 3H) and in one HR array and both LR arrays in 2002 (Fig. 1B, 1D, Table 3K–L). We saw a similar pattern when we considered bee visits alone (closed circles in Fig. 1).

At the patch level, pollinators made more visits to female flowers as the flowering sex ratio became more female-biased (Fig. 2; GLM LR $\chi^2 = 34$, $DF = 1$, $p < 0.0001$). However, the proportionality of these relations differed between the two sex-ratio treatments. In HF (Fig. 2B, 2D), flowering sex ratios and sex-biased foraging varied proportionately, indicated by slopes not significantly different from 1 (HRHF: 0.937 ± 0.476 ; LRHF: 0.843 ± 0.88). In contrast, in LF (Fig. 2A, 2C), slopes were significantly less than 1 (slopes and 95% confidence intervals – HRLF: 0.51 ± 0.363 ; LRLF: 0.443 ± 0.409), indicating more discrimination against female flowers than expected given the flowering sex ratio.

Individual insects showed a bias against female flowers within bouts (Fig. 3) similar to the general insect pool (circles in Fig. 1). Bee bouts were three times as long as fly bouts (bees: 12.3 visits per bout vs flies: 4.2 visits per bout; $\chi^2 = 16.6$, $DF = 1$, $p < 0.0001$). Insects made more total visits per bout in HR ($\chi^2 = 6.4$, $DF = 1$, $p = 0.012$), and LF treatments ($\chi^2 = 5.6$, $DF = 1$, $p = 0.018$), but there were no significant interactions. Resources did not affect the proportion of female flowers visited per bout for either insect group ($\chi^2 = 1.8$, $DF = 1$, $p = 0.18$), but this proportion was affected by sex-ratio treatment ($\chi^2 = 53$, $DF = 1$, $p < 0.0001$) differently between bees and flies

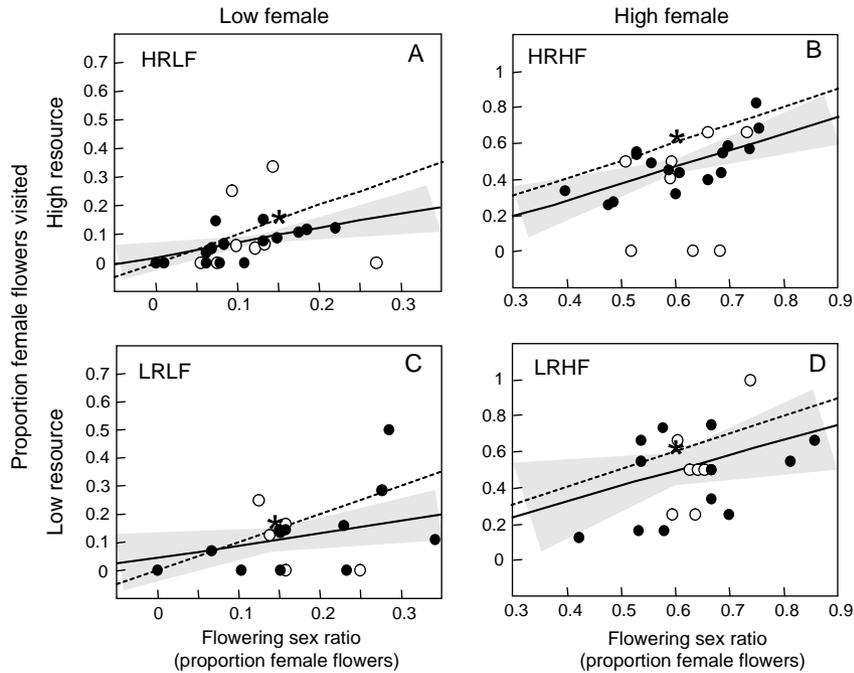


Figure 2. Relations between flowering sex ratios and sex-biased insect visitation in four combinations of resources (high vs low) and plant sex ratios (60% female plants vs 15% female plants; asterisks). Each point represents the mean proportion female flowers open and mean proportion female flowers visited during a single observation period in 2001 (white circles; $n = 10$ per treatment) and 2002 (black circles; $n = 16$ per treatment). Solid gray lines indicate plant sex ratios; dashed lines indicate 1:1 relations; solid black lines show best-fit; grey shading delimits 95% confidence intervals of the regression lines. HR=high resource, LR=low resource, HF=high (60%) female plants, LF=low (15%) female plants. Note the different scales on both axes for each sex-ratio treatment.

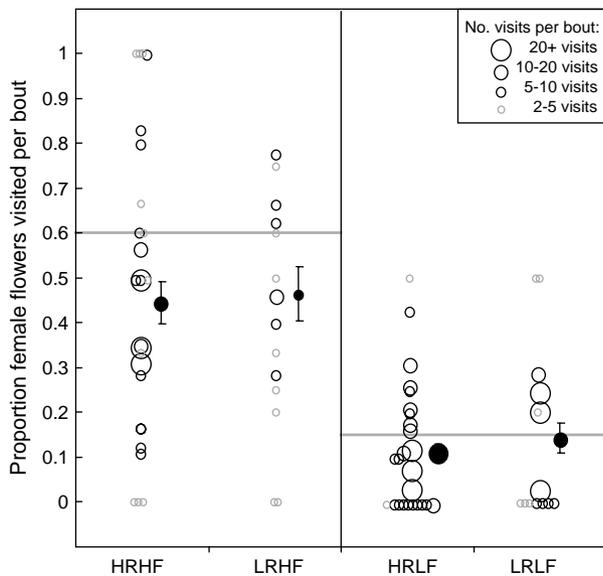


Figure 3. Proportion of visits to female flowers within bouts by individual insects to experimental arrays with varying resources (HR high, LR low) and sex ratios (HF 60% female plants, LF 15% female plants; grey horizontal lines). The position of each data point along the ordinate represents the proportion of female flowers visited during a visitation bout. Horizontal grey lines indicate the proportion of female plants in each array. Open circles show data for each recorded bout; filled circles are means ± 1 SE. The diameter of the circles assigns each to a category based on its length in number of visits (see legend inset).

(sex ratio \times insect type: $\chi^2 = 6.9$, $DF = 1$, $p = 0.008$). Bees visited significantly fewer female flowers per bout than expected (based on flowering sex ratio) in the HF gardens ($42.9\% \pm 0.05$ visits to females per bout) while flies discriminated against female flowers in the LF gardens ($12.2\% \pm 0.06$ visits to females per bout). Single-sex bouts to hermaphrodites occurred in all arrays, but we only observed all-female bouts in HRHF, and none of the bouts we observed in LF included more than 50% visits to female flowers (Fig. 3).

Consequences of visitation bias for pollen limitation

We found no evidence for pollen limitation of seed set in hermaphrodites (all $p > 0.50$ from ANOVAs and paired t-tests). Among females, seed set was significantly pollen-limited in three of the four treatments in 2001 (asterisks in Fig. 4), but seed set was not pollen-limited in HRLF in 2001 or any array in 2002. In 2001, seed set was less pollen-limited in HR relative to LR (resources: $F = 13.3$, $p < 0.0005$), particularly in the two LF gardens (resource \times sex ratio: $F = 17.6$, $p < 0.0001$). There was no significant difference in pollen limitation between sex ratio treatments ($F = 0.04$, $p = 0.80$). Contrary to our initial expectations, females were the most severely pollen limited in LRLF, and there was no difference in mean pollen limitation between LRHF and HRHF. Open-pollinated plants set 15% fewer seeds than hand-pollinated plants in the 2001 HRHF and LRHF gardens, and set 60% fewer seeds in the LRLF garden.

We found no relationship between pollen limitation of female seed set and actual sex ratio ($r = -0.16$, $p > 0.69$), nor flowering sex ratio ($r = -0.12$, $p > 0.77$), nor estimates of mean pollen:ovule ratios (based on direct counts of pollen and ovules per flower on each plant; $r = 0.02$, $p > 0.95$; data not shown). In contrast, pollen limitation of females was better explained by patterns of insect visitation. Mean visitation rates per plant and the ratio of visitation to each sex (F vs H) each explained part of the variation in pollen limitation of females' seed set (Fig. 4). Females were only significantly pollen limited if they were in patches where plants received less than one visit per flower per hour on average; none of the gardens with mean visitation rates above one showed any evidence of pollen limitation (Fig. 4A). However, mean visitation rates did not explain the substantial variation in pollen limitation in 2001 ($r = -0.72$,

$p > 0.25$); that is, it did not explain why females in LRLF had dramatically lower seed set compared to females in HF arrays, despite receiving similar visits per flower per hour. The relative visitation rate to each sex was positively correlated with levels of pollen limitation in both years and significantly so in one year (2001: $r = 0.91$, $p = 0.09$; 2002: $r = 0.96$, $p = 0.036$). Although plant-level visitation rates were never female-biased (i.e. > 1), pollen limitation did increase as females received higher rates of visitation relative to hermaphrodites (Fig. 4B).

Discussion

Our data in *Fragaria virginiana* indicate that resource availability and context-dependent pollination can both complicate predictions about how population sex ratios contribute to frequency-dependent reproductive success. First, resource treatment significantly affected daily flower production and visitation rate per plant, while both resource and sex-ratio treatments contributed to biased flowering sex ratios. Second, low resource conditions significantly increased the proportion of available female flowers, particularly where female plants were in the minority. Third, insect visitors were biased against females in all treatments, whereas flowering sex ratios were sometimes less female-biased than expected. Fourth, sex-biased pollinator foraging explained patterns of pollen limitation better than flowering sex ratios.

Although some of our results met our initial expectations, e.g. resources increased daily flower production, and visitors preferred hermaphrodite flowers, we did not expect females in the LF treatments to exhibit more pollen limitation than the HF. Only the combination of low total visitation and female-biased relative visitation rate accounted for severe pollen limitation in LRLF females in 2001. Below, we discuss these results and their potential to explain why only HRLF and LRHF populations are found in nature.

Resource availability contributes to sex-ratio bias

In natural populations of many gynodioecious species, poor resource conditions appear to favor the maintenance of female plants (reviewed by Ashman 2006). Several authors have suggested that harsh environments may either support different pollinator fauna (Ganders 1978, Delph 1990, Weller et al. 1990) or alter plant phenotype in ways that change mating system (Case and Barrett 2004, Vaughton and Ramsey 2004), pollen movement or mode of selfing. To date, our study is the first to examine these possibilities using an experimental rather than a correlative approach. It is especially important to examine the direct effects of abiotic stress on the details of pollination if we are to understand why empirical studies do not consistently support prediction of insufficient pollination when females are at high frequency (reviewed by Shykoff et al. 2003, Knight et al. 2005, Ashman 2006).

In our experiment, populations having the same proportion of female plants appeared more female under LR conditions. High resources altered the flowering sex ratio

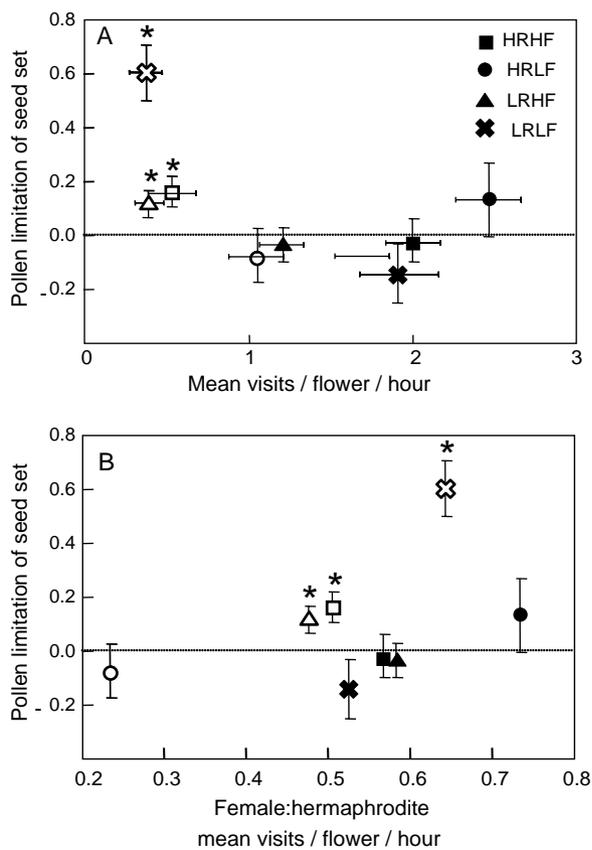


Figure 4. Effect of visitation rates on pollen limitation of female seed set in four experimental arrays varying in resource availability (HR vs LR) and plant sex ratio (HF vs LF) in 2001 (open symbols) and 2002 (closed symbols). The number of visits per flower per hour in each observation period was averaged to produce a single visitation rate for each plant. These values were then averaged among all plants within a garden (A) or averaged by sex within gardens (B) and plotted against mean pollen limitation per plant (see Methods text for calculation of pollen limitation). Asterisks indicate means for pollen limitation that are significantly different from zero based on t-tests ($\alpha = 0.05$). In panel (B), a ratio of one means that females received the same numbers of visits per flower per hour as hermaphrodites, while a value of 0.5 indicates that the average female received half the visitation rate of the average hermaphrodite.

because hermaphrodites increased daily flower production more than females in response to resource supplementation. Adding perfect flowers to a population's daily display has much less of an effect on the pollen:ovule ratio than adding pistillate flowers. Each pistillate flower substantially decreases the pollen:ovule ratio. In our study, we show that female-biased flowering sex ratios can also increase pollinator discrimination against females. As we discuss below, the likely outcome of both of these effects is pollen limitation of females.

Flowering sex ratio had a direct effect on visitation bias. Pollinators more strongly discriminated against female flowers as they became more frequent. This is evident in significantly sex-biased foraging in HF, and in the increasing bias of insect visitors against female flowers in LF. Pollinator visitation patterns to individual plants may vary with the overall quality of the patch. For example, Robertson et al. (1999) found that pollinators discriminated against plants producing low-quality pollen only when they occurred in patches that were dominated by low-quality pollen producers, but not when they were in patches of high-quality pollen producers. They suggested that the level of discrimination exerted by pollinators was likely context-dependent, and that lower-quality phenotypes (in our case, non-pollen-producing females) may be visited more frequently when they are in the minority (in our case, LF). Interestingly, the bias against females in our experiment was evident at the patch level (Fig. 1), but not when we looked at visitation rates to individual plants (Fig. 4). Eckhart et al. (2006) report a similar difference in the 'scale' of pollinator discrimination to two color morphs of *Clarkia*. They detected frequency-dependent preferences only at the level of experimental arrays, and suggested that might reflect within-patch foraging decisions, specifically a tendency to visit aggregates of a single type of flower or multiple flowers on a given plant of a particular morph. In our study, this phenomenon might account for a low proportion of female flowers visited at the patch level but a high relative visitation rate to females at the plant level (e.g. LRLF in 2001). Regardless, it is visitation at the plant level that likely contributes most to patterns of pollen limitation.

Sex-biased visitation explains frequency-dependent pollen limitation

The pattern of variation among treatments in the level of pollen limitation did not match our initial expectations. Given that pollen limitation of females should be negative frequency dependent in all contexts, our finding of pollen limitation only in 2001, and it being most severe in the LRLF context, was not easy to explain. This result is inconsistent with pollen limitation being caused by a low frequency of compatible mates. Studies in a variety of plant breeding systems have shown that fecundity is affected by local mate availability (McCauley and Brock 1998, Stehlik et al. 2006, Busch and Schoen 2008). These studies recognized that the effects of mate limitation on fecundity may be a general feature of the ecology of mating systems, but that the strength of these effects can also be context dependent (Stehlik et al. 2006). In particular, studies should focus on the activity of pollinators in conjunction

with estimating the frequency of compatible mates (Busch and Schoen 2008), as pollinators regulate access to mates. Although this seems obvious, it has never been tested experimentally as an explanation for the correlation between sex ratio and resource availability commonly reported in gynodioecious plants (Ashman 2006).

When we compared pollen limitation to visitation data, rather than plant or floral sex ratios, a clear pattern emerged. Mean visitation per flower per hour among observation days represents the likelihood of visits to each flower produced by a given plant at any point during the season. Low values should increase pollen limitation if the cause of pollen limitation is too few visits. We found that females were only pollen limited in contexts where all plants received, on average, less than 1 visit per flower per hour. This explains why only 2001 plants were pollen limited – plants in 2002 received an average of 1–3 visits per flower per hour. It does not explain why similar (low) overall visitation rates to HRHF, LRHF, and LRLF resulted in very different levels of pollen limitation in 2001.

The second potential cause of pollen limitation is too little pollen per visit. With respect to females, this should relate to the relative number of visits made to each sex, as pollinators can only pick up pollen by visiting hermaphrodites. If hermaphrodites receive fewer visits, then pollinators may carry smaller pollen loads and deposit less pollen per visit onto stigmas. When we compared relative visitation rates per flower per hour to females versus hermaphrodites in each garden, we found positive relationships to pollen limitation in both study years. This means that if the average female received similar levels of visitation compared to the average hermaphrodite (e.g. LRLF 2001 in Fig. 4), those females were more likely to be pollen limited than if hermaphrodites received twice the number of visits as the average female. This suggests that, at the low levels of visitation we observed in 2001 (less than one visit per flower per hour), multiple visits to hermaphrodites for each visit to a female increased pollen loads per visit and reduced pollen limitation. Relative visitation rate may have had less of an effect on pollen limitation in 2002 because overall visitation rates were higher.

One recent study taking a similar dynamic approach found no evidence of frequency-dependent pollen limitation in females of *Kallstroemia grandiflora* (Zygophyllaceae), even though pollinators were biased against visiting females, and delivered less pollen to female flowers (Cuevas et al. 2008). Their measure of relative visitation rate is equivalent to our measure of sex-biased insect visitation at the patch level, and in our study we reached the same conclusion – no relationship between patch-level visitation bias and pollen limitation of females. It was only when we analyzed visitation bias at the plant level that we found evidence for increased pollen limitation with increasingly female-biased visitation. Low resource availability increased pollen limitation in 2001 through an indirect effect on visitation bias, mediated by female-biased patch-level flowering sex ratios. We would not have understood this pattern if we had not assessed the potential for sex-ratio bias at both the garden level (which affects the behavior of pollinators) and the plant level (which is important for understanding natural selection) simultaneously. The value of this dual

perspective should be particularly relevant to future studies of frequency-dependent fitness in animal-pollinated plants.

Linking our findings to the gradient between LRHF and HRLF in nature

Our original motivation for this study was to understand the causes of sex ratio variation in gynodioecious plants, particularly why females become more common under stressful conditions in nature (reviewed by Ashman 2006). The prediction that pollen limitation should reduce the fecundity of females when they are common should not necessarily be resource dependent – it does not explain how high-female frequencies are maintained under low-resource but not high-resource conditions.

We have shown that resource availability can significantly bias sex ratios in *F. virginiana* through its effects on both daily flower production and sex discrimination by pollinators. Because we conducted this study in experimental gardens, these effects are not confounded by variation in plant density or microsite variation in soil conditions. HR conditions significantly reduced apparent female frequency compared to LR, particularly in the LF gardens. In addition, greater discrimination against females by pollinators under HR compared to LR conditions may contribute to the maintenance of low female frequencies under HR in nature. If HRLF populations shifted to LR conditions, they may be converted to LRHF populations over time, largely because of resource effects on floral sex ratios and visitation bias.

Pollen limitation is not an inevitable outcome of being surrounded by few compatible mates, as is often predicted, but is likely to be context-dependent (Shykoff et al. 2003, Knight et al. 2005, Busch and Schoen 2008). Low resource availability is frequently associated with high female frequency in gynodioecious plants, and has been assumed to exert its influence primarily through resource-limitation of reproductive allocation and fecundity (reviewed by Ashman 2006), rather than directly affecting the likelihood of pollen limitation. Detailed studies of how pollinators respond to resources directly, and indirectly to changes in reproductive allocation by plants under varying resource conditions, will continue to increase our understanding of sex-ratio evolution in gynodioecious plants, and the role of pollen and mate limitation in the ecology and evolution of plant mating systems (Busch and Schoen 2008).

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Supplementary material (available online as Appendix O17520 at www.ekol.lu.se/appendix). Appendix 1. Appendix 2.