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Gynodioecy and Biotic Interactions: Plant Traits, Insect Preferences, and Population-Level Consequences

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GYNODIOECY AND BIOTIC INTERACTIONS: PLANT TRAITS, INSECT PREFERENCES, AND POPULATION-LEVEL CONSEQUENCES

A Dissertation Presented

by

LAURA A. D. DOUBLEDAY

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University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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Organismic and Evolutionary Biology/Entomology

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EPIGRAPH

“They come in airy flotillas
on each stem, little flower-
blimps, propellers
of petals at their back ends,”

– Fleda Brown, in her poem Bladder Campion (*Poetry*, November 2005)

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ABSTRACT

GYNODIOECY AND BIOTIC INTERACTIONS: PLANT TRAITS, INSECT PREFERENCES, AND POPULATION-LEVEL CONSEQUENCES

MAY 2018

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In species with distinct sexes, differences between the sexes often affect interspecific interactions. In gynodioecious flowering plants, where individuals are female or hermaphrodite, both pollinators and herbivores tend to prefer hermaphrodites. Because pollinators and herbivores affect plant fitness, their preferences have consequences for plant mating patterns, natural selection on mating-related traits, and plant breeding system evolution. Being sessile, the spatial arrangement of females and hermaphrodites in gynodioecious plant populations alters conspecific density and sex ratio locally, which can also have important fitness effects.

My dissertation combines observational studies in natural *Silene vulgaris* populations and simulation modeling to address questions about how females and hermaphrodites experience intraspecific and interspecific interactions, with consequences for reproductive success, selection on traits, and population sex ratio evolution. Chapter 1 is an introduction. Chapter 2 addresses effects of plant sex and floral and vegetative traits on a recently described interaction between *S. vulgaris* and *Hadena ectypa*, a moth that pollinates plants but also deposits eggs in flowers with developing larvae feeding on plant reproductive tissues. Moth oviposition was hermaphrodite-biased and associated with plants having deeper flowers and more stems. However, moth oviposition had limited fitness consequences for host plants, as plants that received moth eggs lost relatively few fruits to predation, receiving eggs did not affect fruit production at the plant level, and oviposition was not associated with enhanced pollination. Chapter 3 demonstrates scale-dependent effects of conspecific density and sex ratio on reproduction and phenotypic selection in *S. vulgaris*. Fine scale density variation had opposite effects on reproduction in females and hermaphrodites, both sexes experienced enhanced reproductive success with increasing hermaphrodite frequency at high densities, and females and hermaphrodites experienced different effects of density on phenotypic selection. Chapter 4 uses simulation models to assess how pollinator sex bias intensity affects female maintenance and sex ratio evolution in gynodioecious plant populations, finding that even small preferences for hermaphrodites can lead to the loss of females from populations. Taken together, my work sheds new light on the patterns and processes that affect reproduction, selection on floral and vegetative traits, and the maintenance of females in gynodioecious plant populations.

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CHAPTER 1

INTRODUCTION

Most angiosperms are hermaphrodite, with both male (anthers and filaments) and female (stigmas, styles, and ovaries) sex organs in every flower they produce (Barrett 2002). The relative proximity of male and female reproductive structures affects mating patterns, especially the likelihood of self-fertilization (Barrett 2002). Self-fertilization can be beneficial in certain circumstances, like when individuals have genotypes that are extremely well adapted to stable environmental conditions, when pollinators are rare, or when colonizing new habitats with few potential mating partners, but selfing can also be detrimental as it can lead to inbreeding depression.

Angiosperms display a fascinating array of floral adaptations that reduce the likelihood of self-fertilization. Some angiosperms separate male and female function temporally, like protandrous flowers that begin their lives in male phase, shedding pollen before the female structures become receptive to pollen receipt, and protogynous flowers that start out in female phase before transitioning to male phase. Other angiosperms separate male and female sex functions spatially. Simply increasing the distance between the male and female structures (herkogamy) reduces the likelihood of self-pollination, but many plant species go even farther than this and place male and female sex organs in separate flowers or inflorescences (monoecy) or on separate individuals (dioecy). In dioecy, where individuals produce flowers of only one sex type, self-fertilization is impossible.

Dioecy is a relatively rare breeding system, found in 6% of angiosperm species (Renner and Ricklefs 1995), but it has evolved in at least 38% of angiosperm families

(Renner and Ricklefs 1995), begging the question of why it arises frequently but remains rare. Dioecy typically evolves from an ancestral hermaphrodite breeding system, and there are a number of pathways by which this can occur (Barrett 2002, Dufay et al. 2014). The most common intermediate stage between hermaphroditism and dioecy is gynodioecy, where individuals are either female or hermaphrodite (Barrett 2002). For gynodioecy to arise from hermaphroditism, a mutation causing male sterility is required, which creates females (Charlesworth 1999). Once females exist alongside hermaphrodites, those females must produce more seeds than hermaphrodites in order to be maintained, rather than having the gynodioecious population revert to hermaphroditism (Charlesworth 1999). Gynodioecy can be a stable breeding system, but if a gynodioecious population is to shift towards dioecy, another mutation is required, this time creating pure males by eliminating female function in some hermaphrodites (Charlesworth 1999). If pure males sire more offspring than hermaphrodites, hermaphrodites should not be maintained in the population, which will reach dioecy (Charlesworth 1999).

Introducing a second sex morph into a hermaphroditic population, as when females appear alongside hermaphrodites in gynodioecy, fundamentally changes interactions between individuals because females require pollen from hermaphrodites in order to produce seeds, but hermaphrodites have both male and female functions. Because of this, conspecific density and sex ratio are expected to have substantial effects on reproduction in focal females and hermaphrodites in gynodioecious populations (McCauley and Brock 1998, Gunton and Kunin 2009). When females grow in patches with high frequencies of hermaphrodites, they have access to more potential pollen donors and are also exposed to less competition from other pollen-seeking females. When hermaphrodites grow in patches with high hermaphrodite frequencies, they experience more competition to fertilize available ovules, but are also exposed to more potential pollen donors. Growing in high densities introduces more competition among individuals for nutrients and resources, but can be beneficial to both females and hermaphrodites, as pollinators are typically more attracted to denser patches of plants (Kunin 2012).

Having separate sexes also alters interactions between plants and other species, like pollinators that mediate outcrossing and herbivores that feed on plants. Pollinator and herbivore preference for the pollen-bearing sex (hermaphrodites in gynodioecy and males in dioecy) relative to females has been demonstrated in several gynodioecious and dioecious taxa (Bell 1985, Ågren et al. 1999, Ashman 2000, 2002, Asikainen and Mutikainen 2005, Varga and Kytöviita 2010, Doubleday and Adler 2017, Stone and Olson 2018). In addition to only one sex morph producing pollen, which many flower visitors seek, there is often sexual dimorphism in other traits between the pollen-bearing sex and females, such that the pollen-bearing sex often has a larger floral display (Eckhart 1999). Sexual dimorphism in attractive traits like floral display size could explain the tendency for pollinators and herbivores to prefer the pollen-bearing sex.

My dissertation focuses on interspecific and intraspecific interactions in gynodioecious plant populations, seeking to understand the causes and consequences of sex-biased interspecific interactions and how the local social context affects reproduction and phenotypic selection in focal females and hermaphrodites. I present three data chapters (chapters 2–4), which combine empirical and theoretical approaches.

In chapter 2, I examine whether a recently established interaction between gynodioecious *Silene vulgaris* plants and *Hadena ectypa* moths, which pollinate but also lay eggs in flowers, shows evidence of sex bias, what plant traits correlate with oviposition, and whether there are positive or negative fitness consequences associated with egg receipt for host plants. To do this, I use observational data collected in 2014 and 2015 from six natural *Silene vulgaris* populations in the northeastern US. I find that hermaphrodites are more likely to receive eggs than females, which can be explained by hermaphrodites having deeper flowers than females and more stems than females. Plants that received eggs lost more fruits to obvious predation than plants that did not receive eggs, but fitness costs were small and there were no apparent fitness benefits associated with receiving eggs. The moth's ancestral host plant (*Silene stellata*) is hermaphrodite and, taken together, these results suggest that sex bias does not require a long coevolutionary history between the plant and the insect. Rather, recently shifting from a hermaphrodite host plant to a gynodioecious host plant may result in sex bias if sexual dimorphism exists in traits that mediated the interaction on the native host.

In chapter 3, I explore the effects of sex ratio and conspecific density at three spatial scales on reproduction and phenotypic selection in focal female and hermaphrodite *S. vulgaris* plants. For this study, I mapped and sexed all of the individuals in a 45 x 10 m section of an *S. vulgaris* population in Sheffield, MA, USA. I assigned 57 of these plants to be focal individuals and calculated the frequency of hermaphrodites and density of conspecifics neighboring each focal plant at three spatial scales: within 0.5 m, 0.5 – 2 m, and 2 – 5 m. Density within 0.5 m decreased fruit production by focal females, but increased fruit production by focal hermaphrodites. An interaction between density and sex ratio within 0.5 m revealed a positive relationship between hermaphrodite frequency and seeds per fruit for females and hermaphrodites growing in high-density patches. I also found evidence of positive directional selection on flower width in females and stem number in hermaphrodites. In females, selection on flower width was modified by density within 0.5 m, such that females in high-density patches had a positive relationship between seeds per fruit and flower width, but no fitness benefit associated with wider flowers for females growing in low-density patches. For hermaphrodites, selection on stem number was modified by density within 0.5 m, such that increasing stem number conferred fitness benefits to hermaphrodites growing in low-density patches, but not to those growing in high-density patches. These results highlight the importance of considering effects of the local social context on reproduction and phenotypic selection in focal individuals, as well as the need to choose the appropriate spatial scale at which to conduct research.

In chapter 4, I use a computer simulation model to address how the intensity of pollinator sex bias affects the evolution of population sex ratios and the maintenance of females in gynodioecious plant populations. Pollinator sex bias intensity, female fertility advantage relative to hermaphrodites, and female and hermaphrodite progeny sex ratios interact to predict the frequency of hermaphrodites at equilibrium and whether females will be maintained in populations. My results indicate that small changes in pollinator sex bias intensity can shift equilibrium sex ratios and destabilize gynodioecy, but other factors can compensate and allow females to be maintained.

Together, my work speaks to three areas ripe for further research. First, continued study of sex-biased interspecific interactions will allow us to develop a richer understanding of the circumstances that favor shifts between hermaphroditism, gynodioecy, and dioecy as well as how sexual dimorphism in plant traits evolves. In particular, we currently know very little about the intensity of pollinator and herbivore sex bias in gynodioecious plants. Documenting patterns of sex-biased interactions will allow us to develop clearer pictures of the selective forces acting on females and hermaphrodites. Second, being spatially explicit will help us develop a richer understanding of how organisms experience their environments, allowing us to refine our understanding of how the ecological context affects reproduction and selection on traits in focal individuals. Finally, the results presented in this dissertation and in similar studies, especially those concerning the effects of the local social context and the traits important in mediating interactions with pollinators and herbivores, could have agricultural applications. Several crop species, like kiwis, pistachios, persimmons, cannabis, hops, and asparagus, have separate sexes. Understanding how density and sex ratio affect focal individual reproduction and attraction of pollinators and herbivores could allow us to develop planting strategies for farmers that would maximize yields and minimize losses to pests.

CHAPTER 2

SEX-BIASED OVIPOSITION BY A NURSERY POLLINATOR ON A GYNODIOECIOUS HOST PLANT: IMPLICATIONS FOR BREEDING SYSTEM EVOLUTION AND EVOLUTION OF MUTUALISM

Abstract

Dioecy, a breeding system where individual plants are exclusively male or female, has evolved repeatedly. Extensive theory describes when dioecy should arise from hermaphroditism, frequently through gynodioecy, where females and hermaphrodites coexist, and when gynodioecy should be stable. Both pollinators and herbivores often prefer the pollen-bearing sex, with sex-specific fitness effects that can affect breeding system evolution.

Nursery pollination, where adult insects pollinate flowers but their larvae feed on plant reproductive tissues, is a model for understanding mutualism evolution but could also yield insights into plant breeding system evolution because host plants often have separate sexes and insects often prefer to oviposit on one plant sex. We studied a recently established nursery pollination interaction between native *Hadena ectypa* moths and introduced gynodioecious *Silene vulgaris* plants in North America to assess whether oviposition was biased towards females or hermaphrodites, which traits were associated with oviposition, and the effect of oviposition on host plant fitness.

Oviposition was hermaphrodite-biased and associated with deeper flowers and more stems. Sexual dimorphism in flower depth, a trait also associated with oviposition on the native host plant (*Silene stellata*), explained the hermaphrodite bias. Egg-receiving plants experienced more fruit predation than plants that received no eggs, but relatively few fruits were lost, and egg receipt did not significantly alter total fruit production at the plant level. Oviposition did not enhance pollination; egg-receiving flowers usually failed to expand and produce seeds. Together, our results suggest that *H. ectypa* oviposition does not exert a large fitness cost on host plants, sex-biased interactions can emerge from preferences developed on a hermaphroditic host species, and new nursery pollination interactions can begin as negative or neutral rather than as mutualistic for the plant.

Key words: gynodioecy, *Hadena ectypa*, nursery pollination, plant breeding systems, sex-biased interactions, *Silene vulgaris*

Introduction

Flowering plants have diverse reproductive strategies. Although most are hermaphroditic, producing flowers that contain both male and female reproductive structures, many angiosperms have adaptations that reduce the likelihood of self-fertilization. Plants commonly separate female and male sex functions in time (e.g. protandry) and, less commonly, in space (e.g. dioecy, monoecy). In dioecy, the most extreme form of spatial sex separation, individual plants produce only female or only male flowers, making self-fertilization impossible.

One of the most common evolutionary pathways from hermaphroditism to dioecy involves gynodioecy, where female and hermaphrodite individuals coexist, as an intermediate stage (Charlesworth, 1999). For gynodioecy to arise from hermaphroditism, first a mutation causing male sterility must occur in a hermaphroditic population, creating female individuals (Charlesworth, 1999). If females have a large enough seed production advantage over hermaphrodites, they will persist, stabilizing gynodioecy.

The genetics of sex determination affect the conditions that will determine whether females persist among hermaphrodites and what female frequencies will be stable. Sex can be determined by nuclear male sterility alleles or interactions between nuclear and mitochondrial alleles (hereafter "cytonuclear interactions"), where mitochondrial alleles cause male sterility (creating females) but nuclear alleles restore male function to hermaphrodites (Lewis, 1941; Lloyd, 1976; Saumitou-Laprade *et al.*, 1994; Bailey & Delph, 2007). When plant sex is under nuclear control, females must produce at least twice as many seeds as hermaphrodites to persist (Lewis, 1941), but when sex determination is cytonuclear, the female fertility advantage required over hermaphrodites is smaller (Charlesworth, 1981). For example, in specific theoretical conditions females were maintained when they produced only six percent more seeds than hermaphrodites (Charlesworth, 1981). Female reproductive advantage over hermaphrodites is common in gynodioecious species, but the magnitude varies among species as well as sometimes varying among populations or with female frequency within single species (Dufay & Billard, 2012). Female advantage can be expressed through sex differences in fruit number, fruit set (fruits/flowers), seed set (seeds/ovules), seeds per fruit, seeds per plant, seed mass or size, and/or germination rate (Dufay & Billard, 2012). Because cytonuclear gynodioecy can be maintained with a small female seed production advantage, if the initial relative advantage of females compared to hermaphrodites is small, then a minor reduction in female fitness due to biotic or abiotic factors could shift relative fitness below the 1:1 ratio needed to maintain the stability of gynodioecy. Thus, depending on the relative fitness of females and hermaphrodites, small fitness shifts due to abiotic or biotic factors could have large evolutionary implications in systems with cytonuclear gynodioecy.

In dioecious and gynodioecious plants, phenotypic differences between the sexes often affect interactions with pollinators and herbivores (Ashman & Stanton, 1991; Ashman, 2002; Barrett & Hough, 2013). For example, pollinators are frequently more attracted (i.e. make more or longer-lasting visits) to pollen-bearing plants because of larger flowers or floral displays (e.g. Ashman 2000, Williams et al. 2000, Asikainen and Mutikainen 2005). Herbivores also prefer the pollen-bearing sex. In 17 of 21 dioecious species from 15 families, male plants suffered significantly more herbivory than females (Ågren *et al.*, 1999) and damage was biased towards hermaphrodites, rather than females, across several gynodioecious taxa (Ashman, 2002).

Ashman (2002) has demonstrated theoretically that sex-biased damage can promote the evolution of gynodioecy and dioecy from hermaphroditism, especially when the tissues consumed are resource sinks (flowers, fruits, and seeds) rather than sources (leaves). Although Ashman (2002) does not distinguish between nuclear and cytonuclear gynodioecy, she considers the effects of sex-biased damage on seed production, pollen fitness, and hermaphrodite mating system parameters, which could be important in both nuclear and cytonuclear gynodioecy. Because damage to flowers and fruits directly affects plant reproduction, it is likely to have a stronger effect on female and hermaphrodite fitness (both in terms of pollen and seeds) than leaf damage (Ashman, 2002). Because of their direct and often extreme effects on plant reproduction, nursery pollination interactions (also known as brood pollination), where an insect species pollinates but also lays eggs in flowers and larvae feed on the plant's reproductive tissues, are good candidates for improving our understanding of how sex-biased interactions affect the relative fitness of females and hermaphrodites and the maintenance of gynodioecy.

In this study, we evaluated sex bias in a recently established nursery pollination interaction between native *Hadena ectypa* (Morrison) moths and their introduced gynodioecious host plant, *Silene vulgaris* (Moench) Garcke. We addressed the following questions:

1. Is there sex bias in oviposition and damage to plants among and within populations?
2. What plant traits are associated with oviposition?
3. How does receiving eggs affect female and hermaphrodite host plant fruit and seed production?

Methods

Study system

Species in the plant genus *Silene* (Caryophyllaceae) engage in diverse nursery pollination interactions, with outcomes ranging from negative to positive with moths from two genera (*Hadena* [Noctuidae] and *Perizoma* [Geometridae]) (Kephart *et al.*, 2006). *Hadena* moths can have significant fitness effects on their *Silene* host plants, with *Hadena rivularis* (F.) damaging up to 100% of the available ovules in some European populations of *Silene latifolia* Poir. (Wolfe, 2002). *Hadena ectypa*, a species native to North America, was discovered in western Massachusetts in 2002 (Nelson, 2012). This was the first record of the moth in New England, as its range had previously been thought to stretch no further north or east than southeastern New York state (Nelson, 2012). *Silene stellata* (L.) W. T. Aiton, a hermaphroditic species native to North America, is the known host plant for *H. ectypa* (Nelson, 2012), but *S. stellata* does not occur in Massachusetts (Cullina *et al.*, 2011), with the northern edge of its range historically occurring in Connecticut (Nelson, 2012). Since at least 2002, *Hadena ectypa* has been using *Silene vulgaris* (Figure 2.1) as its host in western Massachusetts (Nelson, 2012). *Silene vulgaris* was introduced from Europe around 200 years ago and is now widely naturalized throughout North America, including in the southeastern US where *S. stellata* also occurs (Nelson, 2012). *Silene vulgaris* is gynodioecious with cytonuclear sex determination (Charlesworth & Laporte, 1998) and has nursery pollination interactions with several *Hadena* moth species in Europe (Pettersson, 1991b).

Sampling

To assess sex bias in *H. ectypa* oviposition on *S. vulgaris*, we surveyed six natural populations in 2014 (Table S1), examining all of the flowers on one *S. vulgaris* stem every 5m along a transect at each site. We examined single flowering stems because individual plants can have hundreds of stems and plants grew densely at our study sites, making it difficult to identify which stems belonged to particular individuals. Transects traversed populations and ranged from 100–600 m in length. At each point along the transect we examined the nearest stem bearing an open flower. For each stem, we recorded the sex of the flowers (female or hermaphrodite), the number of open flowers, and the number of *H. ectypa* eggs and caterpillars present. Late-instar *H. ectypa* caterpillars have a distinctive dorsal chevron pattern (Nelson, 2012) that allows them to be discerned from other species likely to occur in most of our study areas (M. W. Nelson, personal communication). As *Hadena capsularis* Guenée is known to occur in Vermont (M. W. Nelson, personal communication), it is possible that either or both *H. capsularis* or *H. ectypa* eggs and caterpillars were observed in our Vermont populations (VBE and VBR). Because we were simply interested in whether oviposition and different forms of damage were sex-biased in our multi-population surveys, rather than the effects or preferences of particular interacting species, the potential presence of *H. capsularis* in our Vermont populations does not affect our interpretation of the multi-population surveys. We also recorded whether each stem had leaf or flower damage, although for this damage we did not know herbivore or florivore identity.

To assess whether oviposition was associated with plant traits other than sex, we focused on our largest *S. vulgaris* population (MSH) in western Massachusetts in 2015 and monitored 80 females and 80 hermaphrodites across the flowering season, using whole plants rather than single stems. We chose these focal plants haphazardly based on having at least one open flower at the time of selection (June 22 – July 6, 2015). We checked each plant for eggs and late-instar caterpillars four times over the flowering season (June 22 – July 6, July 20 – 22, July 31 – August 6, and August 17 – 19) and measured plant and floral traits that might influence oviposition (Kula *et al.*, 2013 and references therein): number of open flowers, plant size (projected area, number of stems, and height of tallest flower), and flower size (floral face width and flower depth; Figure S1). Projected area was calculated by multiplying plant length and width obtained by measuring the plant from above along its longest axis for length and at 90 degrees from the length axis for width. For plant-level floral traits, we averaged the mean of the measurements from two flowers to obtain mean floral trait measurements for each plant. We also assessed damage to floral tissues at the 1st and 3rd census dates by examining plants for bud, calyx, petal, and ovary damage.

To assess the effect of within-plant floral variation on oviposition decisions, we collected detailed measurements of floral traits for age-matched pairs of flowers on individual plants where one flower received an egg but the other did not at MSH in 2015. *Silene vulgaris* flowers progress through predictable stages of sex expression and maturity (Jolls *et al.*, 1994), so we used sex expression to assess the developmental stage of flowers. We assessed the egg-receiving flower's developmental stage and chose another flower on the same plant that most closely matched this stage, but contained no eggs, as the non-egg-receiving flower. We measured the width of the floral face, flower length, calyx width, calyx length, and the diameter of the floral tube opening (Figure S1) for the pairs of egg-receiving and non-egg-receiving flowers. A single observer made all of the measurements and each measurement was made twice. We averaged the two measurements to obtain a single measurement for each trait for each flower.

To determine the effect of oviposition on host plant reproduction, we counted the number of expanded and damaged fruits on each focal plant at the third census date. We counted fruits and assessed the number of predated fruits at this time because it appeared that most plants had finished flowering for the season. We observed new eggs on plants after the fruit count, but did not include these oviposition events in our analyses of traits affecting fruit production and predation. We also counted the number of seeds produced by the egg-receiving and non-egg-receiving flower pairs described above. To assess whether egg-receiving flowers produced more seeds than non-egg-receiving flowers, indicating that they were pollinated effectively, we also counted seeds produced by 10 additional flower pairs at MSH in 2016 from which we removed the egg from the egg-receiving flower and performed a sham egg removal from the non-egg-receiving flower. We removed the eggs from these egg-receiving flowers because developing larvae would consume fruits and seeds, precluding comparison of seed production. A single observer counted all the seeds.

Statistical analyses

We conducted all statistical analyses in R, version 3.3.1 (R Core Team, 2017). Several of our response variables were binary (i.e. whether plants received eggs or damage), for which we report 95% binomial confidence intervals for these response variables along with observed proportions of outcomes. We used the binom package (Dorai-Raj, 2014) to calculate binomial confidence intervals with the Pearson-Klopper exact method. Error bars for figures with binomial response variables are not equal in length above and below the observed proportion because binomial confidence intervals are not symmetric.

Sex-biased oviposition and damage

In testing for sex bias in oviposition and damage, our null hypothesis was that females and hermaphrodites would receive eggs or damage in proportion to the population sex ratio (at the individual, stem, or flower level, depending on the analysis). For example, in a population that was 10% female and 90% hermaphrodite with no sex bias, we would expect females to receive 10% of the eggs and hermaphrodites to receive 90% of the eggs. If oviposition were female-biased, we would expect females to receive significantly more than 10% of the eggs and if oviposition were hermaphrodite-biased, we would expect females to receive significantly less than 10% of the eggs.

We used binomial generalized linear models (GLMs) to test for sex bias in oviposition and damage. The sex term in the model estimates the likelihood of a female or a hermaphrodite receiving an egg. If the sex term is significant, it indicates that one sex is receiving eggs or damage significantly more often than expected based on the underlying sex ratio in the sample. For all GLMs, we used likelihood ratio (LR) tests to assess the significance of the sex term and other predictors of interest by comparing two GLMs that only differed in the presence of the predictor of interest. For our 2014 surveys, we used binomial GLMs to test the effect of plant sex, population, and a sex by population interaction on oviposition. For our 2015 monitoring study, we tested for sex bias in the likelihood of a plant ever receiving an egg using the same binomial GLM approach, with sex as the only predictor.

Because sex at the flower level, rather than the stem or plant level, could be more important to ovipositing insects, we also assessed whether oviposition was sex biased at the flower level for the 2014 multi-population dataset. We used both binomial glms (as above) and a permutation test for the flower-level analyses. To conduct the permutation test, we reshuffled whether each flower received an egg among all of the flowers within each population 10,000 times, calculated the number of hermaphrodites that had received eggs for each of those randomizations, and compared the actual number of hermaphrodite flowers that had received eggs to the distribution of simulated hermaphrodite egg receipt. We calculated the permutation *P*-value (two-tailed) as twice the number of simulated values that were more extreme than the observed value. We were unable to assess flower level sex bias in oviposition at MSH in 2015 because of our study design: we checked plants for eggs four times throughout the growing season, but only obtained a single flower count for each plant, and 20 of the 47 plants that received eggs did not have any open flowers at the time of the flower count.

Traits associated with oviposition

We used a binomial GLM to assess whether particular plant traits were associated with oviposition. We used all measured plant traits and plant sex as predictors. If plant sex were significant along with other plant traits, it would indicate that sexual dimorphism in unmeasured traits was involved in the observed sex bias. If sex were not significant, but other plant traits were, it would indicate that sexual dimorphism in the measured traits explained any observed sex bias. We tested the significance of each predictor using LR tests and took a backwards regression approach to model selection, removing predictor terms from the model one by one until we were left with a model including only the significant predictor variables.

We used paired t-tests to assess differences in traits and seed production in age-matched pairs of flowers on plants collected in 2015 where one flower received an egg and the other did not. For 10 additional age-matched pairs of flowers from 2016, we performed permutation tests, where we reshuffled the number of seeds produced randomly within each pair 10,000 times and took the differences between egg-receiving flowers and controls each time to obtain a distribution of differences against which to compare the difference between egg-receiving and control flowers that we actually observed. Our observed difference would be significantly different from 0 if less than 5%

of the randomized differences were more extreme than the observed difference. We performed permutation tests on number of seeds produced and fruit mass because of the small sample sizes.

Flower and leaf damage

We used binomial GLMs to test for sex bias in flower and leaf damage across populations where we observed *H. ectypa* eggs in 2014 and in bud, calyx, petal, and ovary damage in the MSH population twice in 2015.

Results

Hadena ectypa oviposition

We found eggs and caterpillars in five of the six populations in 2014, with eggs on 18–36% of stems surveyed (Table S1). Caterpillars were quite rare (Table S1), so we did not assess plant traits associated with their presence. In 2014, oviposition was hermaphrodite-biased at both stem ($LR\ X^2_1 = 9.72$, $P = 0.0018$; Figure 2.2) and flower levels ($LR\ X^2_1 = 4.90$, $P = 0.027$, randomization test $P = 0.016$) and oviposition frequency varied among populations ($LR\ X^2_4 = 12.70$, $P = 0.013$; Figure 2.2), but there was no interaction between plant sex and population ($LR\ X^2_4 = 4.46$, $P = 0.35$). Oviposition was also hermaphrodite-biased at the plant level in the MSH population in 2015 ($LR\ X^2_1 = 6.87$, $P = 0.0088$; Figure 2.3). However, when plants received eggs, there was no difference between the sexes in the number of eggs received in either year (2014: $LR\ X^2_1 = 1.38$, $P = 0.24$; 2015: $LR\ X^2_1 = 1.26$, $P = 0.26$), probably because moths usually deposited only one egg per stem (2014) or plant (2015) at a time (percent of observations with only one egg at a time: 71% in 2014 and 73% in 2015).

Some plant traits were associated with oviposition. In the 2015 study, plants with more stems ($LR\ X^2_1 = 5.61$, $P = 0.018$) and deeper flowers ($LR\ X^2_1 = 4.61$, $P = 0.032$) were more likely to receive eggs (Figure 2.4), but height, projected area, number of open flowers, flower width, and sex did not predict oviposition (Table S2). Within a plant, calyx width was the only measured trait that differed significantly between egg-receiving and non-egg-receiving flowers ($t_{35} = 3.15$, $P = 0.0033$), with egg-receiving flowers having wider calyces (mean ± 1SE: 8.16 ± 0.22mm) than non-egg-receiving flowers (7.66 ± 0.21mm) (Figure S2).

Hermaphrodites had significantly deeper flowers than females ($LR\ F_{1,\,126} = 60.76$, $P < 0.0001$; Figure 2.4A), but there was no difference between the sexes in number of stems ($LR\ F_{1,\,126} = 1.11$, $P = 0.29$; Figure 2.4B). Sexual differences in calyx width could also potentially explain the hermaphrodite-biased oviposition we observed. Because we only have calyx width measurements for egg-receiving plants from MSH in 2015, we were unable to directly assess the effect of sexual dimorphism in calyx width on oviposition among plants. However, we tested whether sexual dimorphism existed in calyx width among the egg-receiving plants on which we tracked the outcome of egg-receiving and non-egg-receiving flowers at MSH, and among 22 females and 18 hermaphrodites grown in a greenhouse from MSH-collected seed (see Appendix C for methods details). There was no sexual dimorphism in calyx width among either of these groups of plants (egg-receiving: $LR\ F_{1,\,34} = 1.28$, $P = 0.27$; greenhouse-grown: $LR\ F_{1,\,38} = 0.070$, $P = 0.79$).

Consequences of oviposition

Plant level

For the plants monitored at MSH in 2015, number of stems, height, number of flowers present at time of fruit count, and average flower depth predicted fruit production, but flower width, plant area, plant sex, and oviposition status did not (Table 2.1). Plants that received eggs at MSH in 2015 lost significantly more fruits to apparent *H. ectypa* caterpillar predation than plants that never received eggs ($LR\ F_{1,\,133} = 5.36$, $P = 0.022$) indicating a fitness cost associated with oviposition. There was also a significant effect of plant sex on fruit loss when oviposition status was accounted for ($LR\ F_{1,\,133} = 6.58$, $P = 0.011$), such that females lost more fruits than hermaphrodites. However, the sex effect was no longer significant ($LR\ F_{1,\,132} = 1.57$, $P = 0.21$) when a single extreme fruit loss value was excluded from the analysis, while the oviposition effect remained significant ($LR\ F_{1,\,132} = 6.75$, $P = 0.010$). The number of fruits lost was relatively small (mean ± 1SE: 3.85 ± 0.92 fruits for egg-receiving plants vs. 1.84 ± 0.32 for non-egg-receiving plants) compared to the total number of fruits plants produced (mean ± 1SE: 30.75 ± 3.03). Thus, the number of fruits lost to predation was apparently insufficient to affect total fruit production.

Flower level

In 2015, 61% of egg-receiving flowers and 39% of non-egg-receiving flowers failed to produce seeds. For 29 flower pairs where we were able to collect both flowers, neither flower made any seeds in 28% of the pairs, while both flowers made seeds in 31% of the cases. In pairs where both flowers made seeds, there was no difference in fruit mass ($t_8 = 0.61$, $P = 0.57$) or number of seeds produced ($t_8 = 0.10$, $P = 0.31$). A permutation test showed no significant difference ($P = 0.16$) in the number of seeds produced by 10 additional pairs of egg-receiving vs. non-egg-receiving flowers from which we removed eggs in 2016, although the tendency in our sample was for controls to produce seeds more frequently than egg-receiving flowers. There was also no difference in fruit mass ($P = 0.12$) between egg-receiving and non-egg-receiving flowers from which eggs had been removed.

For the 2015 flower pairs, when flowers produced seeds, there was no difference between the sexes in how many seeds were produced (non-egg-receiving flowers: $t_{18} = 0.76$, $P = 0.46$; egg-receiving flowers: $t_{11} = 0.63$, $P = 0.54$; Table S3). There were also no sex differences in the mass of fruits that produced at least one seed (non-egg-receiving flowers: $t_{18} = 1.5$, $P = 0.15$; egg-receiving flowers: $t_{11} = 1.46$, $P = 0.17$).

Flower and leaf damage

Flower damage occurred on 15–56% of stems at populations where we found *H. ectypa* eggs in 2014, while nearly 100% of stems displayed leaf damage (Figure S3), including in the population (NST) without *H. ectypa*. We included all sites surveyed in our analyses of sex-biased flower and leaf damage, and found that hermaphrodites were more likely than females to have flower damage ($LR\ F_{1,313} = 7.74$, $P = 0.0057$; Figure 2.5A), but there was no sex bias in leaf damage ($LR\ X_1^2 = 0.23$, $P = 0.63$; Figure 2.5B). The frequency of both types of damage varied significantly across populations (flower damage: $LR\ F_{1,317} = 3.24$, $P = 0.0072$; leaf damage: $LR\ X_1^2 = 46.26$, $P < 0.0001$).

In 2015, we examined sex bias in bud, calyx, petal, and ovary damage at MSH. Petal damage was hermaphrodite-biased in July ($LR\ X^2_1 = 7.74$, $P = 0.0054$; Figure 2.6A) and calyx damage was hermaphrodite-biased in August ($LR\ X^2_1 = 12.67$, $P = 0.00037$; Figure 2.6B). We found no evidence of sex bias in bud or ovary damage at either time (Table S4; Figure 2.6).

Discussion

We observed hermaphrodite-biased oviposition by *H. ectypa* moths on gynodioecious *S. vulgaris* host plants. Flower depth and number of stems predicted oviposition among plants, while within plants, flowers that received eggs had wider calyces than flowers that did not receive eggs. Plant sex was not a significant predictor of oviposition when other plant traits were included in the model, indicating that sex differences in traits included in the model, rather than sexual dimorphism in unmeasured traits, accounted for the observed sex bias in oviposition. Although plants that received eggs lost more fruits to damage, fruit loss was relatively small, resulting in no overall effect of oviposition on total fruit production. There was also no difference in fruit production or the number of seeds per fruit between females and hermaphrodites. Below, we discuss the implications of our results for understanding plant breeding systems, the evolution of mutualism, and moth oviposition preferences.

Sex-biased interactions and plant breeding systems

Our observations of hermaphrodite-biased oviposition and flower damage fit the general pattern seen across gynodioecious plant species (Ashman, 2002), but the consequences of hermaphrodite bias for breeding system evolution in our system are not entirely clear. Because females were less likely to receive eggs, we expected them to lose fewer fruits to *H. ectypa* predation than hermaphrodites, but the fruit loss was so minimal that there was no difference in total post-damage fruit production between the sexes. Plants grew close together at our field site and late-instar caterpillars are likely to move among plants to find enough young fruits to feed on as they complete development (Nelson, 2012), so it is possible that some oviposition on hermaphrodite hosts led to fruit losses by neighboring female plants.

We found no difference in the number of fruits or seeds per fruit produced by females and hermaphrodites, which was surprising because Taylor et al. (1999) found that *S. vulgaris* females produced significantly more fruits than hermaphrodites (but had no difference in flower production) in experimental populations and Olson et al. (2006) found that females produced more seeds per fruit than hermaphrodites in one of two natural North American *S. vulgaris* populations. However, another study (Dulberger & Horovitz, 1984) found no difference in number of seeds per fruit between females and hermaphrodites. Olson et al. (2006) and Taylor et al. (1999) both found that females had higher fruit set than hermaphrodites. We were unable to assess fruit set for our study plants because *S. vulgaris* continuously produces flowers and fruits over a period of several months, and monitoring all flowers and fruits produced was logistically impossible. Another caveat regarding our fruit production data is that because *S. vulgaris* is perennial, there are limitations of a single season of data, especially because there may be sex differences in longevity (Delph, 1999). However, our single-season data found surprisingly little difference in reproduction between the sexes, suggesting that our population might be close to the critical 1:1 threshold that is important for the maintenance of cytonuclear gynodioecy. *Silene vulgaris* hermaphrodites are self-compatible (Olson *et al.*, 2006), but individual hermaphrodite flowers are protandrous (Dulberger & Horovitz, 1984; Jolls *et al.*, 1994), so selfing by hermaphrodites is likely to occur via geitonogamy rather than autogamy. If selfing by hermaphrodites reduces seed quality through inbreeding depression, higher selfing rates among hermaphrodites could make it easier for females to be maintained in populations by relaxing the female fertility advantage required for female persistence.

In our system, the ultimate effects of *H. ectypa* on *S. vulgaris* breeding system evolution may also depend on the ecological context. Future work could assess the pollinator and herbivore communities interacting with *S. vulgaris* to determine the relative importance of *H. ectypa* and other non-ovipositing pollinators and herbivores for female and hermaphrodite host plant fitness. We have observed sweat bees (*Halictidae*), thrips (*Thysanoptera*), earwigs (*Dermaptera*), and ants (*Formicidae*) in *S. vulgaris* flowers during the day (L. A. D. D., personal observation), and we have evidence from a temporal pollinator exclusion experiment that seed production is due to nocturnal, rather than diurnal, pollination (L. A. D. D., unpublished data), but studying pollen donation and removal as well as flower and leaf damage by these different taxa would be helpful. It could also be useful to consider the relative frequencies of flower visits by female (ovipositing) vs. male (non-ovipositing) *H. ectypa* as well as the frequency of non-ovipositing visits by female *H. ectypa* moths. However, because oviposition was not associated with increased seed production at the flower level when eggs were removed from flowers, it seems that *H. ectypa*'s role as a pollinator for *S. vulgaris* may be limited, calling the interaction's nursery pollination status into question.

Silene–Hadena **interactions and the evolution of mutualism**

We found a small fitness cost and no apparent benefits associated with receiving *H. ectypa* eggs, suggesting that the recently established *H. ectypa–S. vulgaris* interaction is mildly negative to neutral. Egg-receiving plants lost significantly more fruits to predation than plants that did not receive eggs, but did not differ in the total number of expanded fruits. This could be because plants that received eggs were larger and had more flowers than plants that did not receive eggs, mitigating fruit loss, or because *S. vulgaris* plants generally produced large numbers of fruits (>30) and lost small numbers of fruits (<5). For pairs of flowers where one flower received an egg and the other did not, we were surprised by how frequently both flowers failed to expand and set seed (28% of pairs), suggesting a lack of pollination in spite of oviposition by a nursery pollinator. *Hadena ectypa* may be an ineffective pollinator, or may oviposit in flowers it has not pollinated, suggesting its role in this recently established interaction is as more of an antagonist than mutualist.

Non-ovipositing co-pollinators are often present in *Silene–Hadena* and *Silene–Perizoma* nursery pollination systems, often resulting in negative net fitness effects of nursery pollinators (Pettersson, 1991b; Westerbergh & Westerbergh, 2001; Westerbergh, 2004; Reynolds *et al.*, 2012). For example, in Europe, *S. vulgaris* interacts with several *Hadena* species, including *Hadena bicruris* Hufnagel, *Hadena confusa* Hufnagel, *Hadena perplexa* Denis & Schiffermüller, and *H. rivularis* (Pettersson, 1991b). These

Hadena species only accounted for 7% of pollen deposition on *S. vulgaris* flowers (Pettersson, 1991b), but consumed 10.6–47.9% of *S. vulgaris* fruits (Pettersson, 1991a), suggesting a strongly negative interaction. *Hadena ectypa*'s interaction with its native host plant, *S. stellata*, is also considered to be negative, as non-ovipositing co-pollinators were responsible for the bulk of seed production (Reynolds *et al.*, 2012) and oviposition by *H. ectypa* was associated with flower and fruit destruction (Kula *et al.*, 2013). However, there are also conditions under which the *H. ectypa–S. stellata* interaction may shift towards more positive outcomes for host plants. Reynolds et al. (2012) suggested that the interaction may be mutualistic early in the flowering season and whenever there are high densities of *H. ectypa* moths. Kula et al. (2013) found a link between *H. ectypa* oviposition and *S. stellata* fruit initiation, and that oviposition did not affect the amount of pollen *H. ectypa* delivered to *S. stellata* flowers. Although established *Silene–Hadena* interactions tend to have negative effects on host plant fitness, there are ecological contexts where they can be net positive. Comparing the outcome of the *S. vulgaris–H. ectypa* interaction we describe with these established *Silene–Hadena* systems suggests that nursery pollination interactions can begin as mildly negative to neutral from the host plant's perspective and shift towards parasitism or mutualism, depending on ecological context. Of course, the *S. vulgaris–H. ectypa* interaction described here represents only one data point, and considering additional recently established interactions would strengthen this conclusion. Plant species that, like *S. vulgaris*, have been introduced to new continents or geographic regions relatively recently provide opportunities to shed light on the evolutionary origins of mutualisms.

Plant traits & oviposition preferences

Female moths should experience selection on oviposition preferences such that they prefer to lay eggs in locations that will maximize survival and growth of their offspring (Castillo *et al.*, 2013). Because of the recent establishment of the *S. vulgaris–H. ectypa* interaction, it is likely that *H. ectypa*'s oviposition preferences on *S. vulgaris* were shaped through interactions with *H. ectypa*'s native host plant, *S. stellata*. On *S. stellata*, *H. ectypa* larvae prefer to feed on young *S. stellata* fruits and adult *H. ectypa* preferentially oviposit in flowers that are young and have not been pollinated (Castillo *et al.*, 2013). *Hadena ectypa* also prefers to deposit eggs in deeper *S. stellata* flowers, on plants with fewer flowers, in larger flowers, and on taller plants (Kula *et al.*, 2013).

We found that *H. ectypa* used both among- and within-plant traits in making oviposition decisions on its new host *S. vulgaris*, some of which correspond to preferences on the native host *S. stellata*. Among *S. vulgaris* plants, flower depth and number of stems affected oviposition. Hermaphrodites had significantly deeper flowers than females, accounting for the hermaphrodite-biased oviposition we observed. Within plants that received eggs, egg-receiving flowers had wider calyces than flowers that did not receive eggs. Only flower depth has been consistently associated with *H. ectypa* oviposition on *S. vulgaris* and on *S. stellata*, potentially suggesting that flower depth indicates the extent of floral resources available for adults (nectar) and/or future larvae. Interestingly, the *S. vulgaris* flowers we studied were 6–7 mm deeper on average than *S. stellata* flowers measured by Kula et al. (2013), suggesting that the oviposition preference we observed for deeper flowers was for the deepest available flowers, rather than for *S. vulgaris* flowers that most closely matched preferred phenotypes of the ancestral host plant.

In addition to flower depth, other unmeasured sexually dimorphic qualities might also affect oviposition or be correlated with flower depth. Females and hermaphrodites often have chemical differences (nutrient levels, defenses, attractants, and floral rewards) stemming from divergent life-history strategies (Dawson & Geber, 1999; Eckhart, 1999). *Hadena bicruris* moths use particular floral volatile compounds (lilac aldehydes and phenylacetaldehyde) to locate dioecious *S. latifolia* hosts (Dötterl *et al.*, 2006) and also use floral scent to differentiate between male and female *S. latifolia* plants (Brantjes, 1976). In *S. vulgaris*, hermaphrodites produce more nectar sugar per flower than females (Jolls *et al.*, 1994). Moths may associate sexually dimorphic traits, like flower depth or floral scent, with higher nectar sugar availability, resulting in the hermaphrodite-biased oviposition we observed.

Conclusion

This study adds to the empirical evidence of hermaphrodite-biased biotic interactions on gynodioecious plant species, and identifies plant and flower traits that are associated with hermaphrodite bias. It also highlights that oviposition preferences formed on a hermaphrodite host plant species can lead to sex-biased oviposition after a shift to a gynodioecious host plant species, and shows that both among- and within-plant traits are associated with oviposition. We also find that oviposition did not affect host plant reproduction in terms of fruit number or number of seeds per fruit, suggesting that *H. ectypa* oviposition does not exert a substantial fitness cost on host plants. Further work on this and other *Silene–Hadena* nursery pollination interactions could yield a better understanding of the factors that promote the evolution of mutualism vs. parasitism in nursery pollination interactions. Finally, we found no difference between females and hermaphrodites in fruit number or seeds per fruit, suggesting that female and hermaphrodite fitness in our study population may be close to the 1:1 ratio below which cytonuclear gynodioecy would destabilize. Therefore, if biotic interactions cause even small decreases in female fitness, such that female fitness drops below hermaphrodite fitness, these interactions would have the potential to play an important role in shaping future breeding system stability in this system.

Table 2.1. Plant traits associated with *S. vulgaris* fruit production from Poisson GLM using quasi-likelihood. Non-significant traits were removed one by one from the model to arrive at a final model containing only traits that were significant predictors of fruit production. After the final model was determined, a test statistic (LR F) and *P*-value for each non-significant predictor was obtained by comparing the final model (with all of the significant predictors) to a model containing the significant predictors and the non-significant term of interest; these values are reported in the table below for non-significant terms. A one-unit increase in the value of a predictor corresponds to multiplying the response (number of fruits) by the exponentiated coefficient value for that predictor. Degrees of freedom = 1, 120 for each predictor.

Predictor	**Coefficient**	**Exponentiated Coefficient**	**Likelihood Ratio F**	***P***
Flower number	0.029	1.029	36.08	< 0.0001
Stem number	0.020	1.020	17.65	< 0.0001
Height	0.029	1.029	18.66	< 0.0001
Flower depth	–0.14	0.87	14.62	0.00021
Plant area	0.00011	1.00011	4.74	0.031
Flower width	–0.036	0.96	2.71	0.10
Plant sex	–0.099 (if hermaphrodite)	0.91	0.39	0.53
Oviposition status	–0.10 (if received eggs)	0.90	0.32	0.57

Figure 2.1. Hermaphrodite *Silene vulgaris* in flower. Photo by L. Doubleday.

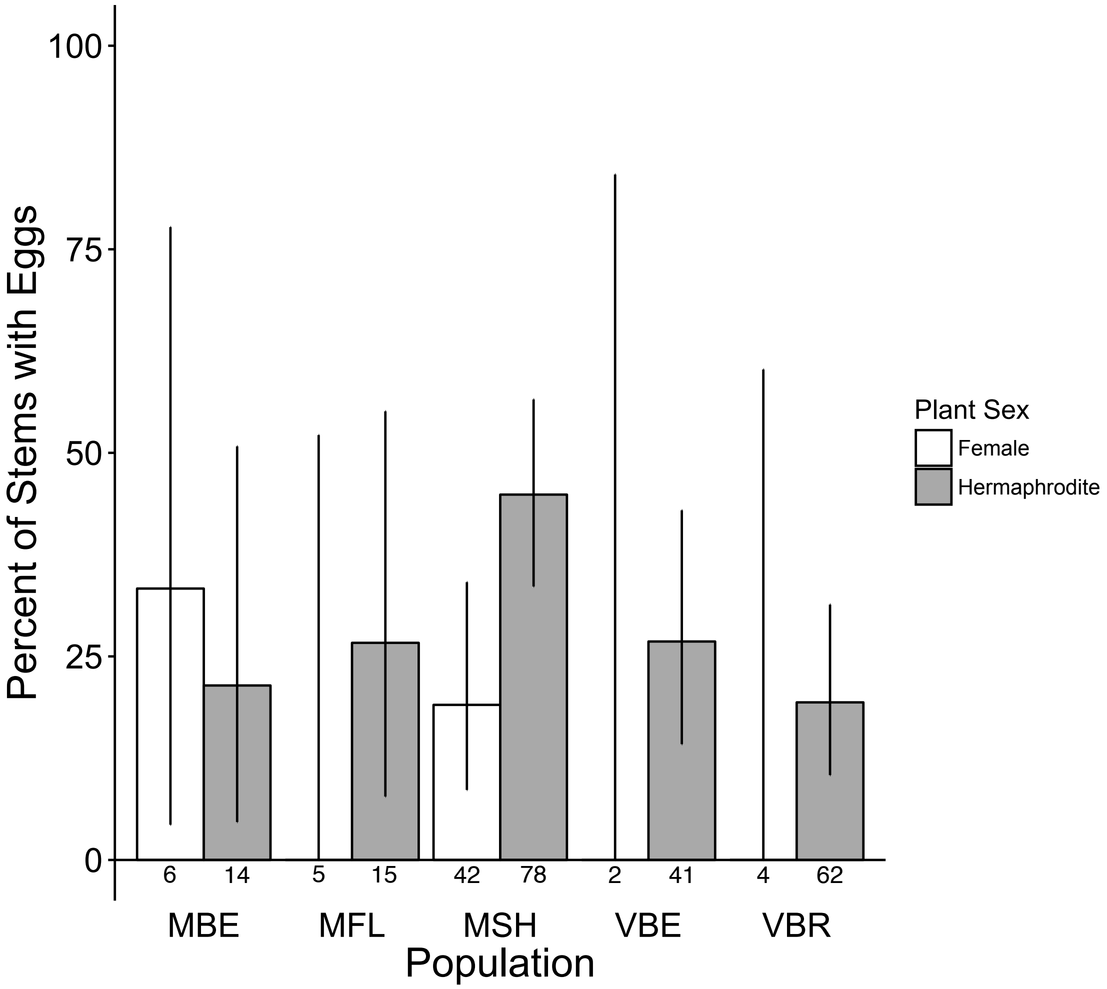

Figure 2.2. Hermaphrodite *S. vulgaris* were significantly more likely to receive *H. ectypa* eggs than females across populations in 2014. Bars represent observed proportion of female or hermaphrodite stems that received eggs in each population, letters are population codes, and numbers beneath the bars are sample sizes. Error bars are 95% binomial confidence intervals.

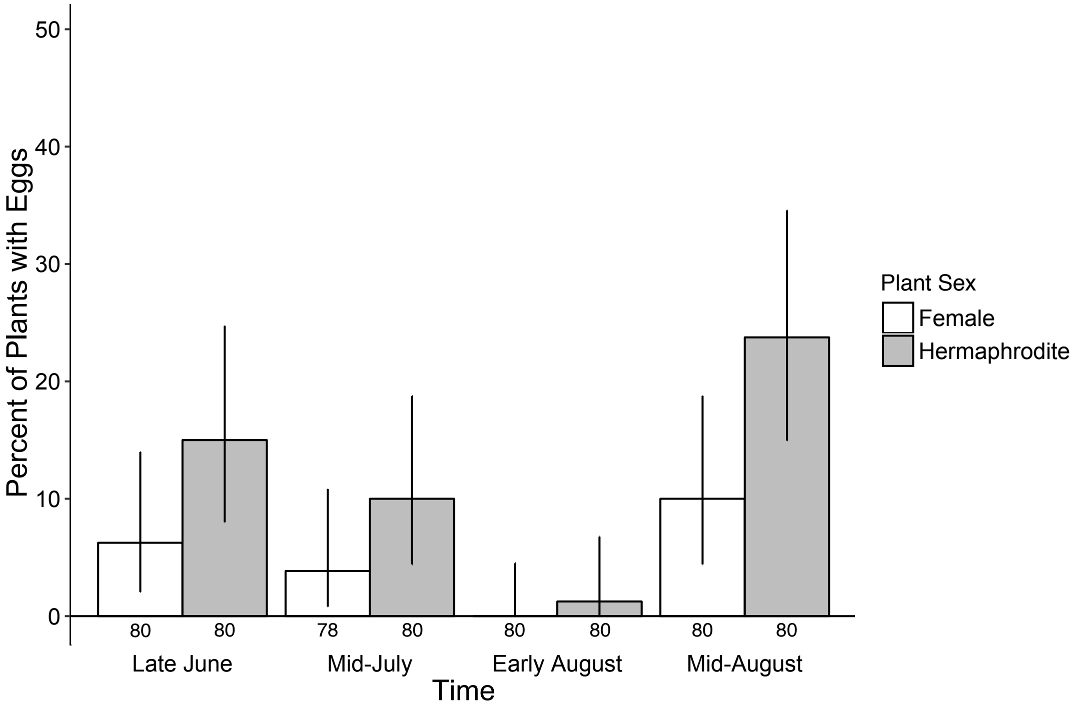

Figure 2.3. Hermaphrodite *S. vulgaris* were significantly more likely to receive *H. ectypa* eggs than females in 2015 at population MSH. Bars represent observed proportion of female or hermaphrodite plants that received eggs at the time of each census and numbers beneath the bars are sample sizes. Error bars are 95% binomial confidence intervals.

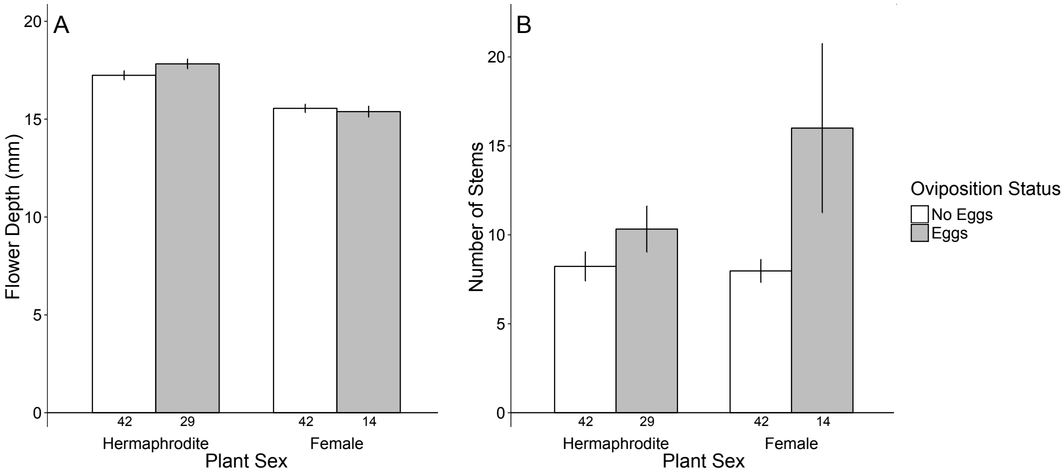

Figure 2.4. *Silene vulgaris* plants that received *H. ectypa* eggs at site MSH in 2015 had deeper flowers (A) and more stems (B) than plants that did not receive eggs. Hermaphrodite *S. vulgaris* plants had deeper flowers than females (A), but there was no sex difference in stem number (B). Numbers beneath bars are sample sizes. Error bars are standard error of the mean.

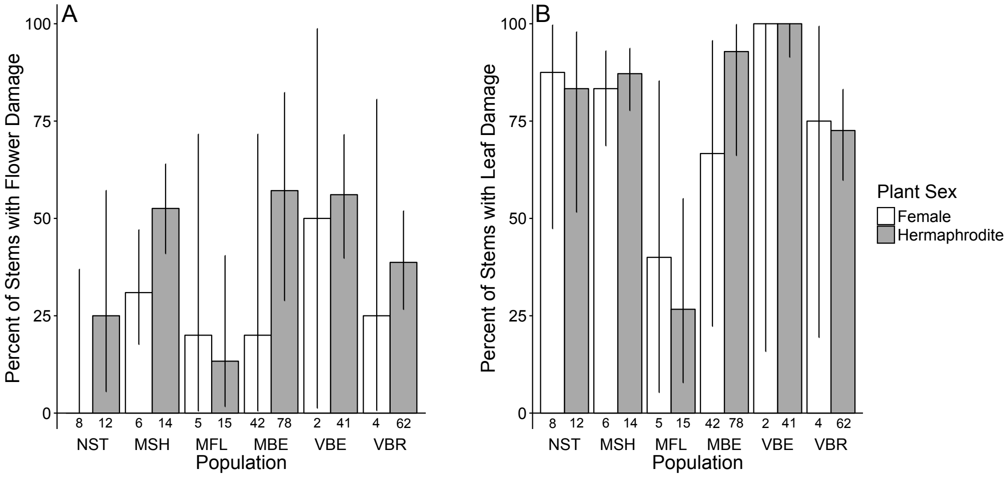

Figure 2.5. Hermaphrodites were significantly more likely to have flower damage than females (A), but there was no sex bias in leaf damage (B) across populations in 2014. Bars represent the observed proportion of females or hermaphrodites with flower or leaf damage in each population and letters are population codes. The numbers beneath the bars are the number of stems of each sex sampled in each population (Table S1). Error bars are 95% binomial confidence intervals.

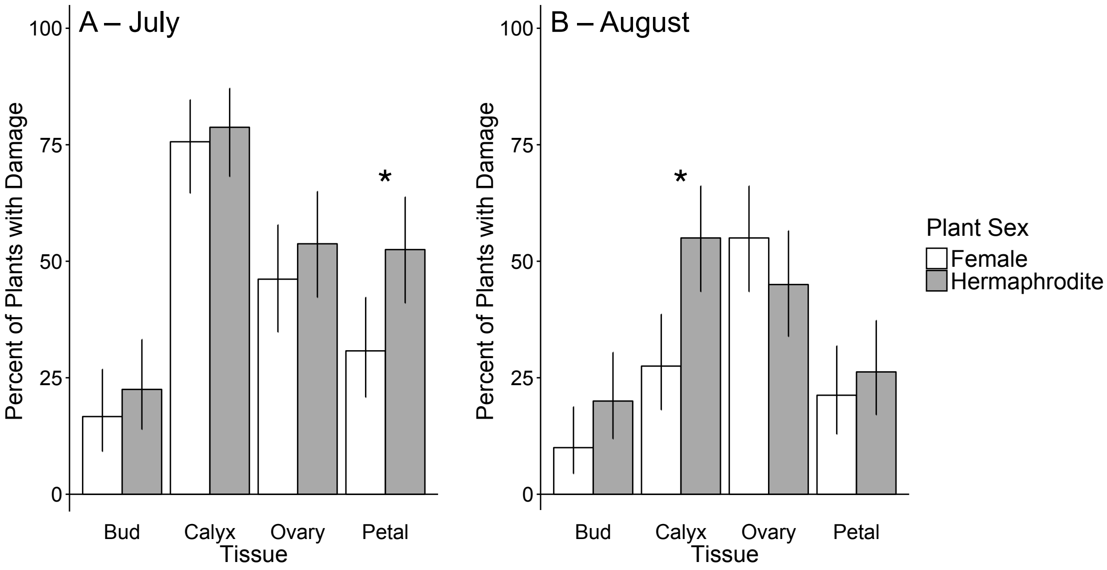

Figure 2.6. Hermaphrodites at site MSH were more likely than females to have petal damage in July (A) and calyx damage in August (B) 2015, but there was no sex bias in damage to buds or ovaries at either time. Error bars are 95% binomial confidence intervals. Sample sizes: July = 78 females and 80 hermaphrodites; August = 80 females and 80 hermaphrodites. Asterisks indicate significant ($P < 0.05$) differences between females and hermaphrodites.

CHAPTER 3

SCALE-DEPENDENT EFFECTS OF DENSITY AND SEX RATIO ON REPRODUCTION AND PHENOTYPIC SELECTION IN A GYNODIOECIOUS PLANT

Abstract

Conspecific density and local sex ratio are key demographic factors that affect fitness via mating opportunities, and that modulate selection on traits. However, understanding density and sex ratio effects requires determining the relevant spatial scale. Different organisms experience spatial variation at different scales, and spatial variation at multiple scales may affect fitness. For plants with distinct sexes, the combination of sexual dimorphism and spatial variation in density and sex ratio creates patches with different trait distributions, affecting individual attractiveness to fitness-altering interaction partners like pollinators and herbivores.

We used an observational approach in a population of gynodioecious *Silene vulgaris* plants to assess the relationship between reproduction and four traits likely to affect pollinator attraction, and how density and sex ratio at three spatial scales (0–0.5, 0.5–2, and 2–5 m from focal individuals) affect reproduction and phenotypic selection. Female fruit production decreased with increasing fine-scale (within 0.5 m) density, while hermaphrodite fruit production increased with fine-scale density. Sex ratio did not affect fruit production at any scale in either sex. In both sexes, seeds per fruit increased with fine-scale hermaphrodite frequency in high-density patches. Both sexes experienced significant selection on plant size and flower width; hermaphrodites also experienced selection on stem number. Furthermore, conspecific density within 0.5 m modified selection on flower width in females and stem number in hermaphrodites. We demonstrate that scale-specific spatial variation in density and sex ratio affects reproduction and selection, highlighting the immediate environment's importance and emphasizing the need to conduct studies at appropriate spatial scales.

Key words: density dependence, frequency dependence, gynodioecy, phenotypic selection, spatial scale

Introduction

Environmental heterogeneity produces variation in selection regimes in nature (Gómez 2003). Such variation occurs within and among populations, on spatial scales ranging from centimeters or meters to hundreds or thousands of kilometers. For example, microhabitat variation in surrounding vegetation affected selection on leaf length, flower height, and stalk length in pink lady's slipper orchid (*Cypripedium acaule*) (O'Connell and Johnston 1998), while spatial variation in herbivore pressure at a continental scale altered expression of chemical defenses in *Arabidopsis thaliana* populations across Europe (Züst et al. 2012). Moreover, organisms may simultaneously experience environmental heterogeneity at multiple scales; for example, murres (*Uria* spp.) track their capelin (*Mallotus villosus*) prey at 3 km, 50 km, and 300 km scales (Fauchald et al. 2000). Furthermore, resource availability, interspecific interactions, and intraspecific interactions may all vary (and covary) spatially, creating multi-scale mosaics of selection. For example, the statistical interaction effects of bison (*Bos bison*) grazing and fire on tallgrass prairie plant community composition were scale-dependent, but the main effects of grazing and fire did not depend on the measurement scale (Collins and Smith 2006).

For species with distinct sexes, conspecific density and sex ratio are two factors with important fitness effects. Conspecific density can have positive and negative effects on individual fitness; neighboring individuals compete for resources and mating opportunities, but low densities may make potential mates unavailable. For flowering plants that require insect pollination, conspecific density can furthermore affect pollinator behavior, with plants in high-density patches typically receiving greater pollinator service than plants growing in low densities (Kunin 2012). However, density effects may be sex-specific: one sex may experience positive density-dependent pollinator visitation, while the other sex experiences negative density-dependent pollinator visitation (Castillo et al. 2002). Given size and other differences among plant taxa, it is not surprising that previous work has shown a large range (0.28 – 70 m) of spatial scales at which density affects reproductive fitness in flowering plants (Spigler and Chang 2008; Gunton and Kunin 2009). Conspecific density can also alter phenotypic selection, because traits may have different fitness effects under different densities (Lankau and Strauss 2011).

Sex ratio can have substantial effects on fitness and selection on traits related to reproductive success by altering mating opportunities for each sex (McCauley and Brock 1998; Ashman and Diefenderfer 2001). Biased sex ratios typically intensify competition for mates in the common sex, leading to higher fitness variation. Meta-analysis of the effects of sex ratio on competition for mates in animals demonstrated that sex ratio changes are accompanied by predictable changes in behaviors like aggression, courtship rate, mate guarding, and copulation duration (Weir et al. 2011). Sex ratio could also modulate the strength of selection on traits (Ashman and Diefenderfer 2001). For example, male water striders (*Gerris odontogaster*) with long abdominal processes that aid in overcoming female mating reluctance had higher relative mating success when females were common but not when females were rare, because female copulation reluctance decreased as females became more rare (Arnqvist 1992). In plants with unisexual flowers, flower size is often sexually dimorphic. Among gynodioecious taxa where individuals are female or hermaphrodite, hermaphrodites tend to have larger flowers than females (Shykoff et al. 2003). The pattern is more complex in dioecious plants, among which males of insect-pollinated species tend to have larger flowers than females with the dimorphism more pronounced for temperate than tropical taxa (Delph et al. 1996). Larger flowers in the sex with male function is frequently proposed as an evolutionary response to stronger competition among males to export pollen than among females for pollen receipt, necessitating greater investment in pollinator attraction by males (Eckhart 1999). It follows from these hypotheses and observations that, among insect-pollinated species with distinct sexes, the more common sex would be expected to have larger flowers than the rare sex because increased intrasexual competition due to increased frequency selects for exaggeration of secondary sexual traits that improve reproductive success.

Few studies have manipulated sex ratios to alter the intensity of intrasexual competition and examined effects on flower size evolution in plants with dimorphic breeding systems, but we are aware of two such studies in gynodioecious *Fragaria virginiana*. In the first, there was selection for larger flowers in females when they were common, and smaller flowers when females were rare (Ashman and Diefenderfer 2001), demonstrating that increased intrasexual competition due to sex ratio variation can result in selection on flower size in the expected directions. Investing in larger flowers increases pollinator visitation (Conner and Rush 1996) and it would be especially valuable to be highly attractive to pollinators when intrasexual competition is intense. The second study found a positive selection gradient for flower size in females when they were rare in one of two years of study, and no significant selection on flower size in hermaphrodites (Case and Ashman 2007).

Although these previous studies demonstrate the importance of neighborhood sex ratio for reproduction and for selection on traits, sex ratio was only manipulated at one spatial scale, leaving the possibility of different results if interacting partners experience sex ratio at different spatial scales than the chosen manipulation, or if each interacting partner experiences neighborhood sex ratio at a unique spatial scale. The effect of density could also differ with spatial scale, and consideration of environmental context at different spatial scales can result in very different conclusions. For example, the diversity and abundance of three guilds of pollinators (solitary bees, bumble bees, and honey bees) was related to habitat composition, but different pollinator guilds responded to habitat variables at different spatial scales (Steffan-Dewenter et al. 2002) and a significant positive relationship between body size and foraging distance in bees suggests that body size is directly related to the spatial scale at which bees respond to landscapes (Greenleaf et al. 2007). For plants and their interacting pollinators, spatial heterogeneity would be expected to affect interactions with different pollinator guilds in a scale-dependent way. Determining the spatial scale at which sex ratio and density affect plant fitness, interactions with mutualists and antagonists, and phenotypic selection is essential to understanding how and why plant traits and breeding systems evolve at the levels of populations, communities, and landscapes, and how to effectively conserve plants and their pollinators.

We used an observational approach in a natural population of the gynodioecious plant species *Silene vulgaris* (Caryophyllaceae) to assess the sex-specific effects of sex ratio and conspecific density on fitness and phenotypic selection, and to identify the spatial scale(s) at which sex ratio and density are important.

We addressed the following specific questions:

1. Does the local sex ratio or conspecific density affect reproduction, and at what spatial scales?
2. Is there selection on floral and vegetative traits and, if so, do females and hermaphrodites experience different directions or intensities of selection?
3. At what spatial scales do sex ratio and conspecific density affect selection on females and hermaphrodites?

Methods

Study system

Silene vulgaris (Moench) Garcke is a gynodioecious perennial native to Europe and widely naturalized throughout North America (Nelson 2012). Previously, Olson et al. (2006) found effects of fine-scale (10 m and smaller) spatial structure of sex ratio on female fitness in two North American *S. vulgaris* populations, and McCauley and Brock (1998) found that female fruit set was positively related to hermaphrodite frequency when sex ratio was manipulated in artificial populations of 10 plants within a 2 m diameter.

In gynodioecious species, shifts in sex ratio affect the abundance of potential fathers, but not mothers. Higher hermaphrodite frequencies increase pollen or sperm availability, so females should experience higher fitness when hermaphrodites are common and females are rare (McCauley and Brock 1998; Graff 1999). A high hermaphrodite frequency induces stronger competition among hermaphrodites to sire offspring, and may also maximize the number of hermaphrodite ovules that are fertilized simply because higher hermaphrodite frequencies equate to higher pollen or sperm availability (McCauley and Brock 1998). In gynodioecious plants, both females and hermaphrodites tend to produce more fruits and seeds when hermaphrodites are common and females are rare (McCauley and Brock 1998; Graff 1999; Rivkin et al. 2015).

Study design and data collection

In summer 2015, we established a 45 x 10 m study area at Mass Audubon's Lime Kiln Farm Wildlife Sanctuary in Sheffield, MA, USA (42.082654, -73.362669) within which we tagged, mapped, and sexed all of the 239 *S. vulgaris* plants. We used triangulation to map the relative positions of plants in the population. We started with the individual in the southeast corner of the population and recorded its location using a GPS unit. Plants grew too densely to simply use GPS to obtain coordinates for each individual. Instead, we measured the distance and bearing from the first plant to the next plants. We continued this procedure, obtaining distances and bearings with reference to at least two other individuals whenever possible. After we had mapped the locations of all of the plants in the study area in this fashion, we used trigonometric relationships to calculate each plant's location in Cartesian space. When more than one estimate of a plant's location was available, we averaged the estimates to determine its position.

Fifty-seven of the mapped plants were "focal plants," on which we measured flower depth (distance from ovary to floral face) and width (width of floral face), plant size (projected area), stem number, and fruit production. We chose to measure these traits because they often mediate interactions with pollinators and herbivores (Klinkhamer et al. 1989; Conner and Rush 1996; Strauss and Whittall 2006) and previous work demonstrated significant relationships between stem number, plant size, flower depth and fruit production, while flower width was associated with hermaphrodite-biased oviposition by a nursery pollinating moth (Doubleday and Adler 2017). Although plant size is frequently implicated in pollinator attraction, we note that it would be less surprising to observe selection on plant size or stem number than on flower depth or flower width because measures of plant size are often positively correlated with the numbers of flowers and fruits produced. We measured flower depth and width for two flowers per plant once, between June 22 and July 16, 2015. Focal plants were selected haphazardly based on having at least one open flower at the time of tagging (June 22 – 6 July, 2015). To estimate plant size, we measured each plant's aerial length (distance along longest axis occupied by the plant from above) and aerial width (distance along axis perpendicular to aerial length) and multiplied aerial length by aerial width. We chose fruit number as a fitness measure, rather than fruit set, for three reasons: fruit number should be directly related to fitness; it was logistically impossible for us to track the fates of all of the flowers produced by the focal plants; and *S. vulgaris* fruits generally remain attached to the maternal plant even after they have shed seeds (LAD Doubleday, pers. obs.), enabling us to evaluate fruit number on a single date. However, fruit number is likely to be affected by plant size and stem number, and as *S. vulgaris* is perennial, may also reflect a plant's age. We counted all the fruits on each focal plant once between July 31 and August 6, 2015, after most plants in the population had stopped flowering for the season. To estimate a plant's average seed production per fruit, we collected up to three fruits from each focal plant, counted the seeds, and averaged the seed counts. Because receiving more compatible pollen should lead a flower to produce more seeds (until saturation) (Bertin 1990), seeds per fruit may better reflect pollinator service to plants than fruit number. We therefore conducted analyses with both number of fruits and average seeds per fruit as fitness measures. We used seeds per fruit and fruit number as two fitness estimates rather than multiplying average seeds per fruit by fruit number to obtain an estimate of total seed production to limit the amount of noise associated with fitness estimates, especially because our seeds per fruit estimates were based on a small sample of the total number of fruits plants produced.

We considered only female fertility (i.e. seed production) in this study, but hermaphrodites accrue fitness through both female (seed production) and male (seed siring) functions. It was not feasible for us to estimate male fertility for hermaphrodites, and we acknowledge that failing to do so yields an incomplete picture of hermaphrodite reproductive success and selection on hermaphrodites. In gynodioecious populations, selection on hermaphrodites through seed production is likely to be more important when females are rare because hermaphrodites will be more likely to serve as mothers (Case and Ashman 2007). At least one study has assessed selection on hermaphrodites through both female and male function and found selection operating differently on female vs. male components of hermaphrodite fitness (Morgan and Ashman 2003). Some other studies (Eckhart 1999; Case and Ashman 2007; Caruso and Yakobowski 2008) have examined selection on gynodioecious plants through female fitness components only, as we do here.

Data analysis

We calculated the density (number of plants per unit area) and sex ratio of neighboring plants within varying radii (0–0.5, 0.5–2, and 2–5 m) of each focal plant. We chose radii based on preliminary analyses showing that sex ratio varied substantially close to focal plants (within 1 m) and stabilized around the population sex ratio within 5 m of focal plants (Figure S4), similar to the pattern reported for *S. vulgaris* populations in Virginia, USA (Sanderson et al. 2016). Density patterns were similar to sex ratio, with considerable variation within 1m of focal plants (Figure S5). We used non-overlapping annuli to ensure statistical independence among different radii. Before conducting any statistical tests, we excluded extreme observations, which we defined as focal plants for which number of fruits produced fell more than three interquartile ranges above the median (three female plants and one hermaphrodite).

Before assessing relationships between density and fruit production, we first evaluated potential confounding of plant size and density. Bigger plants tend to produce more fruits, and growing in high-density patches could limit plant size due to competition for resources. If increasing density reduces plant size, we should observe a negative relationship between the two variables, while a positive relationship could indicate large cohorts of seeds produced by bigger plants. We assessed whether we should include plant size as a covariate in density analyses by conducting a linear regression of plant size on total conspecific density within one meter of focal plants. We chose the 1 m radius because it is a scale at which the roots of neighboring plants should experience variation in soil resources (Jackson and Caldwell 1993) and if density affects plant size due to resource competition, this is a scale at which such effects should be observable. We did this separately for females and hermaphrodites. To better satisfy the assumption of normality, we square root-transformed plant size for females and log-transformed plant size for hermaphrodites. We found no relationship between density and plant size for either sex (females: slope = –1.215, $F_{1,24} = 0.25$, $P = 0.62$; hermaphrodites: slope = 0.05, $F_{1,25} = 0.08$, $P = 0.78$), and thus did not control for plant size in further analyses.

To assess the spatial scale at which conspecific density and sex ratio affect focal plant fitness, we conducted analyses with fruit number or seeds per fruit as the response and density within a given distance from focal individuals, sex ratio within a given distance, and the two-way interaction between density and sex ratio at that distance as predictors. We used generalized linear models with quasi-Poisson errors to account for overdispersion for these models.

We tested for linear directional selection on the four measured plant traits (plant size, stem number, flower depth, and flower width) by conducting multiple linear regression on plant traits (standardized to a mean of 0 and variance of 1) with relative fruit number or relative seeds per fruit as the response to obtain linear directional selection gradients for each trait while accounting for the other traits (Lande and Arnold 1983). Relative fitness measures (fruit number or seeds per fruit) were calculated as the raw fitness measure for each individual divided by the group mean. Because we expected selection to vary between the sexes, we conducted separate analyses for females and hermaphrodites. There were no issues with multicollinearity; variance inflation factors for all of the traits were less than 2 in models for each sex. However, we note that there were positive correlations between plant size and stem number for both sexes (Pearson's r: 0.40 for females and 0.57 for hermaphrodites), but the correlation was only significant for hermaphrodites ($P = 0.002$; females: $P = 0.06$).

To check for sexual dimorphism in any traits under sex-specific selection, we used generalized linear models and conducted likelihood ratio tests comparing a model using plant sex to predict the trait in question with a null model containing only the trait in question and an intercept. We used a quasi-likelihood model with a Poisson error distribution to analyze stem number and a likelihood model with a Gaussian error distribution to analyze square root transformed plant size.

We examined the effects of density and sex ratio on selection (i.e. whether density or sex ratio interacted with traits to predict fitness) only for spatial scales where we found significant effects of density and/or sex ratio on fitness. To do this, we conducted additional analyses (a separate analysis for each trait) where we considered the effect of the standardized phenotypic trait, the main effect of sex ratio or density at a given spatial scale, and two-way interactions between the trait and sex ratio or density on relative fruit number or relative seeds per fruit. Significant interactions between a trait and the sex ratio or density for a given radius indicate that the sex ratio or density of neighbors within that radius alters selection on that trait. We conducted these analyses separately for females and hermaphrodites because density and sex ratio are expected to affect females and hermaphrodites differently.

For all analyses, except the directional selection gradient analyses where all four traits were retained as predictors regardless of their significance, we took the following approach to model building. First, we removed terms from the full model sequentially to arrive at the minimum adequate model where all remaining terms were either significant main effects or interactions, or a main effect that was part of a significant interaction. To obtain slope estimates and significance values for terms that did not remain in the final model, we added them to the final model one by one and used likelihood ratio tests to compare models differing by a single predictor to assess whether that predictor significantly improved the explanatory power of the model. For interaction terms where one or both of the main effects were not significant, we compared the explanatory power of a model including both main effects and the interaction term with a model with only the main effects using likelihood ratio tests as above. We omitted influential observations (0–5 observations, depending on analysis) that created statistical relationships between variables if these relationships disappeared when those observations were excluded.

Because our data are spatially explicit, we checked for spatial autocorrelation in the residuals of all models using Moran's I and Mantel tests. We did not detect significant spatial autocorrelation in any of the models that yielded significant results ($P > 0.05$ for both Moran's I and Mantel tests). All analyses were conducted using R version 3.3.3 (R Core Team 2017).

Results

Density and sex ratio effects on fruit number and seeds per fruit

Conspecific density at a fine spatial scale had significant and opposite effects on fruit production in females and hermaphrodites (Table 3.1). Increasing conspecific density within 0.5 m reduced fruit production for females (Figure 3.1). In contrast, there was a positive relationship between density within 0.5 m and fruit production for hermaphrodites, but only when two high-density, high-fruit number plants were included in the analysis (Table 3.1, Figure 3.1). Without these extreme points, there was no significant relationship between density within 0.5 m and fruit number for hermaphrodites (Table 3.1). Because we are confident that the influential hermaphrodite data points were accurately observed and our sample size is relatively small, more data would be required to draw conclusions about whether the influential points truly reflect underlying processes or represent random noise. There were no significant density effects on fruit number at coarser spatial scales for either sex (Table 3.1). We did not detect effects of sex ratio on fruit number at any of the spatial scales, nor were there any significant interactions between sex ratio and density affecting fruit number (Table 3.1).

We detected a significant interaction between density and sex ratio 0–0.5 m from focal individuals on seeds per fruit for both sexes and for 0.5–2 m for hermaphrodites only (Table 3.2). No main effects of density or sex ratio on seeds per fruit at any distance from focal plants were significant for either sex ($P > 0.05$; Table 3.2). For females, the interaction between density and sex ratio within 0.5 m of focal plants resulted in females in high-density patches experiencing the expected positive relationship between hermaphrodite frequency and seeds per fruit, while hermaphrodite frequency had negative to neutral effects on seeds per fruit for females in low-density patches (Figure 3.2a). For hermaphrodites, the density-by-sex ratio interaction at 0–0.5 m also indicated the expected positive relationship between hermaphrodite frequency and seeds per fruit at high densities, but no clear relationship between sex ratio and seeds per fruit for plants in low-density patches (Figure 3.2b). Similarly, for 0.5–2 m from focal plants, the interaction between density and sex ratio reflected a positive relationship between hermaphrodite frequency and seeds per fruit for hermaphrodites in high-density patches and no clear relationship between hermaphrodite frequency and seeds per fruit for hermaphrodites in low-density patches (Figure S6).

Sex-specific selection and sexual dimorphism

Females and hermaphrodites experienced different phenotypic selection on traits. Using fruit number as the fitness measure, females experienced significant selection for increased plant size (Figure 3.3a Table 3.3). Using seeds per fruit as the fitness measure, females experienced selection for increased flower width (Figure 3.3b; Table 3.3). With fruit number as the fitness estimate, hermaphrodites experienced significant selection for increased plant size (Figure 3.4a) and stem number (Figure 3.4b), and decreased flower width (Figure 3.4c), but no selection on flower depth (Table 3.3). The plant size and stem number results were robust to eliminating two extreme high-fruit hermaphrodites, but the flower width result was not (Table 3.3). Using seeds per fruit as the fitness estimate, no hermaphrodite traits were under significant selection (Table 3.3). Hermaphrodites had significantly wider and deeper flowers than females, but there were no significant differences in plant size or stem number between the sexes (Table 3.4).

Fine-scale density effects on selection

In females, density modified selection on flower width with seeds per fruit as the fitness measure (Table 3.5), such that there was positive selection for flower width in high-density patches and negative selection in low-density patches (Figure 3.5a). Density did not modify selection on any traits for females with fruit number as the fitness estimate (Table 3.5). In hermaphrodites, density within 0.5 m of focal individuals modified selection on plant size, stem number, and flower width in hermaphrodites regardless of fitness measure used (Table 3.5). However, only the stem number effect (fruit number as the fitness measure) was robust to removing the two large hermaphrodites. Density modified selection on stem number in hermaphrodites such that there was greater positive selection for stem number at lower density (Figure 3.5b).

Discussion

We found effects of fine-scale spatial variation in density and sex ratio on fruit number and seeds per fruit in female and hermaphrodite *S. vulgaris*, sex-specific phenotypic selection on floral and vegetative traits, and that conspecific density within 0.5 m of focal individuals modified selection on some traits.

Density and sex ratio effects on reproduction

Conspecific density within 0.5 m of focal plants affected fruit production by females and may have affected fruit production in hermaphrodites. There were no effects of density on fruit production or seeds per fruit at the other scales considered. A few studies have examined the effects of conspecific density at multiple spatial scales on plant fitness. In dioecious *Silene latifolia*, conspecific density affected survival and fitness in a variety of complex ways at multiple scales (0.28–70 m) through interactions with plant size and variable effects of male vs. female vs. total flower density at a given scale (Gunton and Kunin 2009). There were contrasting effects of density within 1 m of focal plants and 1–4 m from focal plants in *Sabatia angularis*; when growing in denser neighborhoods at a 1 m scale, plants had lower seed set, while increases in neighborhood density between 1 and 4 m corresponded to higher seed set (Spigler and Chang 2008).

A general expectation in gynodioecious species is that the reproductive success of females should be positively correlated with the frequency of hermaphrodites, due to increased pollen availability (McCauley and Brock 1998). We found evidence of this in the effects of interactions between density and sex ratio on seeds per fruit, but the expected positive relationship between hermaphrodite frequency and seeds per fruit was only evident at higher densities. Previous studies, including two studies of *S. vulgaris*, have demonstrated similar effects of sex ratio on plant reproduction. McCauley and Brock (1998) experimentally manipulated sex ratio in *S. vulgaris* at a 2 m spatial scale and found that female reproductive success increased with hermaphrodite frequency, while Olson et al. (2006) demonstrated the same relationship in an observational study of natural populations. In *Lobelia cardinalis*, flower-level sex ratio affected fruit production at a 1 m scale, but not at 0.1, 0.2, or 0.5 m scales (Bartkowska and Johnston 2014). These studies and our results highlight the importance of considering spatial scale when studying the patterns and processes of plant mating; as these studies demonstrate, one spatial scale may yield a different pattern than another scale.

Although we observed the expected positive relationship between hermaphrodite frequency and seeds per fruit under high densities at a 0–0.5 m scale, we found no effect of sex ratio on fruit number at any scale. Several factors could have contributed to the lack of a consistent relationship between sex ratio and fruit number in our study. Fruit production by female and hermaphrodite *S. vulgaris* plants may not have been not pollen-limited, even in patches where hermaphrodite pollen donors were less common. If fruit production were pollen-limited, increasing the frequency of pollen donors would be expected to increase fruit production. Assessing pollen limitation was not feasible as part of this study, but would be a valuable addition to future studies. Another possibility is that other components of fitness, like fruit set or seed set, would be more affected by sex ratio than fruit number. McCauley and Brock (1998) and Olson et al. (2006) chose fruit set (number of fruits/number of flowers) as their fitness measure and both studies found sex ratio effects. Fruit number is more likely to be affected by factors outside of our design, like plant size, while fruit set may be less size-dependent. However, fruit number is more closely related to fitness (number of offspring) than fruit set. We were also unable to assess the effects of density and sex ratio at different spatial scales on siring success of hermaphrodites, which would be expected to increase as hermaphrodites became rare. Future studies should consider both male and female aspects of hermaphrodite fitness and assess the composition of local pollinator fauna to better understand how different pollinator taxa respond to plant density and sex ratio and the resulting effects on female and male aspects of reproductive success.

Sex-specific selection on vegetative and floral traits

Females and hermaphrodites both experienced selection for larger plant size, hermaphrodites experienced selection for more stems, and females experienced selection for wider flowers. Hermaphrodites accrue fitness through both seeds and pollen, while females accrue fitness solely through seeds, which means that certain alleles might be exposed to selection when they are expressed in one sex but not the other. Traits that enhance male reproduction (seed siring success) have traditionally been thought to be under stronger selection by pollinators than traits that enhance female reproduction (seed production) (Bell 1985; Burd and Callahan 2000), but there is only limited evidence in support of this paradigm (Ashman and Morgan 2004). Recently, attention has been paid to the importance of ecological context, especially the degree to which seed production is pollen-limited, in generating patterns of sex-biased selection (Ashman and Morgan 2004). In gynodioecious plants, considering selection only via seed production ignores the role of male function in selection on hermaphrodite traits, but has yielded useful insights about how ecological context affects sex-specific evolution. For example, in *F. virginiana*, considering sex-specific selection via seed production revealed sex-specific effects of resource availability and sex ratio (ecological context) on phenotypic selection, opportunity for selection, and the degree to which females and hermaphrodites allocate resources to seed production (Case and Ashman 2007). One might expect sex-specific selection on plant traits to promote sexual dimorphism, but we observed no significant sexual dimorphism in stem number or plant size.

Sexual dimorphism in vegetative traits is uncommon among gynodioecious plant species but extremely common among dioecious (individuals are male or female) plants, prompting questions about whether gynodioecious taxa display no dimorphism because they do not experience sex-specific selection, the selection they experience is variable, or because evolutionary responses to sex-specific selection pressures are genetically constrained (Ashman 2005). The evolution of sexual dimorphism can be constrained by both positive and negative genetic correlations, if positively correlated traits experience selection in opposite directions or if negatively correlated traits experience selection in the same direction (Ashman 2005). In a study of vegetative trait dimorphism in gynodioecious *F. virginiana*, Ashman (2005) suggested that the lack of a response to sex-specific selection (i.e. the predicted change in mean phenotype of offspring relative to the parental generation, calculated from genetic covariances and phenotypic selection gradients), despite significant sex-specific selection gradients, was likely because of low genetic variation and genetic covariation of traits within sex morphs. Another study concluded that high phenotypic integration (i.e. correlation) of vegetative traits constrained the evolution of sexual dimorphism in a gynodioecious orchid, but did not evaluate whether plants experienced sex-specific selection (Lu and Huang 2010). An alternative explanation for more sexual dimorphism in dioecious taxa than in gynodioecious ones is that gynodioecy may arise more easily than dioecy and gynodioecious taxa may be younger on average than dioecious ones, allowing less time for dimorphism to have evolved. Our results add an example of sex-specific selection on vegetative traits without corresponding sexual dimorphism, but further work is necessary to assess the frequency with which gynodioecious taxa experience divergent selection on vegetative traits without displaying dimorphism.

We found evidence of selection on flower width via seeds per fruit in females as well as significant sexual dimorphism in flower width. Interestingly, females were under positive directional selection for flower width via seeds per fruit while hermaphrodites experienced no significant selection on flower width, yet females had narrower flowers than hermaphrodites. This could suggest that wider flowers in hermaphrodites enhance male function (Campbell 1989), which we were unable to measure. Sexual dimorphism in floral traits is common among gynodioecious taxa, with hermaphrodites usually having larger flowers than females (Eckhart 1999).

Density-dependent phenotypic selection

We found that density within 0.5 m modulated phenotypic selection on flower width in females and stem number in hermaphrodites. There were additional density effects on selection in hermaphrodites that were not robust to two extreme points. Many studies have demonstrated that flower density affects pollinator behavior in ways that may affect plant mating patterns, such as altering the length of visits to individual flowers, changing the number of flowers visited on each plant, and affecting the distance travelled between plants, but the direction of results is mixed (Smithson and Macnair 1997; Grindeland et al. 2005; Makino et al. 2007). Local density has also been shown to affect reproductive success across a variety of plant taxa (e.g. Spigler and Chang 2008; Gunton and Kunin 2009; Bartkowska and Johnston 2014), but few studies have explicitly examined relationships between density and selection on plant traits. Pollinators showed density-dependent preferences for plants with particular trait values in *Phyteuma spicatum*, but there was no evidence that density modulated selection on the traits (Weber and Kolb 2013). Bartkowska and Johnston (2014) demonstrated effects of density and plant height on fitness in *Lobelia cardinalis*, but did not explicitly test for interactions between density and phenotype on fitness measures. Our results demonstrate that conspecific density at a fine spatial scale can have significant effects on the relationship between plant traits and fitness, highlighting the need to consider how the local environment modifies selection on traits.

Conclusion

We demonstrated that conspecific density and sex ratio affect reproduction and phenotypic selection in gynodioecious *S. vulgaris* and that these effects vary with spatial scale and plant sex. Our results contribute to the growing body of studies suggesting that the spatial scale at which we conduct experiments can have substantial effects on results. Although it is logistically demanding, assessing the spatial scale relevant to research questions is a critical step in experimental design. Integrating the effects of conspecific density and sex ratio on reproduction and phenotypic selection at multiple spatial scales will provide insights into the evolution of sexual dimorphism in gynodioecious and dioecious taxa.

Table 3.1. Effects of density (Dens) and sex ratio (SR) at three spatial scales on focal individual fitness (number of fruits produced) for female and hermaphrodite *Silene vulgaris* plants. Tests come from quasi-Poisson generalized linear models with fruit number as the response variable.

		Distance from focal plant (m)								
			0 – 0.5			**0.5 – 2**			**2 – 5**	
Plant sex	**Term**	**Slope estimate**	**F_{df}**	***P***	**Slope estimate**	**F_{df}**	***P***	**Slope estimate**	**F_{df}**	***P***
Female	Dens	**–0.3056**	**$14.7000_{1,17}$**	**0.0013**	–0.0810	$0.6295_{1,24}$	0.4353	–1.0950	$3.0970_{1,24}$	0.0912
	SR	–0.1159	$0.1383_{1,16}$	0.7148	0.7950	$0.2359_{1,24}$	0.6316	–0.4906	$0.0313_{1,24}$	0.8611
	Dens*SR	–0.1664	$0.2590_{1,15}$	0.6182	1.0687	$0.3087_{1,22}$	0.5841	–23.5210	$3.0812_{1,22}$	0.0931
Hermaphrodite	Dens	**0.0808**	**$5.0797_{1,17}$**	**0.0377**	0.0606	$0.2302_{1,25}$	0.6355	–0.1389	$0.0321_{1,25}$	0.8593
	SR	0.9885	$2.3559_{1,16}$	0.1444	–0.6221	$0.0819_{1,25}$	0.7771	2.0222	$0.4625_{1,25}$	0.5027
	Dens*SR	0.1525	$1.3829_{1,15}$	0.2579	–1.0578	$0.1381_{1,23}$	0.7136	–12.3837	$0.4016_{1,23}$	0.5325
Hermaphrodite (without two influential points)	Dens	–0.0504	$0.8291_{1,15}$	0.3769	–0.2021	$3.4512_{1,23}$	0.0761	0.8973	$2.2993_{1,23}$	0.1431
	SR	0.3683	$0.4719_{1,15}$	0.5026	0.0136	$0.0001_{1,23}$	0.9939	–2.9931	$1.2474_{1,23}$	0.2756
	Dens*SR	–0.4773	$4.0923_{1,13}$	0.0642	0.4180	$0.0368_{1,21}$	0.8498	5.5847	$0.1292_{1,21}$	0.7228

Table 3.2. Effects of density (Dens) and sex ratio (SR) at three spatial scales on focal individual fitness (number of seeds per fruit) for female and hermaphrodite *Silene vulgaris* plants. These tests come from quasi-Poisson generalized linear models with seeds per fruit as the response variable.

		Distance from focal plant (m)								
		0 – 0.5			**0.5 – 2**			**2 – 5**		
Plant sex	**Term**	**Slope estimate**	**F_{df}**	***P***	**Slope estimate**	**F_{df}**	***P***	**Slope estimate**	**F_{df}**	***P***
Female	Dens	–0.5323	$3.0078_{1,14}$	0.1048	–0.0640	$0.4486_{1,24}$	0.5094	–1.6252	$0.9552_{1,24}$	0.3382
	SR	–2.1298	$0.0131_{1,14}$	0.9106	0.6379	$0.1712_{1,24}$	0.6827	–1.4827	$0.3370_{1,24}$	0.5670
	Dens*SR	**1.0333**	**$8.4582_{1,14}$**	**0.0115**	–0.5707	$0.0998_{1,22}$	0.7550	–11.8850	$0.6861_{1,22}$	0.4164
Hermaphrodite	Dens	–0.2154	$0.1303_{1,12}$	0.7244	0.0970	$0.9642_{1,21}$	0.3373	–0.2256	$0.1317_{1,22}$	0.7201
	SR	–0.7799	$1.1675_{1,12}$	0.3012	–0.2901	$0.0331_{1,21}$	0.8574	–0.5569	$0.0517_{1,22}$	0.8222
	Dens*SR	**0.5973**	**$7.4302_{1,12}$**	**0.0184**	**4.6018**	**$4.7357_{1,19}$**	**0.0424**	–17.6581	$1.1563_{1,20}$	0.2950

Table 3.3. Linear directional selection gradients for flower and plant traits, with relative fruit number and seeds per fruit as fitness measures. We analyzed female and hermaphrodite *Silene vulgaris* plants separately, with all traits in each model simultaneously. Traits were standardized to have a mean of 0 and a variance of 1. We did not remove non-significant terms from these models because we were interested in the relationships between traits and fitness when the other traits were accounted for. Bold font indicates P < 0.05.

Plant sex	**Fitness estimate**	**Trait**	**Slope**	**F_{df}**	***P***
Female	Fruit number	**Plant size**	**0.3005**	**$8.6952_{1,15}$**	**0.0100**
		Stem number	0.1036	$0.8915_{1,15}$	0.3600
		Flower depth	0.0303	$0.0621_{1,15}$	0.8066
		Flower width	–0.1709	$1.9662_{1,15}$	0.1812
	Seeds per fruit	Plant size	–0.0369	$0.0653_{1,17}$	0.8013
		Stem number	0.2016	$1.4691_{1,17}$	0.2421
		Flower depth	–0.0652	$0.1460_{1,17}$	0.7071
		Flower width	**0.3761**	**$5.1031_{1,17}$**	**0.0373**
Hermaphrodite	Fruit number	**Plant size**	**0.4656**	**$14.2186_{1,19}$**	**0.0013**
		Stem number	**0.5951**	**$23.0679_{1,19}$**	**0.0001**
		Flower depth	0.0813	$0.6311_{1,19}$	0.4368
		Flower width	**–0.2158**	**$4.8732_{1,19}$**	**0.0398**
	Seeds per fruit	Plant size	0.3064	$1.8617_{1,17}$	0.1893
		Stem number	–0.1089	$0.2614_{1,17}$	0.6154
		Flower depth	–0.1834	$1.1519_{1,17}$	0.2973
		Flower width	–0.1884	$1.0643_{1,17}$	0.3159
Hermaphrodite (two high-fruit plants omitted)	Fruit number	**Plant size**	**0.4827**	**$15.0668_{1,19}$**	**0.0010**
		Stem number	**0.2783**	**$5.4463_{1,19}$**	**0.0307**
		Flower depth	0.0374	$0.1033_{1,19}$	0.7514
		Flower width	–0.1461	$1.8366_{1,19}$	0.1912
	Seeds per fruit	Plant size	0.1002	$0.2092_{1,16}$	0.6535
		Stem number	–0.2273	$1.1474_{1,16}$	0.3000
		Flower depth	–0.1282	$0.4261_{1,16}$	0.5232
		Flower width	–0.2723	$1.8958_{1,16}$	0.1875

Table 3.4. Tests for sexual dimorphism in plant size, stem number, flower depth, and flower width between female and hermaphrodite *Silene vulgaris* plants. Boldface type indicates significant sexual dimorphism at $P < 0.05$.

Trait	$\mathbf{F_{df}}$	***P***
Plant size	$0.5665_{1,53}$	0.4550
Stem number	$0.0209_{1,53}$	0.8855
Flower depth	$\mathbf{17.1960_{1,50}}$	**0.0001**
Flower width	$\mathbf{27.9930_{1,50}}$	**<0.0001**

Table 3.5. Effects of conspecific density within 0.5 m of focal individuals on selection on four plant traits in female and hermaphrodite *Silene vulgaris* plants with two fitness estimates (fruit number and seeds per fruit). F-tests are for a two-way interaction between density and the trait of interest in a regression on the fitness estimate. A significant F-test (indicated in bold type) indicates that density modified the relationship between the trait of interest and the fitness estimate (i.e. that density modified selection on the trait).

Plant sex	**Fitness estimate**	**Trait**	**F_{df}**	***P***
Female	Fruit number	Plant size	$0.8684_{1,22}$	0.3615
		Stem number	$0.3464_{1,21}$	0.5625
		Flower depth	$0.3898_{1,18}$	0.5403
		Flower width	$0.3278_{1,18}$	0.5740
	Seeds per fruit	Plant size	$0.4310_{1,22}$	0.5183
		Stem number	$1.6917_{1,22}$	0.2068
		Flower depth	$0.7699_{1,18}$	0.3918
		Flower width	**$9.0511_{1,15}$**	**0.0088**
Hermaphrodite	Fruit number	**Plant size**	**$11.3646_{1,23}$**	**0.0026**
		Stem number	**$6.6882_{1,18}$**	**0.0186**
		Flower depth	$2.2434_{1,18}$	0.1515
		Flower width	**$5.6376_{1,20}$**	**0.0277**
	Seeds per fruit	**Plant size**	**$7.0107_{1,20}$**	**0.0154**
		Stem number	**$4.7113_{1,20}$**	**0.0422**
		Flower depth	$2.9629_{1,19}$	0.1014
		Flower width	**$5.4645_{1,19}$**	**0.0305**
Hermaphrodite (two high-fruit plants omitted)	Fruit number	Plant size	$0.1289_{1,21}$	0.7231
		Stem number	**$6.6882_{1,18}$**	**0.0186**
		Flower depth	$0.3145_{1,20}$	0.5811
		Flower width	$0.3237_{1,20}$	0.5757
	Seeds per fruit	Plant size	$0.0245_{1,18}$	0.8774
		Stem number	$0.0018_{1,18}$	0.9671
		Flower depth	$0.1485_{1,17}$	0.7048
		Flower width	$1.5224_{1,17}$	0.2340

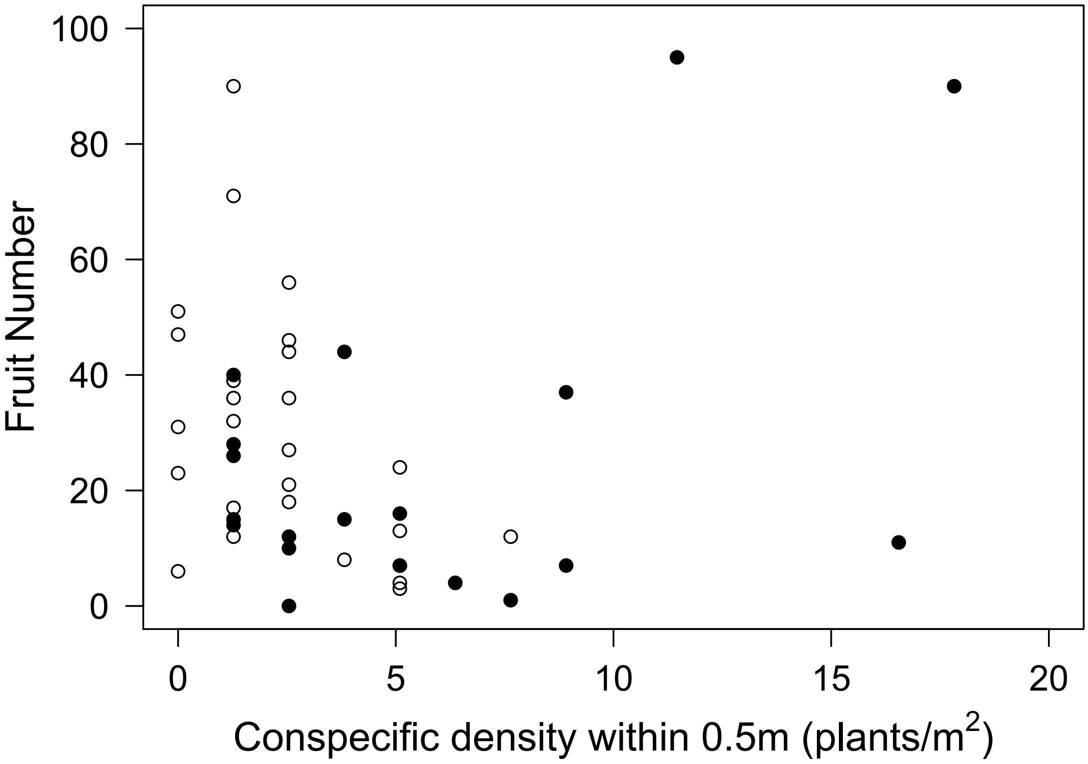

Figure 3.1. Conspecific density within 0.5 m of focal individuals affected fruit production in opposite directions in females (open circles) and hermaphrodites (filled circles). The positive relationship between density and fruit number in hermaphrodites was driven by two individuals at high densities that produced many fruits. Without the two influential points, there was no significant relationship between density and fruit number for hermaphrodites.

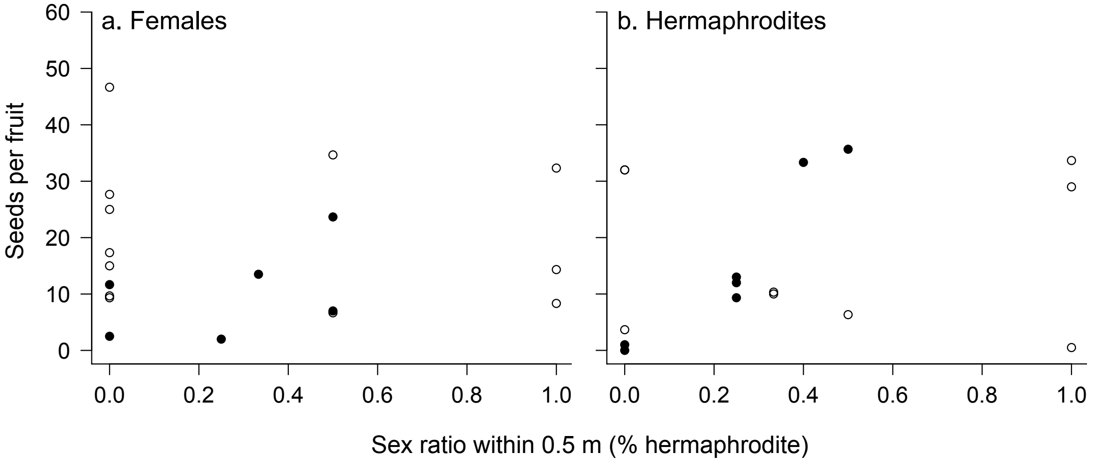

Figure 3.2. Conspecific density and sex ratio within 0.5 m of focal individuals affected seeds per fruit in female (a) and hermaphrodite (b) *Silene vulgaris* plants. Open circles represent individuals in low-density patches (< 2.55 individuals/m^2 for females and < 5 individuals/m^2 for hermaphrodites) and filled circles represent individuals in high-density patches (>= 2.55 individuals/m^2 for females and >= 5 individuals/m^2 for hermaphrodites). High- and low-density cutoffs were chosen to roughly equalize the number of plants in high- and low-density groups for visualization purposes.

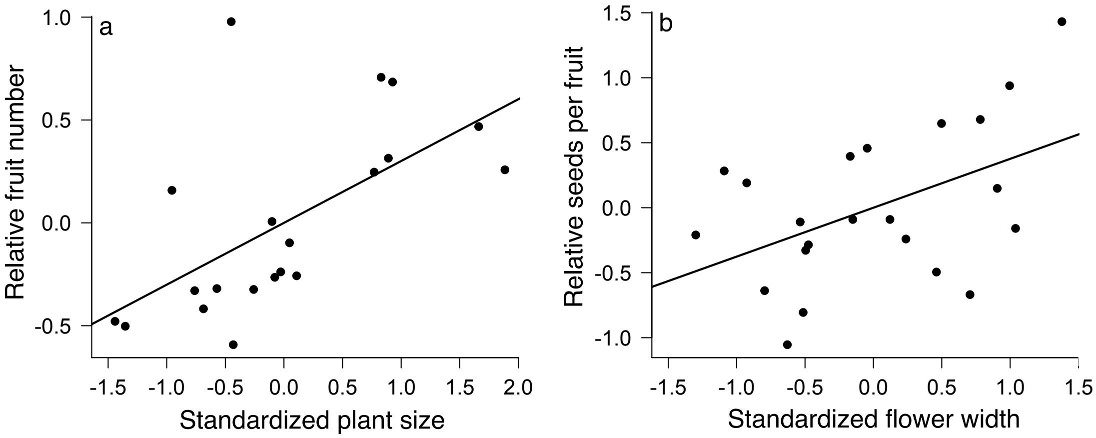

Figure 3.3. Female *Silene vulgaris* plants experienced significant positive directional selection on plant size (a), with fruit number as the fitness estimate, and flower width (b), with seeds per fruit as the fitness estimate. Plant traits were standardized to have a mean of 0 and a variance of 1. Relative fruit number is fruit number divided by mean fruit number and relative seeds per fruit is seeds per fruit divided by mean seeds per fruit. The relationship between each trait and fruit number has been corrected to account for the effects of the other traits on relative fruit number, which allows negative values to arise for relative fruit number. The regression lines represent the corrected linear relationship between the trait and relative fitness.

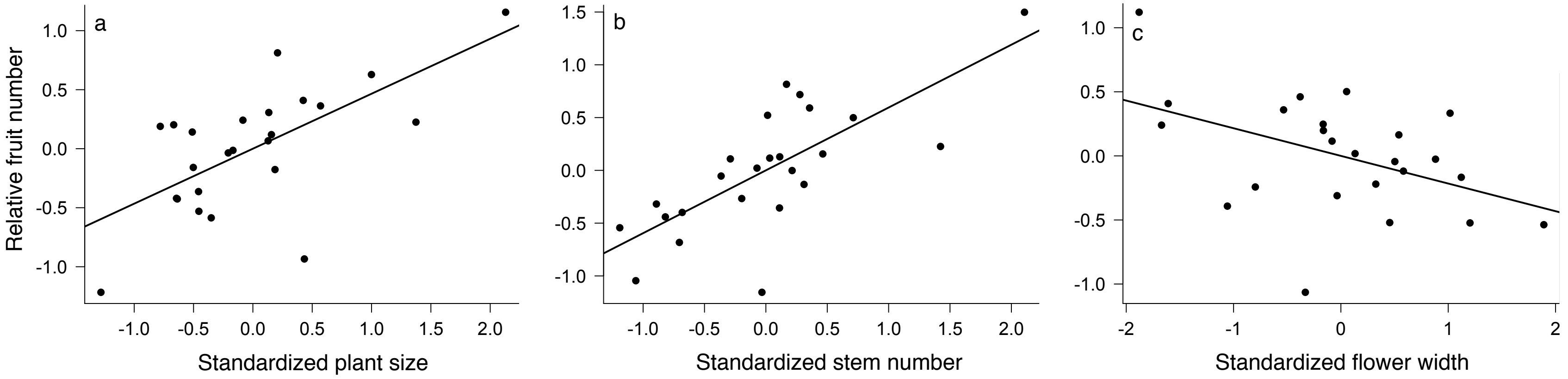

Figure 3.4. Hermaphrodite *Silene vulgaris* plants experienced significant positive directional selection on plant size (a) and stem number (b), and negative directional selection on flower width (c), with fruit number as the fitness estimate. Two influential large hermaphrodites are included in these figures; without them, there is no significant selection on flower width. Plant traits were standardized to have a mean of 0 and a variance of 1, while relative fruit number is fruit number divided by mean fruit number. The relationship between each trait and fruit number has been corrected to account for the effects of the other traits on relative fruit number, which allows negative values to arise for relative fruit number. The regression lines represent the corrected linear relationship between the trait and relative fruit number.

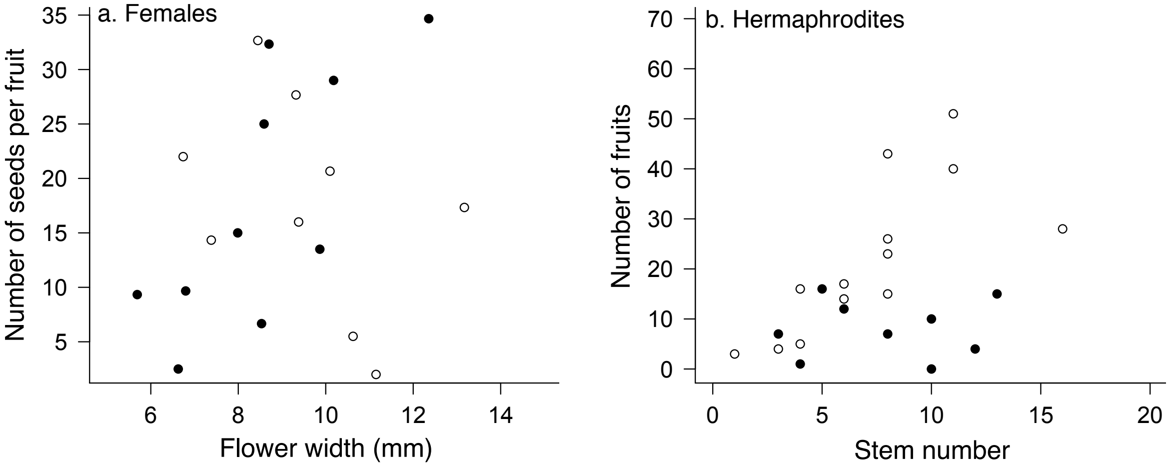

Figure 3.5. Conspecific density within 0.5 m of focal *Silene vulgaris* plants modified selection on flower width for females (a) and stem number for hermaphrodites (b). Open circles represent focal individuals growing in lower density patches (females < 2.5 individuals/m^2; hermaphrodites: < 1.5 individuals/m^2), while filled circles represent focal individuals growing in higher density patches (females: $>= 2.5$ individuals/m^2; hermaphrodites: $>= 1.5$ individuals/m^2). High- and low-density cutoffs were chosen to roughly equalize the number of plants in high- and low-density groups for visualization purposes. Hermaphrodites (b) are shown without two large and influential individuals.

CHAPTER 4

POLLINATOR SEX BIAS AFFECTS EQUILIBRIUM SEX RATIO AND FEMALE MAINTENANCE IN SIMULATED GYNODIOECIOUS PLANT POPULATIONS

Abstract

In sexually polymorphic plant species, pollinators often prefer one sex morph, with consequences for population-level mating patterns. Gynodioecy is a sexual polymorphism where individuals are female or hermaphrodite. In gynodioecious plant species, pollinators tend to prefer hermaphrodites. Because females require hermaphrodite pollen to produce seeds, pollinator preference for hermaphrodites can reduce seed production by females. Female fertility relative to hermaphrodites is likely to affect population sex ratio evolution and, ultimately, whether females can be maintained alongside hermaphrodites. Because gynodioecy is a common intermediate stage between hermaphroditism and the evolution of dioecy, understanding the conditions under which females are maintained with hermaphrodites sheds light on how plant breeding systems evolve.

To investigate how the intensity of pollinator sex preferences affects female maintenance and sex ratio evolution in gynodioecious plant populations, I built a linear discrete time computer simulation model. The model simulates pollination over a fixed number of generations under specified values of pollinator sex bias intensity, female seed production advantage over hermaphrodites, female progeny sex ratio, hermaphrodite progeny sex ratio, and initial population sex ratio. I also extracted parameter values for

three gynodioecious plant species from the literature, ran simulations using these parameter sets, and compared the equilibria attained by the model with sex ratios in natural populations to assess model performance.

Pollinator sex preference, female fertility advantage, female progeny sex ratio, and hermaphrodite progeny sex ratio all had substantial effects on sex ratio equilibria, but initial sex ratio did not. In the parameter space investigated, equilibria ranged from 0% hermaphrodite, representing population extinction, to 100% hermaphrodite, representing the loss of females and breakdown of gynodioecy. Several scenarios led to the loss of females, even when females were preferred by pollinators. Increasing pollinator hermaphrodite preference generally increased the frequency of hermaphrodites at equilibrium. Simulations using parameter values associated with real species yielded equilibrium sex ratios that overlapped with natural population sex ratios for all three species, suggesting that the model predicts realistic outcomes.

Even scenarios with only a slight pollinator preference for hermaphrodites yielded equilibria where females were lost, suggesting that weakly sex-biased pollination can lead to the breakdown of gynodioecy unless other factors compensate. Pollinator preference for females was able to maintain gynodioecy even when the female fertility advantage was less than theoretically required for nuclear gynodioecy. Realistic female fertility advantage values yielded the broadest range of possible equilibria, which is interesting because population sex ratios of gynodioecious plants are often highly variable. Future work could extend this model to include the effects of sex-biased herbivory on sex ratio evolution and female maintenance in gynodioecious plant populations.

Key words: female maintenance, gynodioecy, pollination, pollinator selectivity, sex-biased interactions, sex ratio, simulation model

Introduction

Because pollinators mediate outcrossing for animal-pollinated plants, their foraging preferences directly affect mating patterns in plant populations (Stanton et al. 1989, Galen 1989). While most flowering plants are hermaphrodite, with all individuals capable of producing both pollen and seeds, many plant species are sexually polymorphic (Barrett 2002). For sexually polymorphic plants, differences in attractive traits between sex morphs can result in pollinators preferentially visiting one sex morph. In gynodioecy, where individuals are either female or hermaphrodite, pollinators usually prefer hermaphrodites to females (Ashman 2000). If hermaphrodites have some degree of self-compatibility, as is common among gynodioecious taxa, hermaphrodite-biased pollinator visitation can increase the incidence of self-fertilization, which could increase inbreeding depression (Charlesworth and Charlesworth 1979). Pollinator hermaphrodite preference can also reduce seed production by females, simply by allowing fewer opportunities for ovule fertilization.

Like other trait-based pollinator foraging preferences, sex bias in pollinator visitation affects mating patterns within plant populations, which can affect the stability of breeding systems. Pollinator sex bias has long been implicated in transitions between gynodioecy, dioecy, and hermaphroditism and in the evolution and maintenance of sexual dimorphism (Ashman 2000), but we know little about how the intensity of pollinator sex bias affects sex ratio evolution and the maintenance of females within gynodioecious populations. The strength of pollinator sex bias varies among both plant and pollinator taxa and among plant populations (Table 4.1). Variation in the strength of pollinator sex bias likely stems from multiple factors, including the degree of sexual dimorphism in attractive traits and food rewards in the target plant species (Ashman et al. 2000) and the density and spatial arrangement of the plant sex morphs within populations (Stehlik et al. 2006, Spigler and Chang 2008).

In addition to pollinator sex bias, other factors are also likely to modulate sex ratio evolution in gynodioecious taxa. These factors include the extent of female fertility advantage over hermaphrodites and the genetics of sex determination. A great deal of theory has assessed the extent of the fertility advantage females require relative to hermaphrodites in order to be maintained in a population (Lewis 1941, Ross and Shaw 1971, Lloyd 1974), and many empirical studies have documented female fertility advantages in natural populations (reviewed in Dufay and Billard 2012). Similarly, theory has demonstrated that the genetics of sex determination affect the female fertility advantage required for gynodioecy to be maintained (Lewis 1941, Lloyd 1974). Sex in gynodioecious taxa is typically controlled by nuclear genes or cytonuclear interactions (Lewis 1941, Bailey and Delph 2007). In nuclear gynodioecy where male sterility (femaleness) is dominant, seeds produced by females will be 50% female and 50% hermaphrodite, and seeds produced by hermaphrodites will all be hermaphrodite (Lewis 1941). For nuclear gynodioecy where male sterility is recessive, female mothers would have 50 or 100% hermaphrodite progeny and hermaphrodites would have 75 or 100% hermaphrodite progeny (Lewis 1941). However, it is thought that most gynodioecious species have cytonuclear sex determination (Bailey and Delph 2007), where maternally-inherited mitochondrial alleles (hereafter "CMS factors") create females by inhibiting male function and nuclear alleles (hereafter "nuclear restorers") restore male function in hermaphrodites (Bailey et al. 2003). In cytonuclear gynodioecy, predicting progeny sex ratios for female and hermaphrodite seed mothers is much more complex, as it depends on the number and frequency of CMS factors and nuclear restorers in the population and whether the alleles are at stable equilibrium frequencies (Bailey and Delph 2007).

In this study, I use a simulation model to assess how the strength of pollinator sex bias, female fertility advantage, female progeny sex ratio, and initial sex ratio affect female maintenance and sex ratio evolution. I also compare the results of simulations using data from *Fragaria virginiana* (Bell 1985, Ashman 1999, 2000, 2003), *Geranium sylvaticum* (Ramula and Mutikainen 2003, Asikainen and Mutikainen 2003, 2005a, 2005b, Varga and Kytöviita 2010), and *Silene vulgaris* (Charlesworth and Laporte 1998, Olson et al. 2006, Doubleday and Adler 2017, Stone and Olson 2018, Doubleday, unpublished data) to known population sex ratios for each species to assess whether model results align with empirical data.

I address the following questions:

1. Do pollinator sex bias, female fertility advantage, female progeny sex ratio, and initial sex ratio affect equilibrium sex ratios and the stability of those equilibria?
2. What conditions lead to the loss of females or hermaphrodites from simulated populations?
3. How does the intensity of pollinator sex bias affect equilibrium sex ratios and the stability of gynodioecy?

Methods

Model details

I built a linear discrete-time simulation model in R (version 3.3.3, R Core Team 2017) that simulates pollination and reproduction in a gynodioecious plant population. The code required to run the model is available in Appendix D. The model runs for a specified number of generations and records the sex ratio in each generation. In building the model, I made the following simplifying assumptions:

1. The plants are annual.
2. Plant population size is fixed and constant across generations.
3. Pollinator population size is fixed and constant across generations. I chose the number of pollinators so that about 25% of the plants would be visited in each generation. Allowing only small numbers of plants to be visited would increase the variability of outcomes, making it more difficult to discern general parameter effects, while allowing the majority of plants to be visited would obscure effects of pollinator preference because preference is inconsequential when all or nearly all individuals are visited.
4. The intensity of pollinator sex preference is an innate property of the pollinators that is constant across generations, rather than being influenced by the plant population sex ratio.
5. Females and hermaphrodites make seeds with specified progeny sex ratios. Females producing 50% female and 50% hermaphrodite seeds and

hermaphrodites producing only hermaphrodite seeds represents single-locus nuclear sex determination where male sterility is dominant to male fertility.

6. Hermaphrodites are self-incompatible. All seeds produced in the population are the result of mating between two separate individuals.

When running the simulation model, the first generation starts with a fixed number of plants in a defined sex ratio and a fixed number of pollinators. Within a generation, each pollinator visits a randomly generated sequence of plants of a specified length (reported results use a 20-plant visit sequence). The sequences of plants visited are generated simultaneously for all pollinators. To incorporate pollinator preference for females or hermaphrodites, the model includes a term for pollinator sex bias intensity. A value of one indicates no bias, values less than one indicate pollinators prefer females, and values greater than one indicate that pollinators prefer hermaphrodites. This value is used to adjust the probability of pollinators visiting plants of each sex when generating sequences of plants for pollinators to visit.

In the simulation, each pollinator can only visit an individual plant once, but different pollinators can visit the same plants. For a plant to be pollinated, the previous plant visited by the pollinator must have been hermaphrodite, because only hermaphrodites produce pollen. The pollen from a hermaphrodite is completely deposited on the next plant the pollinator visits. For example, if a pollinator starts by visiting a female plant, she will not be pollinated because the pollinator is not yet carrying pollen. If the second plant in the pollinator’s visit sequence is a hermaphrodite, it will not be pollinated because the pollinator’s previous plant was female (no pollen), but the pollinator will collect pollen. The third plant that the pollinator visits will be pollinated, regardless of its sex, because the second plant visited was hermaphrodite. If multiple pollinators pollinate the same individual plant within a generation, that plant's seed production is only counted once in calculating the composition of the next generation, reflecting an assumption that one visit is sufficient for full seed set. To maintain a constant plant population size across generations, the sex ratio of seeds produced in one generation is multiplied by the specified population size to determine the numbers of females and hermaphrodites in the next generation.

I was most interested in the effects of four parameters: pollinator sex preference, initial sex ratio, female progeny sex ratio (FPSR), and the relative seed production advantage of females over hermaphrodites (F:H seeds). For pollinator sex bias, I chose a range of values that included pollinators preferring females (0.5), no sex preference (1), and three intensities of hermaphrodite bias (1.5, 2, 2.5). For initial plant population sex ratios, I used 5, 10, 25, 50, 75, 90, and 99% hermaphrodite. I used FPSR values of 50, 70, 80, 90, 95, and 99% female combined with only allowing seeds produced by hermaphrodite mothers to be hermaphrodite for the results reported in the main text. An FPSR value of 50% female combined with an HPSR value of 0 (hermaphrodite mothers only produce hermaphrodite seeds) reflects a scenario where plant sex is under nuclear control, male sterility (femaleness) is dominant, and female mothers are heterozygous (Lewis 1941, Ashman 1999). Combinations of FPSR values greater than 50% female and HPSR values greater than 0% female mimic more complex situations that arise in cytonuclear gynodioecy where the number of cytoplasmic male sterility alleles and nuclear restorers are variable. To model cytonuclear gynodioecy, I also performed simulations with FPSR values of 10, 25, 50, 75, and 99% female, and HPSR values of 1, 5, 25, and 40% female. Results for these additional FPSR and HPSR combinations are available in Appendix A (Figures S7–S10). For F:H seeds, I used 0.5, 1, 1.5, 2, and 2.5, which are realistic values based on published studies (Dufay and Billard 2012). An F:H seeds values of 2 is theoretically required for female maintenance in nuclear gynodioecy (Lewis 1941).

I used trial and error to determine an appropriate number of generations for which to run the simulations that would allow equilibria to be reached while also maximizing computational efficiency. The simulations tended to reach equilibrium sex ratios within 200 generations (Figure S11), and I chose to simulate for 1000 generations so that I could compute a mean equilibrium sex ratio value from the last 500 generations of each simulation run. I ran the model with each set of parameter values three times and took the mean of the mean equilibrium sex ratio for each set of parameter values across the three runs. I calculated the standard deviation of the mean sex ratio across the three runs as a measure of equilibrium stability.

Assessing sensitivity of equilibria to changes in pollinator sex bias

To assess how changes in the intensity of pollinator sex bias affect the sex ratio attained at equilibrium by a population, I increased pollinator sex bias by 20%, 50%, or 100% while holding other parameters constant and compared the resulting sex ratio equilibria to the equilibria attained without increasing pollinator sex bias. This manipulation was conducted for a variety of sets of parameter values.

Simulating real species

I obtained estimates of three model parameters (FPSR, pollinator sex bias, F:H seeds) from the literature (Bell 1985, Charlesworth and Laporte 1998, Ashman 1999, 2000, 2003, Ramula and Mutikainen 2003, Asikainen and Mutikainen 2003, 2005a, 2005b, Olson et al. 2006, Varga and Kytöviita 2010, Doubleday and Adler 2017, Stone and Olson 2018) and previously unpublished data for three relatively well-studied gynodioecious species: *Fragaria virginiana*, *Geranium sylvaticum*, and *Silene vulgaris* (Table 4.2). For *G. sylvaticum*, published FPSR values were not available, so I used a range of values between 0.25 and 0.95 (Table 4.2). For all three species, I ran the simulations with initial sex ratio values of 25, 50, 75, and 95% hermaphrodite. I used an HPSR value of 0 (no female seeds produced by hermaphrodite mothers) for *F. virginiana* because it has nuclear sex determination (Bailey and Delph 2007), and HPSR values of 0.01, 0.05, 0.25, and 0.40 for *G. sylvaticum* and *S. vulgaris* because they have cytonuclear sex determination (Bailey and Delph 2007, Varga and Kytöviita 2017). For each set of parameter values, I simulated sex ratio evolution as above, with 1000 generations per run and three runs per set of parameter values. I compared the resulting equilibria with published population sex ratios for each species (Table 4.2) to assess whether model results were realistic.

Results

Effects of initial conditions on sex ratio equilibria

The parameter space explored yielded sex ratio equilibria ranging from 0 (100% female, population goes extinct) to 100% hermaphrodite (no females, gynodioecy breaks down). Certain parameter combinations yielded wider ranges of equilibria (Figure 4.1). Of the four parameters of particular interest (initial sex ratio, pollinator sex bias, FPSR, and F:H seeds), all but initial sex ratio had substantial effects on equilibrium sex ratio (Figure 4.1). Increasing the proportion of female seeds produced by hermaphrodite mothers (HPSR) generally increased the number of females at equilibrium across values of other parameters (Figures S7–S10), therefore I focus on reporting detailed results for the simpler case of HPSR = 0.

Increasing the strength of pollinator bias towards hermaphrodites increased the proportion of hermaphrodites at equilibrium. Stable equilibria for cases where there was no pollinator sex bias ranged from 0 to 100% hermaphrodite, indicating that the effect of pollinator sex bias on sex ratio evolution depended on the values of other parameters in the model. Increasing F:H seeds increased the range of equilibrium sex ratios that could be reached (Figure 4.1). Increasing the proportion of seeds produced by female that were also female (FPSR) expanded the range of pollinator hermaphrodite bias values over which low-hermaphrodite equilibria were reached (Figure 4.1).

Several parameter value combinations led to the loss of females (Figure 4.2). Females could be lost, even when preferred by pollinators (i.e. pollinator hermaphrodite preference = 0.5) except when F:H seeds was greater than 1, or FPSR was greater than 0.7 and F:H seeds was at least 1 (Figure 4.2). Fewer parameter combinations led to the loss of females when F:H seeds and FPSR were both high (Figure 4.2). Some parameter combinations led to the loss of hermaphrodites and extinction of simulated populations (Figure 4.3), but there were fewer scenarios where hermaphrodites were lost than where females were lost. Hermaphrodites were never lost from simulated populations when pollinator hermaphrodite preference was greater than 1 or FPSR was less than 0.99 (Figure 4.3). Increasing F:H seeds when FPSR was high increased the number of scenarios where hermaphrodites were lost (Figure 4.3).

Effects of initial conditions on stability of sex ratio equilibria

Complex relationships among model parameters led to some combinations of parameter values yielding equilibria that were more stable (smaller standard deviations) than others (Figure 4.4). Standard deviations were uniformly low for initial conditions where F:H seeds was 0.5. Standard deviations varied substantially for conditions where F:H seeds was 1.5 or 2 and FPSR was 0.9 or greater (Figure 4.4). For these conditions, the highest standard deviations were generally associated with values of pollinator preference for hermaphrodites of 1.5 or 2.

Sensitivity of equilibria to changes in pollinator preference for hermaphrodites

Certain sets of initial conditions yielded equilibria with different sensitivities to pollinator hermaphrodite preference (Figure 4.5). Under initial conditions that yielded a particular equilibrium sex ratio, increasing pollinator preference for hermaphrodites generally increased the proportion of hermaphrodites present at equilibrium. The strength of increase in pollinator hermaphrodite preference affected the maximum range of possible equilibria produced; the more the strength of pollinator preference for hermaphrodites was increased, the larger that maximum range of equilibria became (Figure 4.5). For example, for initial conditions that yielded equilibria with 20% hermaphrodites, increasing pollinator hermaphrodite preference by 20% led to new equilibria ranging from 30 to 40% hermaphrodite; a 50% increase in pollinator preference led to new equilibria ranging from 50 to 75% hermaphrodite; and a 100% increase in pollinator hermaphrodite preference led to new equilibria ranging from 80 to 98% hermaphrodite (Figure 4.5).

Model performance with parameter values for real species

For the parameter values associated with each species, the model yielded a range of sex ratio equilibria that overlapped with known population sex ratios (Table 4.2). For *F. virginiana,* I observed a 100% hermaphrodite equilibrium, indicating the loss of females and breakdown of gynodioecy, and a 76% hermaphrodite equilibrium. The 76% hermaphrodite equilibrium was associated with F:H seeds of 1.57; higher F:H seeds values led to 100% hermaphrodite populations. For *G. sylvaticum*, the model yielded a larger range of equilibria (0 to 99% hermaphrodite) than has been reported for natural populations (Table 4.2). For *S. vulgaris*, I also obtained equilibria ranging from 0 to 99% hermaphrodite, which corresponded well with the extremely variable sex ratios reported for natural populations (Table 4.2).

Discussion

Pollinator sex bias

Small changes in the strength of pollinator sex bias had substantial effects on equilibrium sex ratios, reflecting how small but stable changes in pollination conditions could alter population sex ratios. In natural populations however, the strength of pollinator sex bias likely fluctuates within or across seasons. Pollinator fauna composition often shifts temporally (Pettersson 1991, Dupont et al. 2009, Burkle et al. 2013) and not all pollinator taxa share the same sex bias intensity (Delph and Lively 1992, Jordano 1993). For example, in *Prunus mahaleb*, bees preferred hermaphrodites but flies preferred females (Jordano 1993). Pollinator fauna also vary spatially, so that different plant populations experience different pollination conditions (Price et al. 2005, Bustamante et al. 2010). The functional sex ratio in sexually polymorphic plant populations also fluctuates in time and space depending on neighborhood sex ratios and individual flowering phenology (Wolfe and Burns 2001, Olson et al. 2006, Caruso and Case 2007, Bartkowska and Johnston 2014), adding an additional layer of complexity to natural systems.

In the present study, even a slight pollinator preference for hermaphrodites yielded scenarios where females were lost from simulated populations. This suggests that, for certain values of other parameters, it would be very difficult for gynodioecy to be maintained if there were any level of pollinator preference for hermaphrodites. For example, when females produced 1.5 times as many seeds as hermaphrodites (F:H seeds = 1.5), more than 70% of the seeds produced by female mothers needed to be female (FPSR > 0.7) for gynodioecy to be maintained. However, even when only half of the seeds produced by females were female (FPSR = 0.5), a sufficiently high female seed production advantage (F:H seeds) allowed gynodioecy to be maintained. Although a slight pollinator preference for hermaphrodites was able to destabilize gynodioecy, the degree of female fertility advantage and value of female progeny sex ratio were able to compensate and shift the outcome.

Female fertility advantage

Considerable theory has shown that, in gynodioecious systems, females must compensate for their lack of male function through an increase in seed production or seed quality relative to hermaphrodites (Charlesworth 1999), and female fertility advantage is a widespread phenomenon among gynodioecious plants (Shykoff et al. 2003, Dufay and Billard 2012). For gynodioecious taxa with nuclear sex determination, females must theoretically produce at least twice as many seeds as hermaphrodites in order to be maintained (Lewis 1941). This was borne out in my simulations. When FPSR was 0.5, females were lost from scenarios with no sex bias unless F:H seeds was greater than 2. If pollinators preferred females, females could be maintained when F:H seeds was only 1.5. Increasing FPSR reduced the F:H seeds value required for female maintenance.

For the majority of gynodioecious plants, sex is determined through cytonuclear interactions (Bailey and Delph 2007) and the fitness advantage required by females to be maintained in a population is much smaller than in nuclear gynodioecy (Bailey et al. 2003). In the simulations presented here, setting F:H seeds to 1.5 resulted in the most variable outcomes, reflected in the highest standard deviations. This is interesting because 1.5 is a realistic female fertility advantage value for plants with cytonuclear gynodioecy (Bailey et al. 2003, Dufay and Billard 2012) and it gave rise to a large range of population sex ratios, as we see across natural populations (Bailey and Delph 2007) and among species (Delph and Carroll 2001, Asikainen and Mutikainen 2003, Caruso and Case 2007, Miller and Stanton-Geddes 2007, Ruffatto et al. 2015). Taken together, results from my simulations suggest that the female seed production advantage that allows females to be maintained in populations allows them to be maintained at a wide range of frequencies.

Comparison with empirical data

For all three species, I obtained some equilibria that matched sex ratios of natural populations, suggesting that the model produces realistic outcomes. For *F. virginiana*, in addition to the equilibrium that corresponded to a natural population sex ratio, I also observed a 100% hermaphrodite equilibrium, indicating a loss of females and breakdown of gynodioecy. Some gynodioecious taxa do have populations that are entirely hermaphrodite (Caruso and Case 2007), but I suspect this result is a consequence of the model necessarily being a simplification of complex natural processes. Factors not included in the model may also be important in maintaining females in certain contexts. For *G. sylvaticum* and *S. vulgaris*, I obtained broad ranges of equilibrium sex ratios from the model, some of which corresponded to observed sex ratios in natural populations. Although I found correspondence between sex ratio equilibria from the model and natural population sex ratios for the three species, it is important to note that it may be rare for natural gynodioecious plant populations to be at stable sex ratio equilibria (Bailey and Delph 2007).

Extending the model to investigate the effects of sex-biased herbivory

The goal of this study was to take a first step towards understanding how the intensity of sex-biased pollination affects sex ratio evolution and female persistence in gynodioecious plant populations. In addition to pollinator sex bias, sex-biased herbivory has also been implicated in the maintenance of gynodioecy (Marshall and Ganders 2001) and the evolution of separate sexes in plants (Ashman 2002). It would be straightforward to modify the simulation presented here to incorporate the effects of sex-biased herbivory on female persistence and sex ratio evolution in gynodioecious taxa. One would add a section of code very similar to the pollinator sex bias section that calculates the number of seeds each visited plant loses to sex-biased herbivores in each generation. If the pollinator sex bias were set to one (no sex bias), the model would then simulate the effects of sex-biased herbivory, but one could also vary the pollinator sex bias to simultaneously investigate the effects of sex-biased pollination and herbivory, giving insight into the effects of multi-species interactions on sex ratio evolution and the maintenance of females.

Plant species commonly have multiple interaction partners, with possibilities including pollinators, leaf herbivores, florivores, pre-dispersal seed predators, mycorrhizal fungi, and parasitic plants (Vega-Frutis et al. 2013). Little is currently known about sex bias in multiple interactions in gynodioecious taxa, but a small number of studies have shown differences in sex-specific fitness effects of different types of interactions. In *Geranium sylvaticum*, pollinators and florivores both preferred hermaphrodites, but pollinators had a stronger hermaphrodite preference, and pre-dispersal seed predators had no sex preference (Asikainen and Mutikainen 2005b). In *Polemonium foliosissimum* a pre-dispersal seed predator was hermaphrodite-biased, but pollen limitation (a proxy for pollinator visitation) did not differ between the sexes (Clarke and Brody 2015). Because sex preferences are likely to be taxon and context-specific, simulation models like the one presented here would be an efficient way to gain preliminary insights and develop testable hypotheses about the effects of multiple sex-biased interactions on sex ratio evolution and the stability of gynodioecy.

Table 4.1. Evidence of pollinator sex bias in gynodioecious plants. H: hermaphrodite; F: female. When possible, I calculated the magnitude of sex bias for each species based on data provided in the cited studies, in terms of number of visits to hermaphrodites relative to females. If values were given for multiple populations, experiments, or specific pollinator taxa I calculated the magnitude of sex bias separately for each.

Family	**Species**	**Evidence of pollinator sex bias?**	**Direction of sex bias**	**Magnitude of sex bias**	**References**
Caryophyllaceae	*Silene vulgaris*	Yes	H > F	Diurnal visitors: 11 Nocturnal visitors: 2.5	(Stone and Olson 2018)
Geraniaceae	*Geranium richardsonii*	Yes	H > F	1.99	Williams et al. 2000
	Geranium sylvaticum	Yes	H > F	0.91 1.5 3.47 4.50	Asikainen and Mutikainen 2005b, Varga and Kytöviita 2010
Iridaceae	*Iris douglasiana*	Yes, but direct test not significant	H > F	1.43 2.86	Uno 1982
Lamiaceae	*Thymus vulgaris*	Yes	H > F	Not available	Assouad et al. 1978
Malvaceae	*Sidalcea oregana* ssp. *spicata*	Yes	H > F	1.40	Ashman and Stanton 1991
Plantaginaceae (formerly Scrophulariaceae)	*Veronica stricta* var. *atkinsonii* (formerly *Hebe stricta* var. *atkinsonii)*	Yes	Overall: H > F Individual taxa, except “other Diptera”: H > F “Other Diptera”: F > H	Overall: 3.54 *Lasioglossum sordidum*: 3.32 *Hylaeus* sp.: H only Hover flies: 16.50 Other Diptera: 6.50 All other visitors: H only	Delph and Lively 1992
Rosaceae	*Fragaria virginiana*	Yes	H > F	1.57 2.33 3.55	Bell 1985, Ashman 2000
	Prunus mahaleb	Yes	Overall: F > H Flies: F > H Bees: H > F	Overall: 0.68 Flies: 0.44 Bees: 1.45	Jordano 1993

Table 4.2. Parameter values used and sex ratio equilibria attained in simulations of three gynodioecious plant species. When values were unavailable for a given parameter, I used a range of possible values. FPSR is the proportion of seeds produced by female mothers that are also female, and F:H seeds is the fertility advantage of females over hermaphrodites. When ranges were given for a parameter value, both the minimum and maximum were used in simulations. For all three species, the initial sex ratios used were 25, 50, 75, and 95% hermaphrodite.

Species	**FPSR**	**F:H seeds**	**Pollinator hermaphrodite preference**	**Natural population sex ratios (% hermaphrodite)**	**Sex ratio equilibria from model (% hermaphrodite)**
Fragaria virginiana	0.5 (Ashman 1999)	4.09 (Ashman 2003)	1.57 (Bell 1985) 2.33 (Ashman 2000) 3.55 (Ashman 2000)	56, 65 (Ashman 2000) 58, 64, 72, 76, 79 (Ashman 1999)	76, 100
Geranium sylvaticum	Not available, used 0.25, 0.5, 0.75, 0.95	1.2 – 1.7 (Asikainen and Mutikainen 2005a) 1.2, 3.3 (Ramula and Mutikainen 2003)	0.91, 3.47, 4.50 (Asikainen and Mutikainen 2005b) 1.5 (Varga and Kytöviita 2010)	72.8 – 99.6 (Asikainen and Mutikainen 2003)	0, 9–16, 19–75, 80–99
Silene vulgaris	0.58 (Olson et al. 2006) 1 (Charlesworth and Laporte 1998)	1.32, 1.87 (Doubleday, unpublished data) 1.39, 2.27 (Stone and Olson 2018)	2.5, 11 (Stone and Olson 2018)	25–100 (McCauley et al. 2000) 65, 70, 75, 94, 95 (Doubleday and Adler 2017)	0, 23–25, 29–31, 33–40, 42–43, 49–54, 56–58, 61–64, 66–67, 69–73, 83–88, 90, 92–95, 97–99

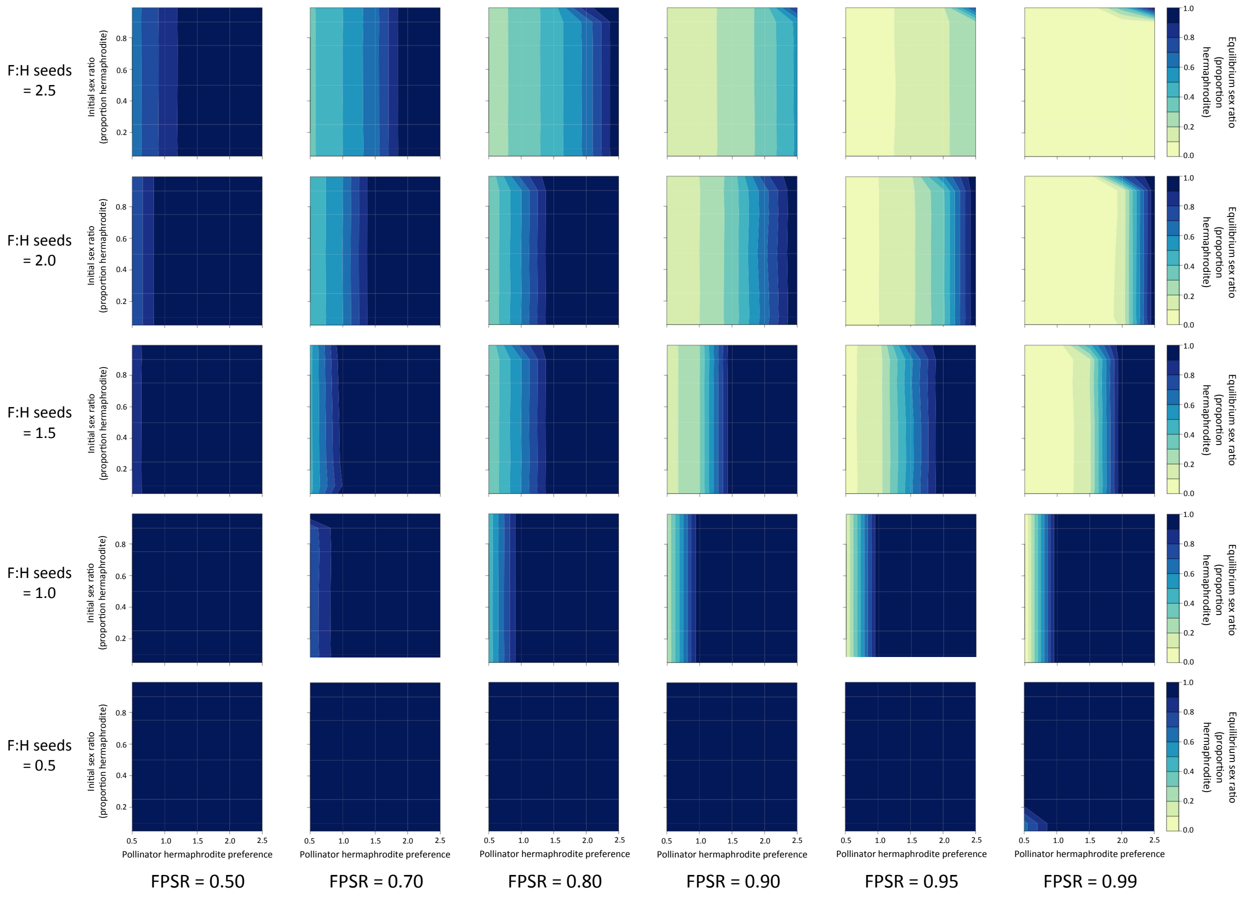

Figure 4.1. Different combinations of pollinator preference for hermaphrodites, initial sex ratio, female relative seed production advantage over hermaphrodites (F:H seeds), and the proportion of seeds produced by female plants that are also female (FPSR) yielded different sex ratio equilibria in simulated gynodioecious plant populations. This figure was made with raw output from simulations under different parameter value combinations. Each sub-panel represents the equilibria reached for combinations of pollinator preference for hermaphrodites and initial sex ratio at a particular value of F:H seeds and FPSR. Colors represent the mean sex ratio over the last 500 generations of simulations that ran for 1000 generations, with darker colors representing higher proportions of hermaphrodites. Each equilibrium is the mean for three runs of the simulation under identical initial conditions. Because each color represents a range of sex ratio values, it is not possible to infer loss of females (breakdown of gynodioecy) or loss of hermaphrodites (population extinction) from this figure. See Figure 3.2 for conditions where females are lost and gynodioecy breaks down, and Figure 3.3 for conditions where hermaphrodites are lost and populations go extinct. Parameter values used were as follows: initial sex ratio: 5, 10, 25, 50, 75, 90, 99% hermaphrodite; pollinator hermaphrodite preference: 0.5, 1, 1.5, 2, 2.5; F:H seeds: 0.5, 1, 1.5, 2, 2.5; FPSR: 0.5, 0.7, 0.8, 0.9, 0.95, 0.99. Pollinator hermaphrodite preference of 1 reflects no sex bias, < 1 reflects preference for females, and > 1 reflects preference for hermaphrodites.

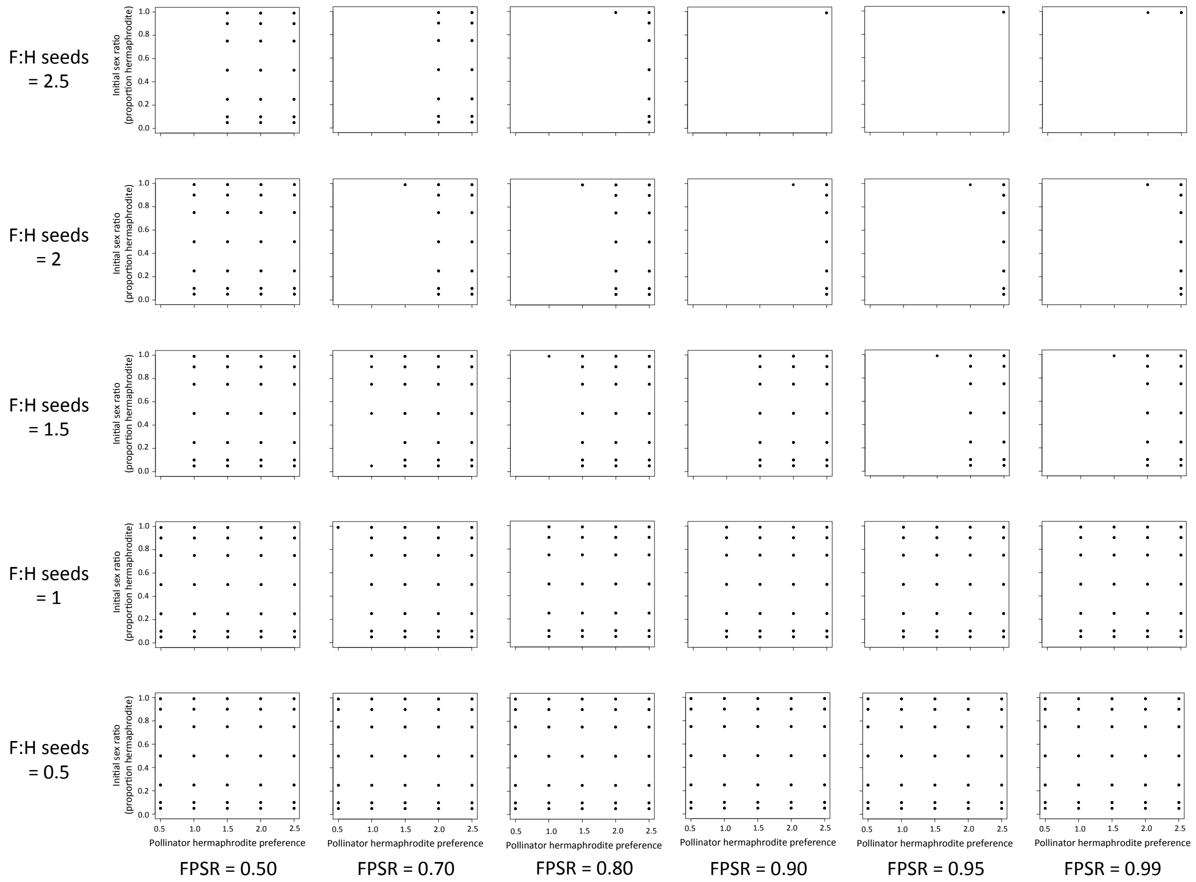

Figure 4.2. Conditions under which females were lost from simulated gynodioecious plant populations, indicating the breakdown of gynodioecy and a shift to hermaphroditism. Each point represents a combination of initial parameter values that resulted in equilibrium sex ratios that were 100% hermaphrodite. A set of discrete values was used for each parameter. F:H seeds is the relative fertility advantage of females over hermaphrodites and FPSR is the proportion of seeds produced by females that are also female.

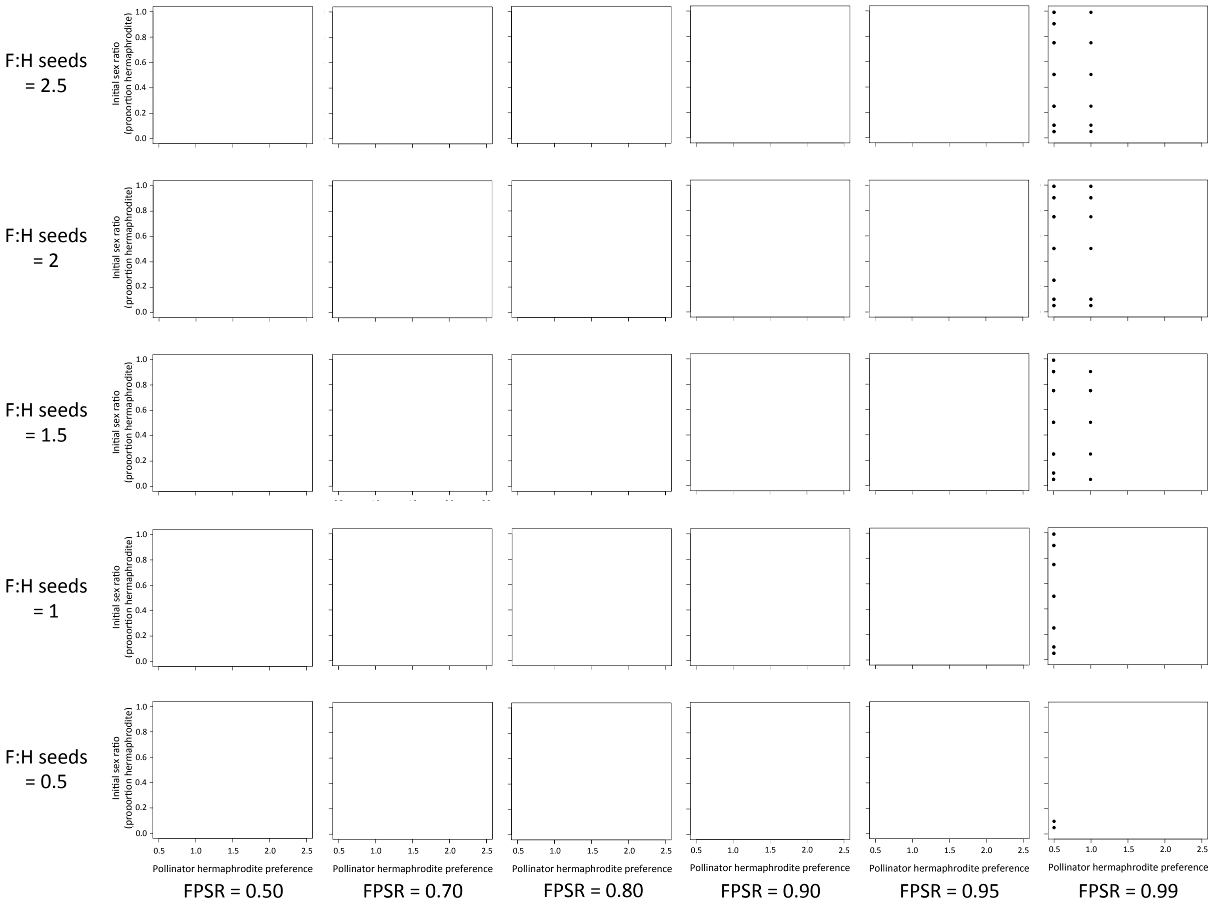

Figure 4.3. Only a small set of parameter value combinations yielded scenarios where hermaphrodites were lost from simulated gynodioecious plant populations. Losing hermaphrodites causes population extinctions because females must be fertilized by hermaphrodite pollen to produce seeds. Each point represents a combination of initial parameter values that resulted in a sex ratio that was 0% hermaphrodite and a corresponding population extinction. A set of discrete values was used for each parameter. F:H seeds is the relative fertility advantage of females over hermaphrodites and FPSR is the proportion of seeds produced by females that are also female.

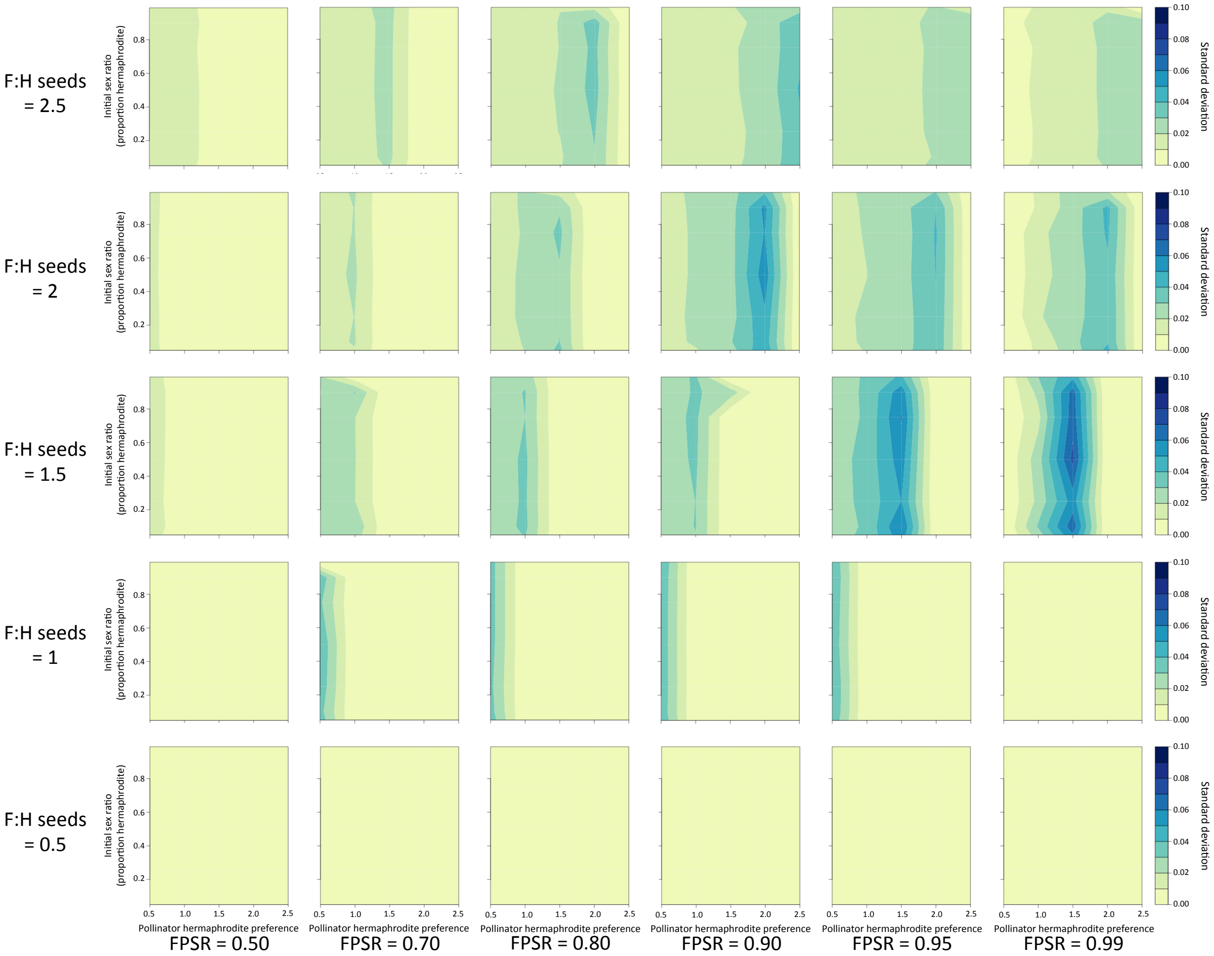

Figure 4.4. Different combinations of pollinator preference for hermaphrodites, initial sex ratio, female relative seed production advantage over hermaphrodites (F:H seeds), and the proportion of seeds produced by female plants that are also female (FPSR) yielded sex ratio equilibria with different stabilities. This figure shows the standard deviation associated with the mean sex ratio equilibrium for three runs of the simulation under the same set of initial conditions for the parameter combinations used to create Figure 3.1. Each sub-panel represents the standard deviations for equilibria reached for combinations of pollinator preference for hermaphrodites and initial sex ratio at a particular value of F:H seeds and FPSR. Colors represent the mean standard deviation of sex ratio over the last 500 generations of simulations that were run for 1000 generations, with darker colors representing higher standard deviations.

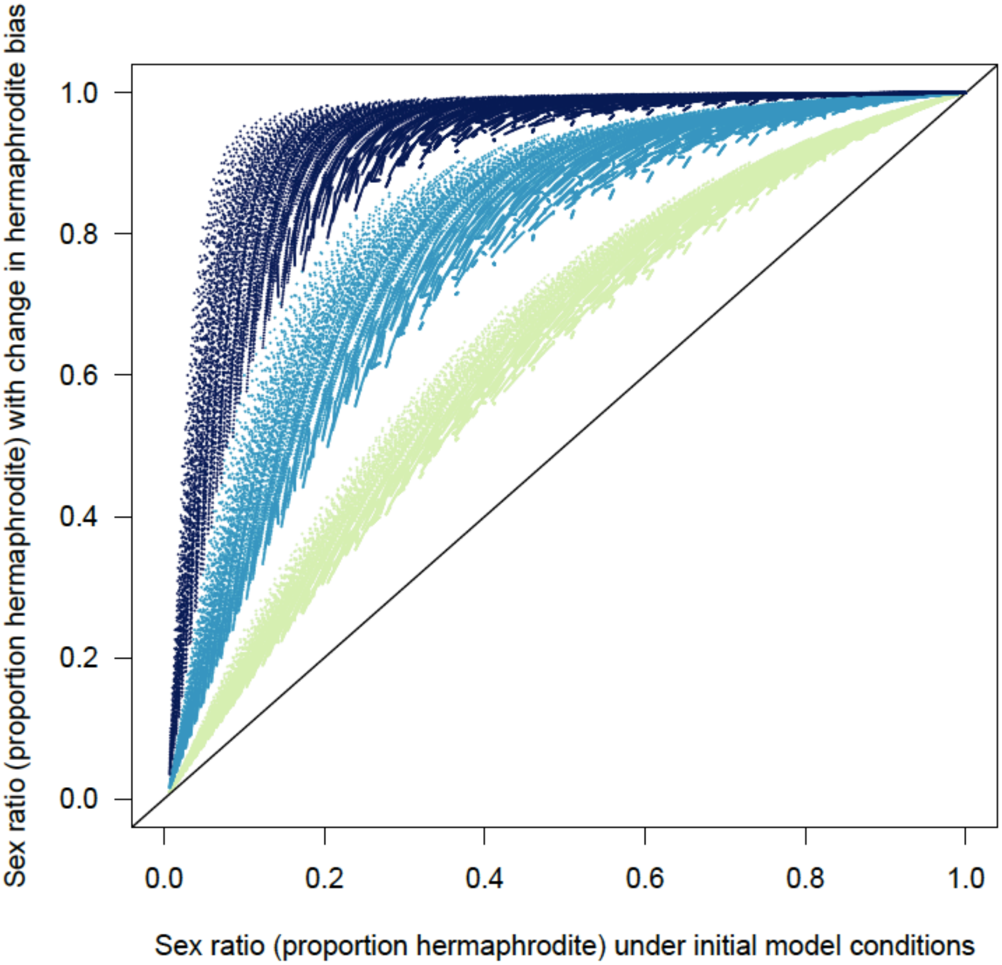

Figure 4.5. Effect of increased pollinator sex bias towards hermaphrodites on sex ratio equilibria. The x coordinate of each point is the sex ratio equilibrium attained under a given set of parameter values, while the y coordinate is the sex ratio equilibrium attained with the same set of parameters except for pollinator hermaphrodite bias, which was increased by 20% (green), 50% (medium blue), or 100% (dark blue). A point would fall on the one-to-one line if there were no change in the sex ratio equilibrium when pollinator sex bias was increased.

APPENDIX A

SUPPLEMENTARY FIGURES

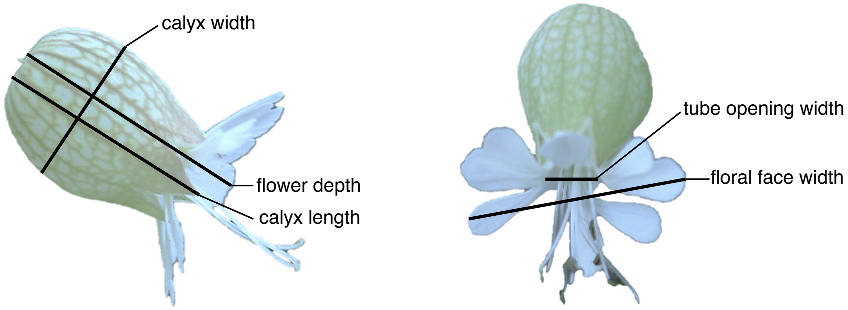

Figure S1. Diagram of floral traits measured. Left: side view of *Silene vulgaris* flower showing calyx width, flower depth, and calyx length measurements. Right: front view of *S. vulgaris* flower showing tube opening width and floral face width measurements.

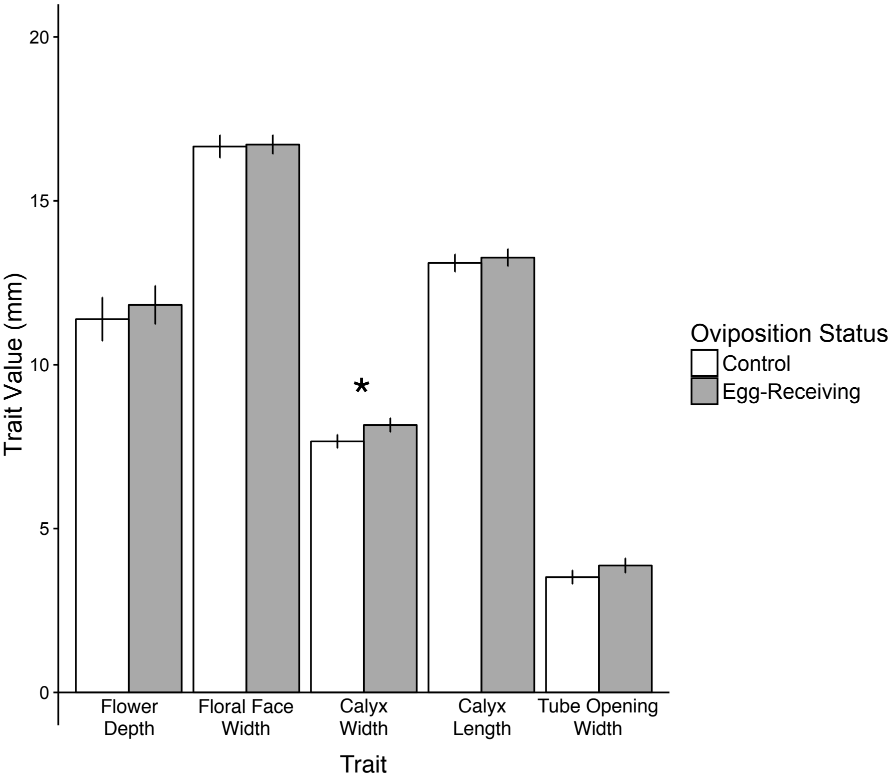

Figure S2. Differences in five floral traits between egg-receiving and control flowers. Egg-receiving flowers had significantly wider calyces than control flowers, but there were no differences between the groups in the other floral traits measured. Sample size = 36 egg-receiving and 36 control flowers. Error bars are ±1 standard error of the mean. Asterisk indicates a significant difference ($P < 0.05$) between egg-receiving and control flowers. See Fig. S1 for details on floral traits measured.

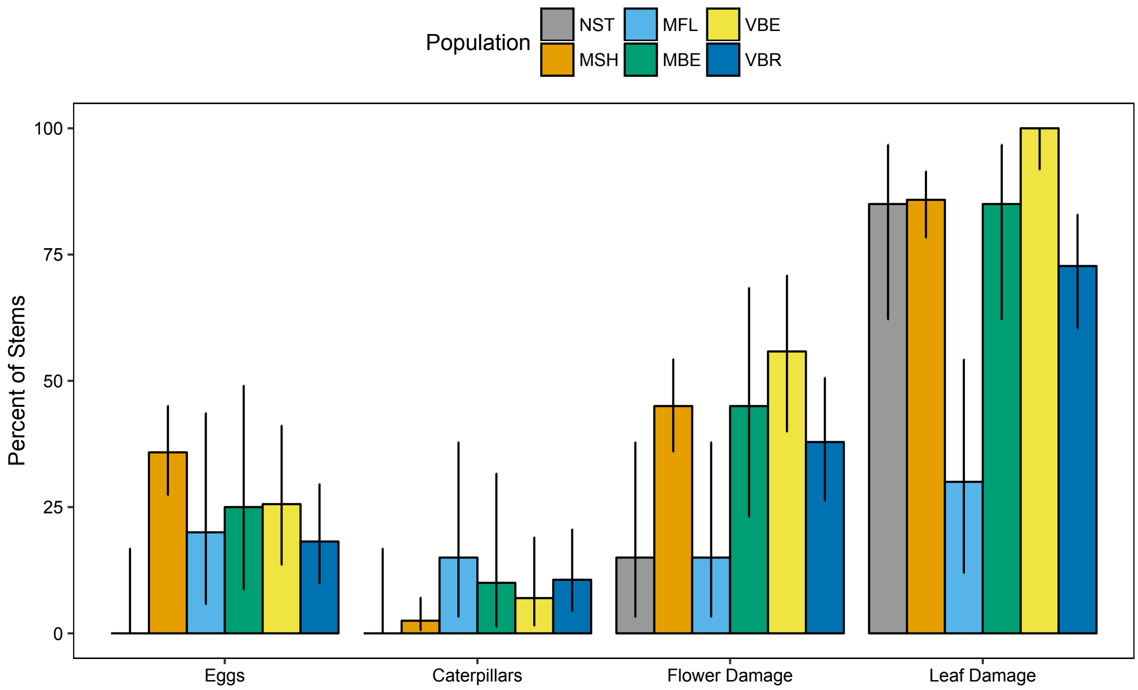

Figure S3. Percent of *S. vulgaris* stems with *H. ectypa* eggs, caterpillars, flower damage, and leaf damage at each of six populations surveyed in 2014. Flower damage was likely due to *H. ectypa* caterpillars, but leaf damage was probably not. Error bars are 95% binomial confidence intervals. Sample sizes: NST = 20; MSH = 120; MFL = 20; MBE = 20; VBE = 43; VBR = 66.

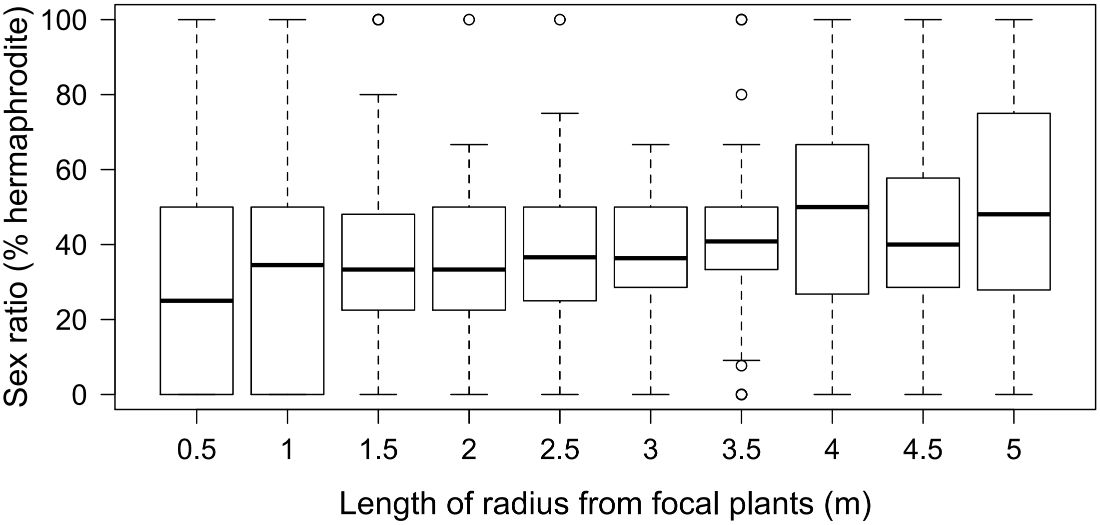

Figure S4. Sex ratio (percent hermaphrodite) of *Silene vulgaris* plants as a function of distance (m) from focal individuals. Boxes represent 25th and 75th percentiles, horizontal lines are medians, upper whiskers are either the maximum or the 75th percentile plus 1.5 times the interquartile range (whichever is less), and the lower whiskers are either the minimum value or the 25th percentile minus 1.5 times the interquartile range (whichever is greater). Points are extreme observations that lie outside the range of the whiskers. Data shown at a radius of 0.5 m from focal plants represent densities 0–0.5 m from focal plants, data at a radius of 1 m represent densities from 0.5–1 m from focal plants, and so on, increasing in 0.5 m increments.

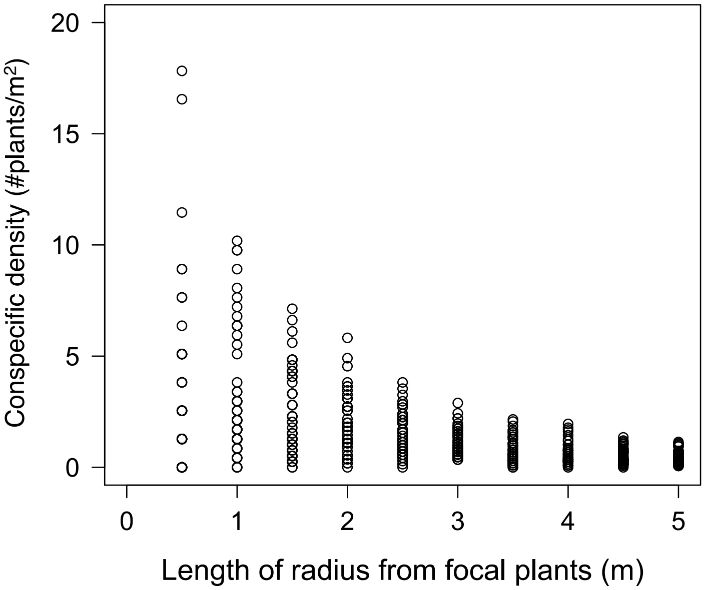

Figure S5. Conspecific density (number of *Silene vulgaris* plants/m^2) as a function of distance (m) from focal individuals. Within a radius, each point represents a single focal plant. Points shown at a radius of 0.5 m from focal plants represent densities 0–0.5 m from focal plants, points at a radius of 1 m represent densities from 0.5–1 m from focal plants, and so on, increasing in 0.5 m increments.

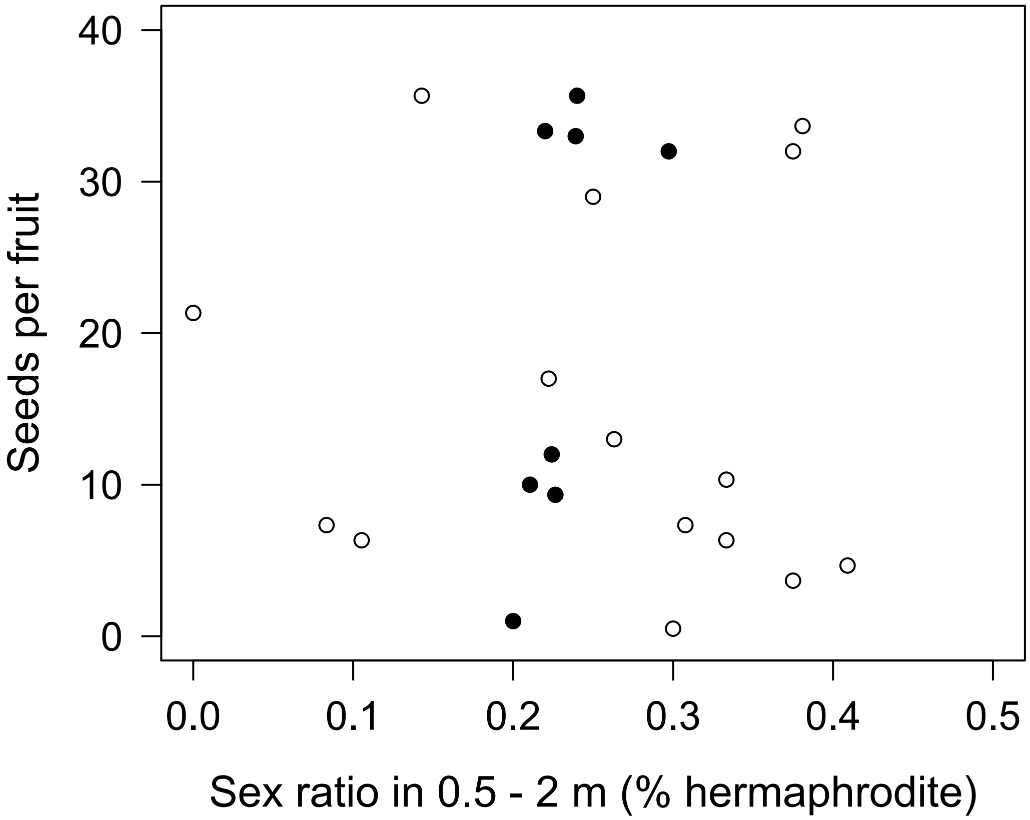

Figure S6. Conspecific density and sex ratio within 0.5 – 2 m affected seeds per fruit in hermaphrodite *Silene vulgaris* plants. Open circles represent hermaphrodites in low-density patches (< 3 individuals/m^2) and filled circles represent hermaphrodites in high-density patches (>= 3 individuals/m^2). High- and low-density cutoffs were chosen to roughly equalize the number of plants in high- and low-density groups for visualization purposes.

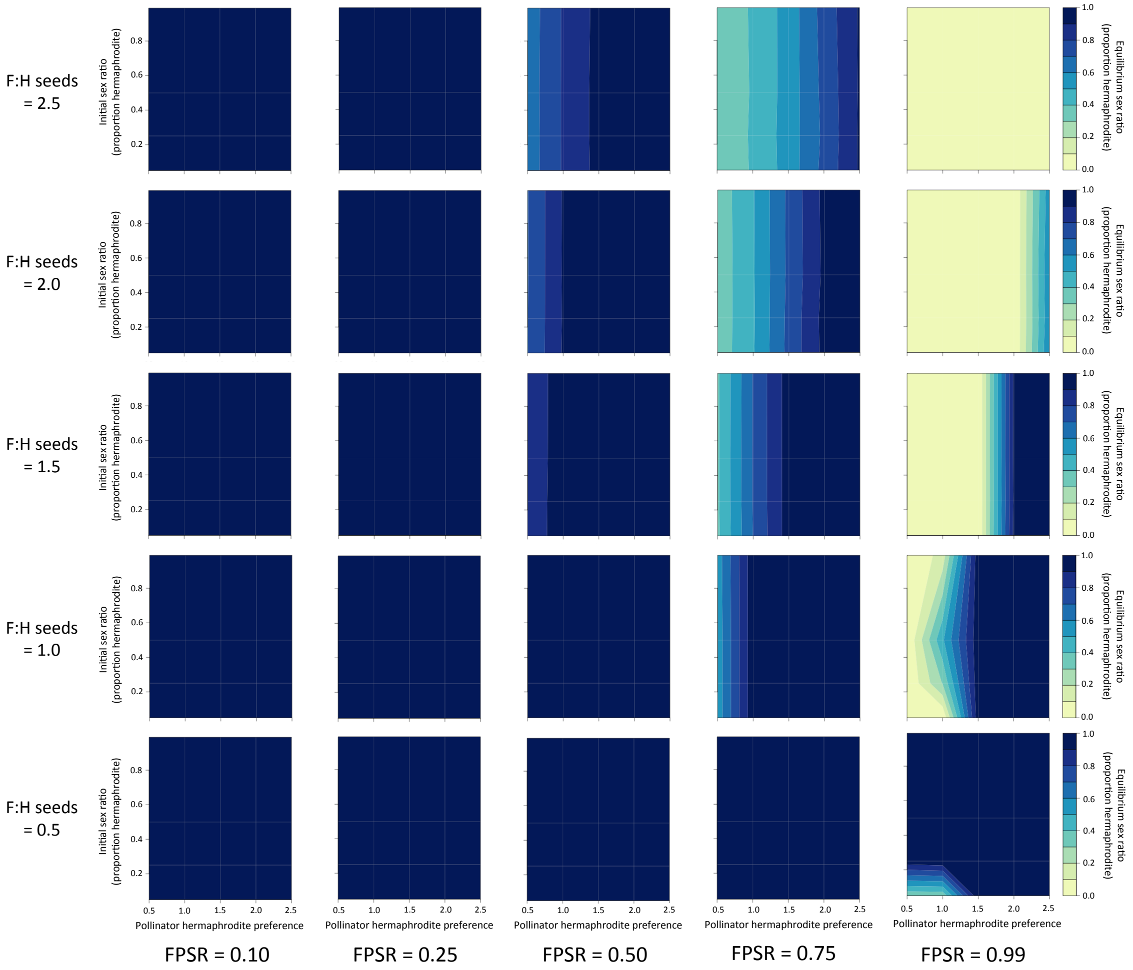

Figure S7. Equilibrium sex ratios produced under different combinations of pollinator preference for hermaphrodites, initial sex ratio, female relative seed production advantage over hermaphrodites (F:H seeds), and the proportion of seeds produced by female plants that are also female (FPSR), when 1% of seeds produced by hermaphrodite mothers are female. Each sub-panel represents the equilibria reached for combinations of pollinator preference for hermaphrodites and initial sex ratio at a particular value of F:H seeds and FPSR. Colors represent the mean sex ratio over the last 500 generations of simulations that ran for 1000 generations, with darker colors representing higher proportions of hermaphrodites. Each equilibrium is the mean of three runs of the simulation under identical initial conditions. Because each color represents a range of sex ratio values, it is not possible to infer loss of females (breakdown of gynodioecy) or loss of hermaphrodites (population extinction) from this figure. Parameter values used were as follows: initial sex ratio: 0.05, 0.25, 0.50, 0.99; pollinator hermaphrodite preference: 0.5, 1, 1.5, 2, 2.5; F:H seeds: 0.5, 1, 1.5, 2, 2.5; FPSR: 0.10, 0.25, 0.50, 0.75, 0.99. Pollinator hermaphrodite preference of 1 reflects no sex bias, <1 reflects preference for females, and >1 reflects preference for hermaphrodites.

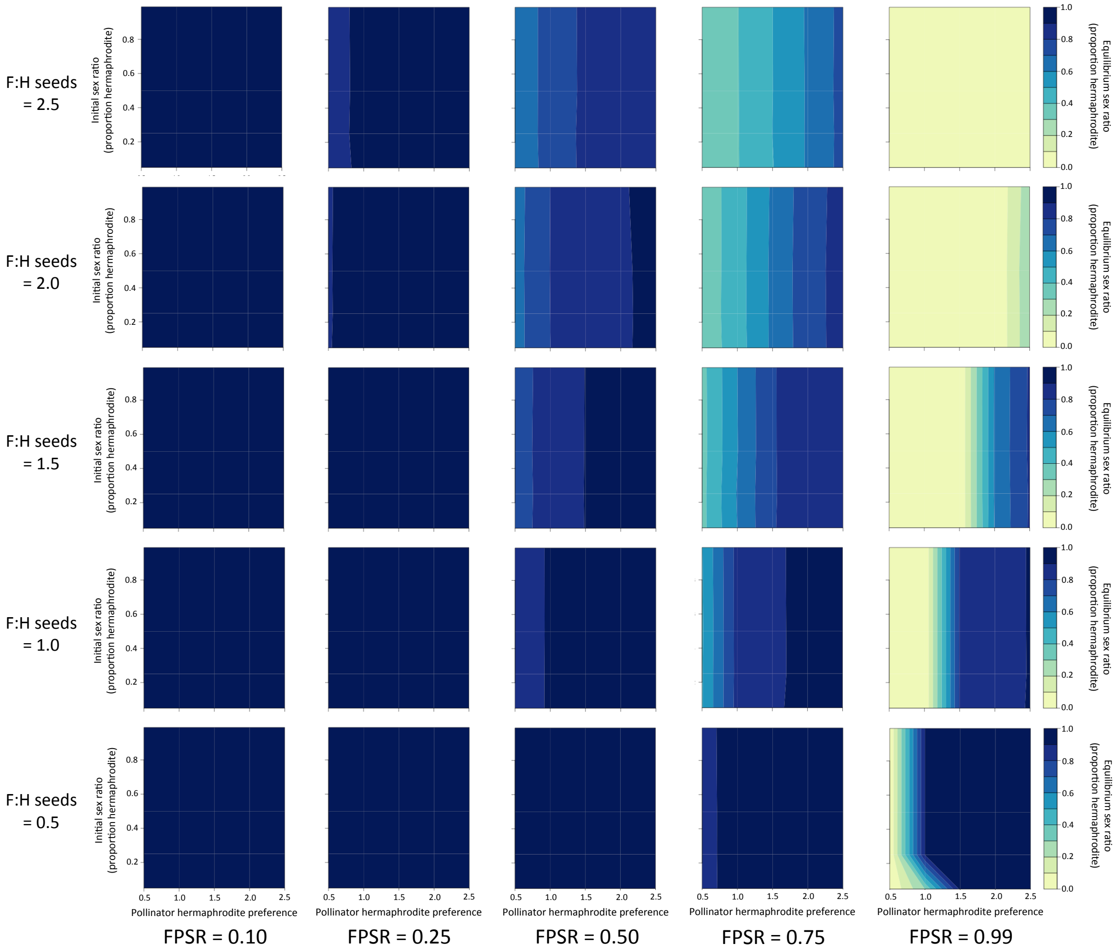

Figure S8. Equilibrium sex ratios produced under different combinations of pollinator preference for hermaphrodites, initial sex ratio, female relative seed production advantage over hermaphrodites (F:H seeds), and the proportion of seeds produced by female plants that are also female (FPSR), when 5% of seeds produced by hermaphrodite mothers are female. Each sub-panel represents the equilibria reached for combinations of pollinator preference for hermaphrodites and initial sex ratio at a particular value of F:H seeds and FPSR. Colors represent the mean sex ratio over the last 500 generations of simulations that ran for 1000 generations, with darker colors representing higher proportions of hermaphrodites. Each equilibrium is the mean of three runs of the simulation under identical initial conditions. Because each color represents a range of sex ratio values, it is not possible to infer loss of females (breakdown of gynodioecy) or loss of hermaphrodites (population extinction) from this figure. Parameter values used were as follows: initial sex ratio: 0.05, 0.25, 0.50, 0.99; pollinator hermaphrodite preference: 0.5, 1, 1.5, 2, 2.5; F:H seeds: 0.5, 1, 1.5, 2, 2.5; FPSR: 0.10, 0.25, 0.50, 0.75, 0.99. Pollinator hermaphrodite preference of 1 reflects no sex bias, <1 reflects preference for females, and >1 reflects preference for hermaphrodites.

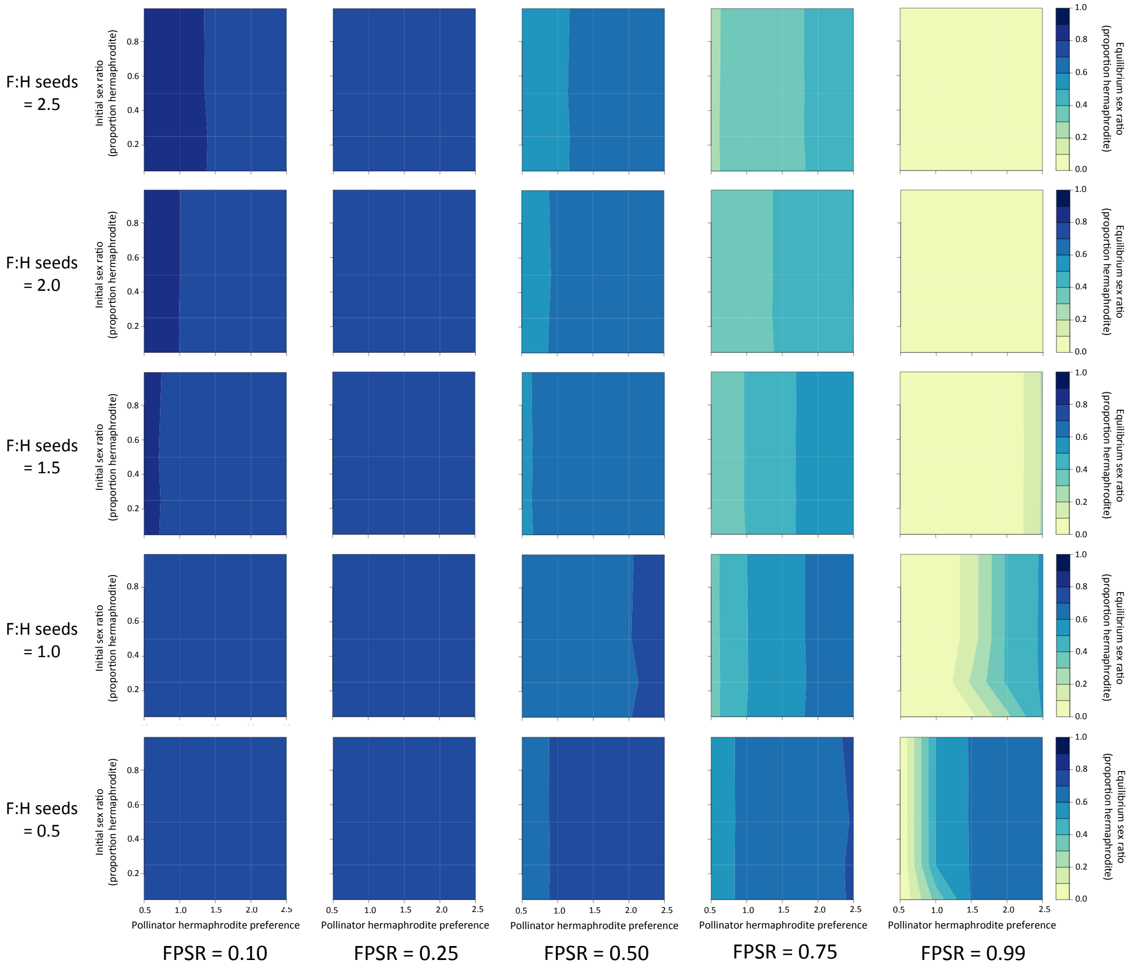

Figure S9. Equilibrium sex ratios produced under different combinations of pollinator preference for hermaphrodites, initial sex ratio, female relative seed production advantage over hermaphrodites (F:H seeds), and the proportion of seeds produced by female plants that are also female (FPSR), when 25% of seeds produced by hermaphrodite mothers are female. Each sub-panel represents the equilibria reached for combinations of pollinator preference for hermaphrodites and initial sex ratio at a particular value of F:H seeds and FPSR. Colors represent the mean sex ratio over the last 500 generations of simulations that ran for 1000 generations, with darker colors representing higher proportions of hermaphrodites. Each equilibrium is the mean of three runs of the simulation under identical initial conditions. Because each color represents a range of sex ratio values, it is not possible to infer loss of females (breakdown of gynodioecy) or loss of hermaphrodites (population extinction) from this figure. Parameter values used were as follows: initial sex ratio: 0.05, 0.25, 0.50, 0.99; pollinator hermaphrodite preference: 0.5, 1, 1.5, 2, 2.5; F:H seeds: 0.5, 1, 1.5, 2, 2.5; FPSR: 0.10, 0.25, 0.50, 0.75, 0.99. Pollinator hermaphrodite preference of 1 reflects no sex bias, <1 reflects preference for females, and >1 reflects preference for hermaphrodites.

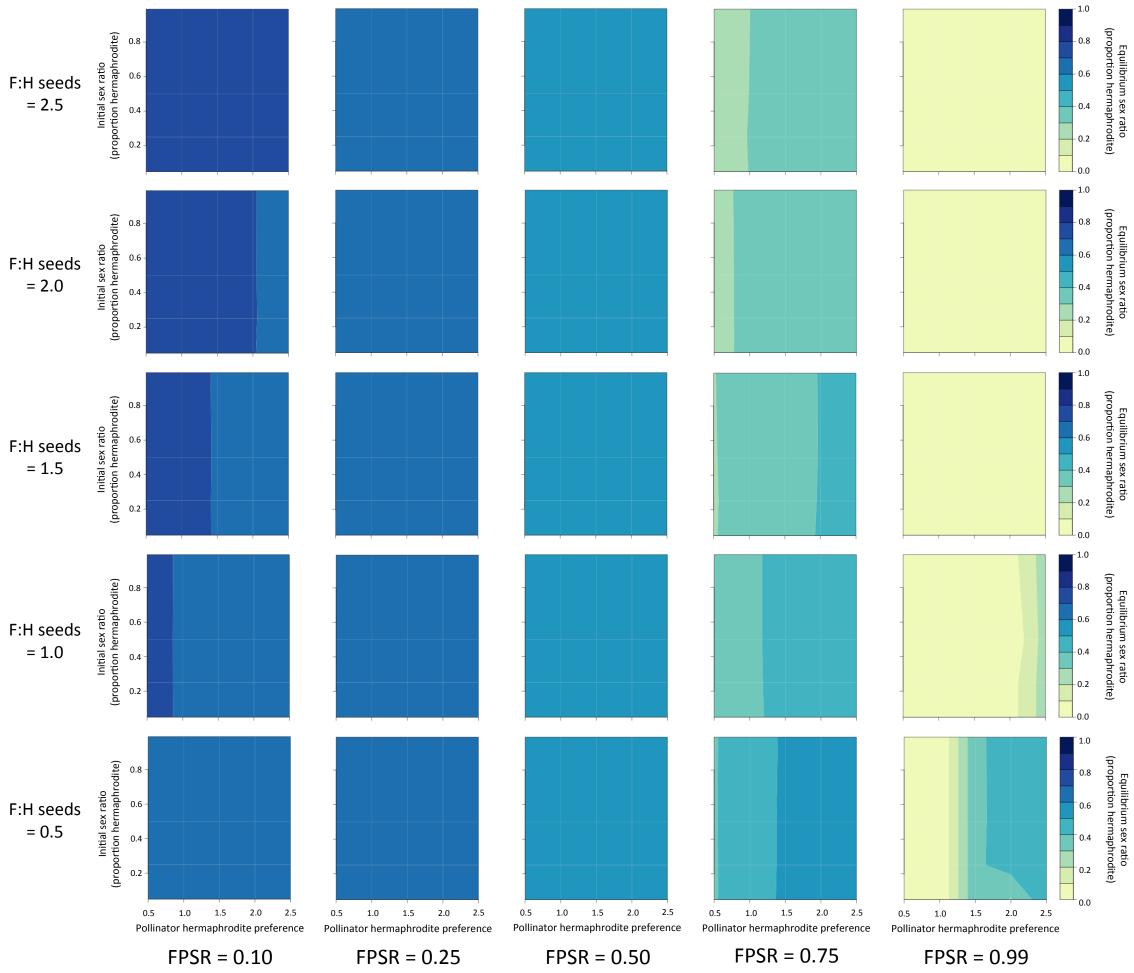

Figure S10. Equilibrium sex ratios produced under different combinations of pollinator preference for hermaphrodites, initial sex ratio, female relative seed production advantage over hermaphrodites (F:H seeds), and the proportion of seeds produced by female plants that are also female (FPSR), when 40% of seeds produced by hermaphrodite mothers are female. Each sub-panel represents the equilibria reached for combinations of pollinator preference for hermaphrodites and initial sex ratio at a particular value of F:H seeds and FPSR. Colors represent the mean sex ratio over the last 500 generations of simulations that ran for 1000 generations, with darker colors representing higher proportions of hermaphrodites. Each equilibrium is the mean of three runs of the simulation under identical initial conditions. Because each color represents a range of sex ratio values, it is not possible to infer loss of females (breakdown of gynodioecy) or loss of hermaphrodites (population extinction) from this figure. Parameter values used were as follows: initial sex ratio: 0.05, 0.25, 0.50, 0.99; pollinator hermaphrodite preference: 0.5, 1, 1.5, 2, 2.5; F:H seeds: 0.5, 1, 1.5, 2, 2.5; FPSR: 0.10, 0.25, 0.50, 0.75, 0.99. Pollinator hermaphrodite preference of 1 reflects no sex bias, <1 reflects preference for females, and >1 reflects preference for hermaphrodites.

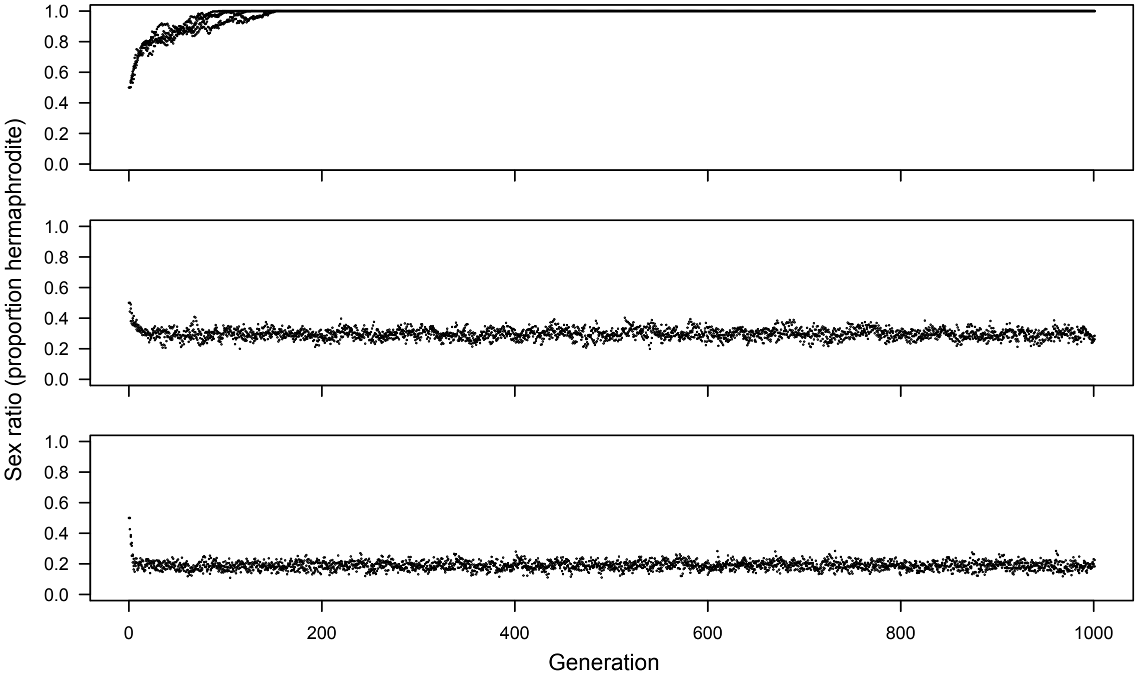

Figure S11. Population sex ratios stabilized in less than 200 generations across sets of parameter values. A different set of parameter values was used in the simulations shown in each panel. Within a panel, the points show population sex ratio trajectories for three runs of the model with the same parameter values.

APPENDIX B

SUPPLEMENTARY TABLES

Table S1. Population codes, locations, sex ratios, and *Hadena ectypa* egg and caterpillar abundance at *Silene vulgaris* study sites.

Population Code	**Location**	**Latitude (° N)**	**Longitude (° W)**	**Year(s) Studied**	**Sample Size**	**Sex Ratio (% Female)**	**# Eggs Observed**	**# Caterpillars Observed**
NST	Stamford, NY	42.39360	74.60000	2014	20	40	0	0
MSH	Sheffield, MA	42.08307	73.36524	2014, 2015	120 (2014); 160 (2015)	35 (2014)	71 (2014)	3 (2014)
MFL	Florida, MA	42.68278	73.01811	2014	20	25	7	3
MBE	Bernardston, MA	42.68178	72.54431	2014	20	30	7	3
VBE	Bennington, VT	42.85460	72.98096	2014	43	5	14	5
VBR	Bristol, VT	44.15323	73.04536	2014	66	6	14	8

Table S2. *Silene vulgaris* traits associated with oviposition by *Hadena ectypa* moths. Results are from likelihood ratio tests comparing binomial generalized linear models that differ in the presence of one predictor (trait). Non-significant traits were removed one by one from the model to arrive at a final model containing only traits that were significant predictors of oviposition. After the final model was determined, a test statistic (LR X^2) and *P*-value for each non-significant predictor was obtained by comparing the final model (with flower depth and stem number as the only predictors) to a model containing the significant predictors and the non-significant term of interest; these values are reported in the table below for non-significant terms. Degrees of freedom = 1 for each test.

Trait	***LR*** X^2	***P***
Flower depth	4.61	0.032
Stem number	5.61	0.018
Height	0.019	0.89
Projected area	0.088	0.77
Number of open flowers	2.38	0.12
Flower width	0.85	0.36
Plant sex	1.22	0.27

Table S3. Mean seed production by eight pairs of female and 21 pairs of hermaphroditic *Silene vulgaris* flowers at population MSH in 2015. Within a pair, both flowers were on the same individual plant and one flower had received an *H. ectypa* egg (egg-receiving) while the other had not (non-egg-receiving). The non-egg-receiving flower was selected as the flower on the plant that most closely matched the egg-receiving flower's developmental stage. Only pairs of flowers where both the egg-receiving and non-egg-receiving flowers produced seeds were included in these calculations.

Flower Sex	**Oviposition Status**	**Mean Seeds Produced (± 1SE)**
Female	Egg-receiving	18.25 ± 5.99
	Non-egg-receiving	20.25 ± 6.05
Hermaphrodite	Egg-receiving	9.67 ± 3.40
	Non-egg-receiving	12.67 ± 3.92

Table S4. Results of statistical tests for sex bias in bud, petal, calyx, and ovary damage in July and August 2015 at population MSH. To obtain a *P*-value for each type of damage in each of the time periods, we used likelihood ratio tests to compare binomial generalized linear models that included plant sex as a predictor or included only an intercept. Petal damage was hermaphrodite-biased in July and calyx damage was hermaphrodite-biased in August. Degrees of freedom = 1 for each test.

Time	**Structure Damaged**	***LR*** X^2	***P***
July	Bud	0.86	0.36
	Calyx	0.22	0.64
	Petal	7.74	0.0054
	Ovary	0.91	0.34
August	Bud	3.19	0.074
	Calyx	12.67	0.00037
	Petal	0.55	0.46
	Ovary	1.60	0.21

APPENDIX C

SUPPLEMENTARY METHODS

Methods for *Silene vulgaris* Greenhouse Study in Chapter 1

Silene vulgaris plants were grown in a greenhouse at the authors' institution from seed collected from population MSH in summer 2013. On December 11 and 12, 2013, seeds were planted into black plastic 128-plug trays, with one seed per cell (T.O. Plastics, Clearwater, Minnesota, USA) and maintained in a propagation house with natural light and a constant temperature of 23.89°C until December 21, 2013, when seedlings were transferred to a greenhouse with 14 hours of supplemental light (0600–2200h) and temperatures of 22.22°C during the day and 18.89°C at night. Between December 21, 2013 and January 26, 2013, seedlings were transferred to another greenhouse with 14 hours of supplemental light (0600–2200h) and temperatures of 23.89°C during the day and 18.33°C at night, where they were maintained for the rest of the study. On January 26 and 27, 2014, seedlings were transplanted to individual 164mL Conetainers (model SC10R; Stuewe & Sons, Inc. Tangent, Oregon, USA). The substrate used at all stages was (by volume) 50% High-Porosity Promix® (Premier Tech Horticulture, Quakertown, Pennsylvania, USA), 25% autoclaved topsoil from the University of Massachusetts South Deerfield Farm, and 25% autoclaved washed, screened sand (Home Depot).

A single observer assessed plant sex and measured calyx width on one flower per plant using digital calipers. Calyx width measurements were repeated twice and averaged for each individual plant. We assessed sexual dimorphism in calyx width by performing a likelihood ratio test in R (R Core Team 2016) on two general linearized models predicting calyx width: model one included a plant sex term and an intercept, while model two included only an intercept. We used a Gaussian error structure for both models.

These plants were part of an experiment on the effects of soil nutrients on plant traits, but we found no difference in calyx width between the high and low nutrient treatment groups (LR $F_{1,40} = 0.13$, $P = 0.72$), nor was there a sex-by-nutrient interaction (LR $F_{1,38} = 2.50$, $P = 0.12$), so we combined the calyx width measurements for high- and low-nutrient plants in our analysis of sexual dimorphism in calyx width. Plants received either 0.32g (low nutrient treatment) or 1.48g (high nutrient treatment) of Osmocote® 14:14:14 controlled release fertilizer (The ScottsMiracle-Gro Company, Marysville, Ohio, USA) once during the experiment.

APPENDIX D

COMPUTER CODE FOR SIMULATION MODEL

Computer Code for Simulation Model Reported in Chapter 4

The following text is R code for a computer simulation modeling sex ratio evolution in gynodioecious plant populations under varying pollinator sex bias intensity, female fertility advantage, female progeny sex ratio, and hermaphrodite progeny sex ratio (in Chapter 4).

```
#Here, I write a function called mysim that performs the simulations
#The user specifies p, gen, inital.sr, pff, seq.length, fem.seeds, herm.seeds, fem.pref, herm.pref, num.runs
# p is population size (# plants)
# gen is number of generations
# initial.sr is initial population sex ratio in terms of proportion hermaphrodite (ranges from 0 to 1)
# pff is female progeny sex ratio in terms of proportion female (ranges from 0 to 1)
# seq.length is the number of plants each pollinator visits
# fem.seeds and herm.seeds indicate relative seed production by females and hermaphrodites. Setting fem.seeds and herm.seeds equal to 1, females and hermaphrodites produce the same number of seeds. Hold herm.seeds equal to 1 and increase fem.seeds to increase the number of seeds females produce relative to hermaphrodites.
# fem.pref and herm.pref values together represent pollinator sex bias. Setting fem.pref to 1 and varying herm.pref allows us to express a preference for hermaphrodites as a number greater than 1 and a preference for females as a number smaller than 1.
```

```
# num.runs is the number of times the simulation should be run over the specified number of generations with the given combination of parameter values


#The mysim function calculates and returns sex ratio in next generation:
mysim<-function(p,gen,initial.sr,pff,phf,seq.length,fem.seeds,herm.seeds,fem.pref,herm.pref,num.runs){
#calculate the proportion of female seeds that are hermaphrodite
pfh<-1-pff #proportion of female offspring that are hermaphrodite
#calculate the proportion of hermaphrodite seeds that are hermaphrodite
phh<-1-phf
#make a matrix where we'll put the sex ratio in each generation, in terms of proportion hermaphrodites
#each column will list the sex ratios in each generation for each run of the model
#each row will contain the sex ratio for that generation, starting with the initial sex ratio in the first row
sex.ratios<-matrix(nrow=gen+1,ncol=num.runs+1)
sex.ratios[1,]<-initial.sr #first generation sex ratio
sex.ratios[,num.runs+1]<-seq(0,gen,1)
sex.ratios.nextgen<-matrix(nrow=gen+1,ncol=num.runs+1)
sex.ratios.nextgen[,num.runs+1]<-seq(0,gen,1)
#set the number of moths
m<-p*0.04
```

```
#for each of the number of runs that we want to repeat the model,
#we'll do the following:
for (irun in 1:num.runs){
#make a vector that will hold the number plants pollinated in each generation
num.polld<-rep(NA,gen+1)
###
#within each generation, need to:
# 1. create new population of plants based on sex ratio of plants pollinated in previous
 generation, combined with female vs. hermaphrodite propensity to make seeds and rules
 about what sex seeds from herms vs females can be
# 2. draw a new sequence of plants for each moth to visit
# 3. for each moth, determine which of those plants were pollinated
# 4. determine the sex ratio of pollinated plants within the generation and use this to
 create the list of plants for the next generation
# 5. store the sex ratio of the next generation

#make a vector to put new sex ratio into for each generation
new.sr<-rep(NA,gen)
#make a vector to put new number of herms in for each generation
herms.nextgen<-rep(NA,gen)

#make a vector to put new number of females in for each generation
fems.nextgen<-rep(NA,gen)
```

```
for (igen in 1:gen){
#make vector of individual plants listing an id number for each plant up to the number of
 plants present in the population
id<-seq(1,p,1)

#make a vector of plant sexes the length of the number of plants in the population
sex<-rep("F",p)

#bind the id and sex columns
d<-cbind.data.frame(id,sex)

d$sex<-factor(d$sex,levels=c("F","H"))
num.herms.polld<-rep(NA,gen)
num.fems.polld<-rep(NA,gen)

#number of hermaphrodites in current generation
herms<-(sex.ratios[igen,irun]*p)

#fill in "H" for to make the right number of plants hermpahrodites
d$sex[1:herms]<-"H"

# 1. draw a sequence of plants for each moth to visit
```

```
#first make a vector containing the female and hermaphrodite numbers (f & h)
 corresponding to the order of sexes in the plant dataframe
d$bias<-rep(0,p)

if (herms>0) d$bias[1:herms]<-herm.pref
if (herms<1000) d$bias[(herms+1):p]<-fem.pref

#make a column that has the fem or herm preference value divided by the total of all the
 preference values
d$bias.prop<-d$bias/(sum(d$bias))

#make a column that cumulatively adds each preference proportion
d$cum.prop<-cumsum(d$bias.prop)

vis.seq.matrix<-matrix(nrow=seq.length,ncol=m)

for (imoth in 1:m){
ivisit<-1
while (ivisit<=seq.length){
chosen<-runif(1)

proposal<-which(d$cum.prop>chosen)[1] #returns first element that had cum.prop >
 choose value
```

```
if (is.element(proposal, vis.seq.matrix[,imoth])==FALSE){
vis.seq.matrix[ivisit,imoth]<-proposal
ivisit<-ivisit+1
}
}
}


#return matrix of plant sexes, to determine which plants were pollinated...
sexes.seq<-as.data.frame(matrix(nrow=seq.length,ncol=m))

for (i in 1:m){
sexes.seq[,i]<-d$sex[vis.seq.matrix[,i]]
}

# within each moth's visit sequence, need to figure out if each plant was pollinated
herm.indicator<-sexes.seq[1:(seq.length-1),]=="H"

polld.candidates<-vis.seq.matrix[2:seq.length,] #first row can never be pollinated

pollinated<-polld.candidates[herm.indicator] #pollinated if previous plant visited was a
 hermaphrodite
```

```
d$polld<-rep(0,length(d$id))
d$polld[pollinated]<-1

#how many plants were pollinated this generation?
num.polld[igen]<-sum(d$polld)

#pull out pollinated plants
next.gen<-d[d$polld==1,]

if (num.polld[igen]==0) break

#what is the sex ratio of hermaphrodites vs. females pollinated?
#number of herms
num.herms.polld[igen]<-sum(next.gen$sex=="H")
new.sr[igen]<-num.herms.polld[igen]/num.polld[igen]

num.fems.polld[igen]<-num.polld[igen]-num.herms.polld[igen]

hh<-sum(rbinom(num.herms.polld[igen],1,phh))
hf<-num.herms.polld[igen]-hh

ff<-sum(rbinom(num.fems.polld[igen],1,pff))
```

```
fh<-num.fems.polld[igen]-ff

herms.nextgen[igen]<-((herm.seeds*hh) + (fem.seeds*fh))

fems.nextgen[igen]<-((fem.seeds*ff) + (herm.seeds*hf))

#put new sex ratio into sex ratio matrix
sex.ratios.nextgen[igen,irun]<-
 herms.nextgen[igen]/(herms.nextgen[igen]+fems.nextgen[igen])
sex.ratios[igen+1,irun]<-herms.nextgen[igen]/(herms.nextgen[igen]+fems.nextgen[igen])

if (sex.ratios[igen+1,irun]==0) break
}
}
return(sex.ratios)
}
```

To call the function (run the simulation), follow this format, specifying values of p, gen, initial.sr, pff, phf, seq.length, fem.seeds, herm.seeds, fem.pref, herm.pref, and num.runs as desired:

```
t.1<-
 mysim(p=1000,gen=1000,initial.sr=0.5,pff=0.5,phf=0.2,seq.length=20,fem.seeds=1.2,he
```

```
 rm.seeds=1,fem.pref=1,herm.pref=1.5,num.runs=1)
```

The t.1 object will then contain the sex ratios across generations of the simulation.

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