

EXPERIMENTAL AND THEORETICAL STUDIES OF
THE POLLINATION ECOLOGY OF GYNODIOECY

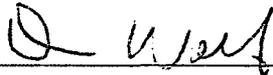
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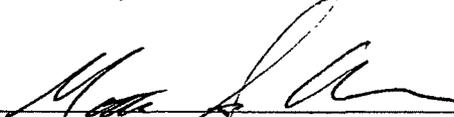
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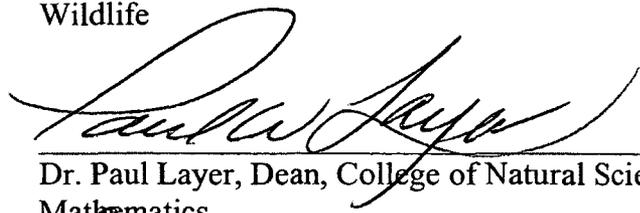


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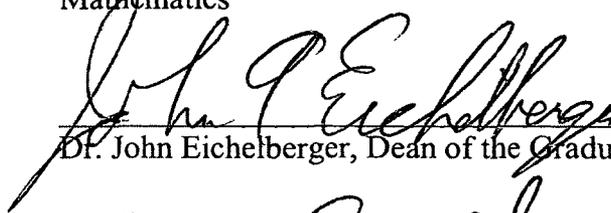


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EXPERIMENTAL AND THEORETICAL STUDIES OF
THE POLLINATION ECOLOGY OF GYNODIOECY

A
DISSERTATION

Presented to the Faculty
of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

By

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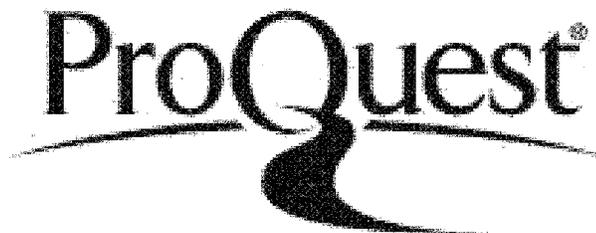


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Abstract

Gynodioecy, a breeding system with females and hermaphrodites, is the most common dimorphic system in plants and found in more species than all other polymorphic systems combined. One unresolved question in gynodioecy evolution is how dramatic sex ratio variation (0-100% female) is maintained among populations. To address this gap, I used complementary empirical and theoretical approaches to elucidate pollinators' role in the evolution and maintenance of gynodioecy and variation in gynodioecious sex ratios. I conducted two field studies restricting the types of pollinators available to artificial populations of *Silene vulgaris*. The first study contrasted pollination by pollen collectors, strongly favoring hermaphrodites, with nectar collectors that readily visit both sexes. Hermaphrodites' relative fitness was greatest in the context of pollen collectors whereas females had dramatically higher relative fitness in the context of nectar collectors, demonstrating pollinators' potential to restrict or facilitate gynodioecy. The second study measured fitness in artificial populations of *S. vulgaris* with either pollen or nectar collecting pollinators in the context of a large natural population, providing access to more pollen sources and more pollinators. Female relative fitness was constant across pollination contexts, unlike in the previous study, as an abundance of pollinators and pollen sources diminished the differences in pollination contexts arising from pollinator bias. On the theoretical front, I developed mathematical models that describe female and hermaphrodite fitness in terms of pollinator abundance and behavior. Then, a single locus nuclear model of gynodioecious sex ratio evolution was used to describe equilibrium female frequency as well as the conditions permitting gynodioecy in terms of pollinator behavior. As in the field studies, I found that pollinators' influence could range from subtle to dramatic. More specifically, under realistic parameter values, where pollinators prefer hermaphrodites to females, incorporation of pollination ecology generally reduces female frequency, and the conditions for the evolution of gynodioecy become more stringent than if pollination processes were ignored. Together, these studies bolster the surprisingly overlooked idea that evolution of gynodioecious populations is directly influenced by pollinator context.

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Dedication

This work is dedicated to the innumerable blind watchmakers unrelentingly and indifferently creating endless forms most beautiful and most wonderful. I look forward to a lifetime of pondering the impressions they have left.

Chapter I

General Introduction

1.1 Introduction

Hermaphroditism, where all individuals act as both males and females, is the most common arrangement among plants, representing more than 80% of angiosperms (Richards, 1997). Dioecy, literally meaning “two houses,” lies at the other extreme with full separation of male and female sexual functions onto separate individuals. Dioecy is relatively rare, found in less than 5% of flowering plants, and disproportionately restricted to island flora and tree growth forms (Bawa, 1980). This suggests that, in general, it is selectively disadvantageous or evolutionarily intractable for individuals to fully specialize in being males or females. There are intermediate possibilities between hermaphroditism and dioecy, however, and these are often portrayed as transitional (Darwin, 1877; Lewis, 1941; Charlesworth and Charlesworth, 1978; Schultz, 1994; but see Lewis, 1942). Gynodioecy, where populations comprise females and hermaphrodites, is the most common polymorphic breeding system. Together with the very similar case of subdioecy, also including females and hermaphrodites, gynodioecy makes up more than 10% of angiosperm species and more than half of all polymorphic species. Thus, gynodioecy is more than a “transitional state,” and a mix of females and hermaphrodites appears to be more stable or achievable than having separate females and males. The stability and prevalence of gynodioecy requires explanation: why would females benefit from losing all reproductive fitness through pollen while hermaphrodites maintain both functions? Why is gynodioecy more common than dioecy- why do pure females more readily evolve than pure males?

Several genetic reasons, described below, offer a basic explanation of why gynodioecy is so prevalent, but these are not sufficient to account for the remarkable breeding system and sex ratio variation seen among natural populations. We do know that gynodioecious hermaphrodites benefit from reproductive assurance through self-

fertilization (Baker, 1967), whereas females benefit from strict outcrossing (Lewis, 1941; Charlesworth and Charlesworth, 1978). We may conclude that gynodioecy is not merely an outcrossing mechanism, as can be argued for dioecy and has been suggested for gynodioecy (Darwin, 1877; Lewis, 1941; Charlesworth and Charlesworth, 1978; Schultz, 1994). In addition to the benefits of outcrossing, gynodioecious females have long been noted to benefit from compensation, elevated seed production due to specializing on seed production (Darwin, 1877). Males, however, cannot benefit by the same mechanism through specializing on pollen export. This asymmetry in selection for specialization on female and male functions is explained below: Specialization on seed production as a female is greatly facilitated through cytoplasmic male sterility genes, *CMS*, transmitted only through seeds. Thanks to compensation increasing seed production, *CMS* genes create a transmission advantage for themselves and spread easily. The same process is not possible through pollen export, as males generally do not transmit cytoplasmic genes, nor do they carry any pollen-specific plasmids for genic selection to favor. Thus, owing to *CMS*, the path to gynodioecy is mechanistically simple and self-selecting; that is to say, evolutionarily tractable.

Although we now know that *CMS* genes are a critical factor for the evolution of gynodioecy in most systems our knowledge of gynodioecy is incomplete and the role of ecological factors remains particularly unclear. For instance, present models cannot fully account for the extreme variability observed in gynodioecious sex ratios, varying from 0-100% female over small geographic scales and within the same species (Widén, 1992; Manicacci et al., 1998; McCauley et al., 2000; Štorchová and Olson, 2004). Pollination ecology is becoming a nascent frontier in gynodioecy research (Ashman, 2006), enriching our understanding of the evolution and maintenance of breeding systems and sex ratio evolution. I here review the early studies of gynodioecy and proceed to recent developments, highlighting the need for greater research effort on pollinators' role in its evolution and maintenance.

Historical perspectives on gynodioecy date to the late 17th century. Prior to the efforts of Kölreuter, Camerarius, and Sprengel, plant sexual reproduction was not a scientifically accepted concept, pollination was poorly understood, and certainly not viewed as sexual reproduction (Mayr, 1986). Camerarius had gathered extensive evidence of plant sexuality by 1694, but it remained an uncouth concept long after its adoption and promotion by Linnaeus during the 18th century. Kölreuter and Sprengel advanced Camerarius' findings and, by the end of the 18th century, had established the basis of our understanding of plant sexuality whereby pollen and ovules contribute male and female gametes, respectively, and often require a vector for transmission (Mayr, 1986). As plant sexuality gained broader acceptance, variation in sexual strategies was discovered. Linnaeus was the first to note that there were several plant breeding systems, from hermaphroditism and monoecy to dioecy and polygamy. A century later, however, Darwin (1877) declared that even this characterization of breeding systems as discrete categories was "artificial, and the groups often pass into one another." Darwin offered the term "gynodioecy" to describe populations of females and hermaphrodites, gave several examples, and was the first to explain the conditions favoring it. Darwin (1877) observed elevated seed production in females of several gynodioecious species. He termed this phenomenon "compensation," attributed it to the reallocation of reproductive resources, and cited it as an important aspect of gynodioecy. Darwin was also aware that inbreeding avoidance could bolster females' fitness (1876) but did not mention this or the contribution of biotic factors such as herbivores or pollinators to gynodioecy. Darwin emphasized (Barrett, 2010) a correlation between female frequency in populations of *Thymus serpyllum* and variation in the physical environment (1877). Since then, elevated female frequency in harsh physical environments has been confirmed in other systems (Delph and Lloyd, 1991; Wolfe and Shmida, 1997; Ashman, 2006) and attributed to differential sensitivities of male and female function to stress. Yet, gynodioecy is not restricted to harsh environments. Additional factors are required to

account for the broad prevalence and stability of gynodioecy under environmentally favorable conditions.

Genetic factors such as resource compensation, inbreeding depression, and the presence of *CMS* genes have long been understood to define conditions favoring gynodioecy (Lewis, 1941). Our theoretical understanding of the role of ecological factors, particularly pollinators, is comparatively recent and remains incomplete (Ashman, 2006; Dufay and Billiard, 2012; Spigler and Ashman, 2012). Lewis (1941) noted that elevated female frequency may result in pollen limitation in females, but did not discuss how pollinators could have the same effect through biased visitation, low visitation rates, or low pollen carryover, regardless of female frequency. Only three studies have directly modeled the influence of pollinator behavior on gynodioecious populations, and their handling of pollination processes was limited to varying the number of pollinator visits received (Lloyd, 1974; Delannay et al., 1981; and Schultz, 1994). Several additional aspects of pollination are potentially influential but remain unstudied, including sex-biased visitation, variation in bout length, pollinator-mediated self-fertilization, and variation in how quickly pollen dissipates from a pollinator. Increasingly complex models of gynodioecy have been constructed (Frank, 1989; Maurice and Fleming, 1995), offering the potential to examine the influence of additional ecological factors in gynodioecy, albeit indirectly, through functional relationships. Although flexible, the lack of biological mechanisms in these models reduces their utility for studying pollinators' direct role in gynodioecy. Thus, the task remains to build models that provide a more complete understanding of how several aspects of pollinator behavior interact with the genetic factors to define conditions favorable to gynodioecy.

Empirical efforts, too, have largely overlooked pollinators' role in gynodioecy, or considered them only indirectly. While Darwin (1877) noted that gynodioecious females of *Thymus vulgaris* managed sufficient pollen receipt, he did not speculate whether pollinators could restrict gynodioecy if services to females were inadequate. We know

that pollinators show biased visitation in gynodioecious populations, generally favoring hermaphrodites over females (Pettersson, 1991; Williams et al., 2000; Vamosi et al., 2006) as the more rewarding sex (Jolls et al., 1994). We know that female pollen limitation increases with distance from hermaphrodite (Widén and Widén, 1990; Taylor et al., 1999; Williams et al., 2000), with female biased sex ratios (Graff, 1999; Miyake and Olson, 2009), and that pollinators mediate these processes.

Direct comparisons of female and hermaphrodite fitness under multiple pollination contexts, however, have been limited to a single study in *Fragaria virginiana* (Ashman and King, 2005), which noted a significant change in components of females' relative fitness between plants selectively exposed to either ants or flying insects for pollination. Several studies documenting pollen limitation in gynodioecious populations (reviewed in Ashman, 2006) make very clear the importance of pollination processes in gynodioecy, but have not directly measured pollinators' role or only quantify pollen limitation in females. The paucity of direct empirical tests of pollinators' influence on gynodioecious populations may be related to the lack of theoretical work to motivate field experiments, described above, or due to the challenges of experimentally varying pollinator context.

The need to more closely examine pollination ecology in gynodioecious systems is further underscored by studies on hermaphroditic plants that demonstrate pollinators' pervasive influence on plant fitness. A study on hermaphroditic *Aquilegia coerulea* (Brunet and Sweet, 2006), for example, shows that variation in pollinator assemblages over the plant's range alters selfing rate- an important parameter in gynodioecious populations. Studies on hermaphrodites have also demonstrated that parameters such as display architecture interact with pollinator behavior to influence selfing rate and plant fitness (Harder and Barrett, 1996). We know, too, that pollinators are highly variable across time and space with broad implications for floral diversity and species distributions, as well as significant economic and conservation impacts (Allen-Wardell et

al., 1998; Vamosi et al., 2006; Burd et al., 2009), but this variation in plant-pollinator interactions, too, remains poorly understood (Ashman et al., 2004; Ollerton et al., 2009). Given pollinators' fundamental role in plant fitness as well as their variability, studies of the direct impact of variation in pollinator behavior on female and hermaphrodite fitness are required.

In this dissertation, I use empirical and theoretical approaches to examine the role that pollination ecology has in elevating female fitness, thereby facilitating gynodioecy. I aim to more directly connect pollinators to female relative fitness and the maintenance of gynodioecy than previous studies have done. I designed two field experiments using the model gynodioecious plant *Silene vulgaris* under multiple pollination contexts in its native and introduced ranges. I also conducted a theoretical investigation that describes gynodioecious plants' fitness in terms of pollination processes and sex ratios. Both the direct empirical tests and the theoretical study explicitly handling pollination ecology are novel, pollinator-focused approaches to studying gynodioecy. This focus is necessary to disentangle the effects of pollination ecology from other influences on plant fitness.

Chapter 2 covers the first field experiment, which compares females' relative fitness when exposed to different sets of pollinators. We established artificial populations of the gynodioecious model system *Silene vulgaris* in a portion of its introduced range, in Virginia, USA. This experiment reduced variation in plant size and condition related to cultivation environment by using greenhouse-raised potted plants, controlled for genetic variation by evenly distributing related plants across replicates, and controlled for population structure, microhabitat, and nutrient levels by spatially shuffling, watering, and fertilizing plants. To impose different pollinator treatments, we selectively exposed plants to diurnal (pollen and nectar collecting) or to nocturnal (strictly nectar collecting) insects, two groups expected to behave differently on female plants. We measured the seed production of females and hermaphrodites under both

conditions, permitting us to test the influence of pollinator community on females' fitness relative to hermaphrodites.

Chapter 3 describes a second field experiment, similar to that in Chapter 2, but conducted with plants from *S. vulgaris*' native range in the Czech Republic, in the context of its native pollinators. European *S. vulgaris* populations have quite different population history and genomic composition (Keller and Taylor, 2010) from the plants used in Chapter 2, with implications for females' relative fitness. The local European pollinator community also differed from that in Chapter 2 in terms of composition, abundance, and behaviors. The contrasts between the experimental plants and local pollinator communities in Chapters 2 and 3 allow us to make additional inferences about the population genetic and pollinator ecological contexts necessary for females' elevated seed fitness. We again selectively exposed greenhouse-raised plants to diurnal and nocturnal segments of their natural pollinator guild and measured the seed production of females and hermaphrodites under both pollination treatments.

Chapter 4 is a theoretical treatment of pollination processes in gynodioecious populations, complementing our empirical data and aiding in their interpretation. As noted earlier in the Introduction, existing models of gynodioecious populations provide only the most basic handling of pollination processes. Accordingly, we derive novel expressions for visitation rates, pollen delivery, selfing rates, and mating frequencies for gynodioecious females and hermaphrodites. These are used to build a model of gynodioecious sex ratio evolution that includes several pollinator behavioral parameters such as visitation bias, pollen carryover rate, and pollinator mediated selfing. This pollinator-focused model of gynodioecy allows us to consider both the independent and interacting roles of several aspects of pollination biology on female relative fitness as well as sex ratios. We compare the conclusions drawn from variations of this model with existing models that do not consider pollination processes directly.

Chapter 5 reviews and summarizes our findings from each chapter and looks for synthetic conclusions to be drawn from this collection of work. In some regards, we find consensus in the outcomes of the field and theoretical studies, suggesting general conclusions that may be made. On multiple occasions, however, the outcomes of the two field experiments differ with each other or with the theoretical study. Contrasting the chapters' findings highlights aspects of pollination ecology that vary in their influence on gynodioecious populations. Lastly, I put our findings in the context of the current gynodioecy literature, suggest how this project's approaches could be applied to similar studies on plant reproductive strategies, and offer additional research directions based on our findings and new questions raised.

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Chapter II

Pollination context alters female advantage in gynodioecious *Silene vulgaris*¹

2.1 Abstract

In gynodioecious plant populations, females typically produce more seeds than hermaphrodites. This elevated seed production, or female advantage, facilitates the maintenance of females and is attributed to genetic and ecological factors. We tested the influence the pollinator community on female advantage by selectively exposing replicate groups of *Silene vulgaris* with 1:1 sex ratio to either diurnal or nocturnal components of the natural pollinator guild in Virginia. We found that female advantage increased seed production to more than twice that of hermaphrodites, but existed only in the context of abundant, effective nocturnal pollinators. In contrast, no female advantage existed when populations were exposed to diurnal, pollen-collecting syrphid flies and halictid bees. Comparison of diurnally visited plants with positive controls indicated that changes in female advantage were caused by sex-specific pollen limitation. Pollen limitation was most acute in diurnally exposed females. Our results provide the first demonstration that the relative fitness of females in a gynodioecious population can change with pollinator context. Finding that pollination context alone can alter female advantage suggests that variation in pollination context may directly contribute to variation in local sex ratios and, over longer timescales, play a central role in breeding system evolution.

¹ Stone, J.D. and M.S. Olson. Pollination Context Alters Female Advantage in Gynodioecious *Silene vulgaris*. Prepared for submission to *The American Journal of Botany*.

2.2 Introduction

Female advantage, the capacity for female plants to mature more seeds than hermaphrodites, is a key feature contributing to the maintenance of gynodioecy (Darwin, 1877; Lewis, 1941; Charlesworth and Charlesworth, 1978; Couvet et al., 1990; Shykoff et al., 2003; Spigler and Ashman, 2012). To persist in a population, females must have higher seed fitness than hermaphrodites. Because females cannot self-fertilize, as hermaphrodites can, pollinators may have a major impact on female fitness, and therefore female compensation. Despite the importance of pollinators to plant reproduction and the centrality of female advantage to gynodioecy, the relationship between female advantage, gynodioecy, and pollination context remains largely undeveloped.

Female advantage is a complex phenomenon determined by the reallocation of resources away from male function, also known as resource compensation, the avoidance of selfing and inbreeding depression, as well as ecological processes favoring seed production in females relative to hermaphrodites (Charlesworth, 1989; Ashman, 1999; Olson and Antonovics, 2000; Ashman, 2006; Bailey and Delph, 2007). The importance of resource reallocation and inbreeding avoidance to female compensation has been apparent since Darwin (1876, 1877). The influence of biotic ecological factors on female compensation, however, was not directly examined until Lewis (1941) noted in his model of gynodioecy that pollinator visit limitation offsets females' fertility advantage, restricting the spread of females. The connection between pollinators and female compensation has become increasingly clear as more and more realistic models capture the influence of pollination ecology on female fitness (Lloyd, 1974; Maurice and Fleming, 1995; Chapter 4). Empirical evidence for pollinators' influence on female compensation, however, is largely unavailable (Ashman, 2006; Dufay and Billard, 2012).

Experimental manipulations of pollinator communities illustrate that variation in pollinator services can be associated with changes in reproductive success.

Hermaphroditic *Silene ciliata*, for example, exhibited greater seed production and

reduced seed predation when exposed to diurnal flies and moths compared to nocturnal pollinator exposure, despite adaptations for nocturnal pollination (Giménez-Benavides et al., 2007). Young (2002) noted that diurnal pollinators significantly reduced seed production in hermaphroditic *S. latifolia* compared to nocturnal pollination. Barthelmess et al. (2006) found that *S. latifolia* seeds pollinated by nocturnal insects were significantly more outbred than diurnally pollinated ones. Similarly, changes in gynodioecious *Fragaria virginiana* seed fitness were attributed to variation in local pollinator communities (Ashman and Diefenderfer, 2001), though only measured in females. Finally, in *F. virginiana*, ant pollination increased female relative seed production over bee and fly pollination (Ashman and King, 2005), the only study to have compared female relative fitness in multiple pollination contexts.

The maintenance of gynodioecy requires that females achieve elevated seed fitness and this requires adequate pollination. The pollination context required for gynodioecy, however, is not yet fully understood (Ashman, 2006). Gynodioecious plants exhibit suites of floral color, morphology, chemoattractant, and phenological patterns that both reflect an evolutionary history, as well as limit compatibility, with particular pollinators. Pollinators, too, may show varied degrees of compatibility toward females and hermaphrodites, influencing the relative seed production of both sexes. Even tightly co-adapted systems are likely to experience variation in pollinator services due to changes in local plant or pollinator abundance. Pollinator variation could, thus, influence the distribution of breeding systems. Over longer timescales, a particular plant species' compatibility with pollinators that readily visit females may be required for gynodioecy to be maintained. For instance, several species in the genus *Silene*, where gynodioecy has evolved multiple times, exhibit a nocturnal pollination syndrome with white flowers that open primarily at night (Kephart, 2006; Kephart et al., 2006). Nocturnal pollinators are primarily nectar-feeding moths (Pettersson, 1991a; 1991b; 1992; 8 references reviewed in Kephart et al., 2006; Taylor et al., 1999; Barthelmess et al., 2006). Diurnal

pollinators, however, are primarily pollen- and nectar-collecting bees, preferring hermaphrodites for pollen rewards. Such differences may be key to understanding the plant-pollinator interactions necessary for the maintenance of gynodioecy.

In this manuscript, we seek to improve our understanding of the conditions necessary for gynodioecy by examining the direct influence of pollinator type on females' relative fitness. We predicted that differences in female and hermaphrodite flowers, as well as differences in pollinator behavior, would influence visitation rates to the two genders. Because hermaphrodites present pollen rewards, they may more easily attract and retain pollen-collecting insects than females. Also, hermaphrodites have the capacity to self fertilize (Miyake and Olson, 2009), whereas females are wholly dependent on pollen vectors and outcross pollen. This generates the prediction that females will be poor at attracting pollinators seeking pollen rewards and when these pollinators predominate females will have lower seed production. However, when pollinators visit both sexes equally, females may have higher seed production due to reduced female-biased pollen limitation. In order to examine the role pollination context plays in female and hermaphrodite seed production, we manipulated pollinator access to experimental populations of *Silene vulgaris*, a gynodioecious plant with a broad geographic range that naturally experiences diverse pollination contexts, both in types of pollinators and their abundance. We exposed replicate groups of gynodioecious *Silene vulgaris* to diurnal and nocturnal segments of the natural pollinator guild, which differed in species composition, behavior, and abundance. We hypothesized that diurnal, pollen-collecting bees and flies would favor hermaphrodites and depress females' relative seed production, whereas nocturnal nectar-collecting moths would be less biased in their visitation resulting in higher female relative seed production.

2.3 Methods

2.3.1 Study system

Silene vulgaris (Caryophyllaceae) is a perennial herb native to Eurasia and introduced to North America several times since the 18th century (Keller and Taylor, 2010). It commonly occurs in meadows and along human-disturbed sites such as roads and fields, in populations ranging from continuous groups with hundreds of individuals to isolated patches with few plants. *S. vulgaris* has a gynodioecious breeding system with sex expression controlled by a cytonuclear polymorphism with multiple *CMS* types and several pairwise-compatible nuclear restorers of male fertility, *Rf* (Charlesworth and Laporte, 1998; Bailey and McCauley, 2005). The sex ratio of populations varies widely, from near zero to 90% female (McCauley et al., 2000; Štorchová and Olson 2004; Glaetli et al., 2006). Hermaphroditic plants are self-compatible (Miyake and Olson, 2009) and selfing occurs primarily through geitonogamy, as flowers are strongly protandrous. Individual plants in the wild may be quite large with over a hundred flowers, many having 10-50 flowers open at a time (personal observation), providing hermaphrodites opportunity for geitonogamous selfing.

Silene vulgaris shows specialization for nocturnal pollination (Marsden-Jones and Turrill, 1957) with white, scented flowers and fringed petals extending from an inflated calyx enclosing nectaries and the gynoecium. The corolla opening is large enough to admit halictid bees, syrphid flies, and small geometrid moths, all of which were seen entering calyces, presumably to collect nectar. Large *Bombus* and sphingid moths hover while feeding or land on the petals and exerted reproductive structures. Both of these animals were observed extending their proboscis inside the corolla and some smaller *Bombus* were observed entering calyces, suggesting all potential pollinators in this study were at least somewhat interested in collecting nectar. All diurnal visitors were observed actively handling hermaphrodites' anthers with their legs or mouthparts. Nocturnal

visitors' contact with the stigmas and anthers appeared incidental, as their abdomens or feet contacted those organs while probing the calyx for nectar, often without landing.

In our experimental populations (described below), flowers opened near dusk and remained open for multiple days. On hermaphrodite flowers, anthers dehisced between 1900 and 2100 hours and remained in male-phase for two days, exerting five anthers the first night and five the second night. On the third night hermaphrodite flowers entered female-phase, exerting stigmas that remained receptive for two to three days, but wilted earlier if pollinated. Female flowers have similar total longevity as hermaphrodites, and may have stigmas receptive for up to five days. Flowers of both sexes varied slightly in floral morphology between individuals but differed most conspicuously in the presence or absence of anthers.

2.3.2 *Experimental populations*

Artificial populations of *Silene vulgaris* were arranged on an abandoned golf fairway (an open field, location: 37.3595°N, 80.5540°W, elevation: 1200 m), extending roughly 1 km by 150 m and surrounded by deciduous forest on the Mountain Lake Conservancy in Giles County, Virginia. The nearest wild *S. vulgaris* population was roughly 4 km to the south. Four replicate artificial populations, each with 20 females and 20 hermaphrodites (160 plants total), were established using greenhouse-grown plants in 2-liter pots that were acclimated to outdoor conditions for one month prior to the start of the experiment. Replicate populations were separated by at least 100 m, a distance beyond which pollen dispersal was minimal in *Silene latifolia*, a congener with similar pollination ecology (Barthelmess et al., 2006). Pollinator exclusion treatments were conducted by placing potted plants into large insect enclosure tents constructed using bridal veil to eliminate pollinators' access to plants during the day and/or night. Pollinator exposure treatments were conducted by placing potted plants under shade tents that provided similar amounts of shade as the insect enclosures, but were only partially

covered with bridal veil to allow pollinators to visit flowers. Within each replicate population, five female and five hermaphrodite plants were assigned to one of four pollinator exposure treatments: 1) diurnal pollinators only (shade tent from 0800 to 2030 hours, otherwise in enclosure), 2) nocturnal pollinators only (shade tent from 2030 to 0800 hours, otherwise in enclosure), 3) both diurnal and nocturnal pollinators (positive control, always in shade tent), and 4) neither diurnal nor nocturnal pollinators (negative controls that were always in the enclosure). All plants were moved daily between enclosure and shade tents (1-2) or within the shade tent or enclosure (3 and 4, respectively) to control for handling and spatial effects. The mesh tents were effective at restricting pollinator access. In two of the replicate populations, both female and hermaphrodite negative controls began to show several fruits after day 10 of the experiment; while this indicates the presence of some pollinator infiltration to the enclosures, fruit set on negative controls was far less than in any other treatment group, indicating that the majority of pollination occurred when plants were exposed in shade tents. Because our artificial populations were comprised of greenhouse-grown plants of known parentage, we were able to control for plant age, size, relatedness, microhabitat, and spatial effects that may contribute to variation in seed production in natural populations. All open flowers were removed prior to the experiment to ensure that all pollination occurred during our treatments. The controlled exposure to pollinators began on 15 June and ended on 28 June.

2.3.3 Pollinator observations

In order to better understand the relationship between insect visitors and the pollination services received by female and hermaphrodite plants, we monitored nocturnal and diurnal insect visitation patterns using video recorders. Up to four plants were individually monitored simultaneously using four separate video cameras placed roughly 50 to 150 cm from the focal plant. The nocturnal video recordings were made

just after dusk from approximately 21:15-22:15 with near-infrared illumination (λ roughly 800-1000nm) and the diurnal recordings were conducted at 08:15-09:15 using natural light. The nocturnal observation period coincided with observed periods of high moth activity. The diurnal observation period occurred after populations were fully exposed to daylight but before ambient air temperatures reached their mid-day highs. Very cool, very hot, rainy, or windy conditions were expected to reduce insect foraging activity and were avoided as observation periods. Every open flower on a focal plant was identified at the beginning of the recording. If the focal individual was a hermaphrodite, the gender-phase of each individual flower was noted. Video recordings were reviewed in the lab to collect data on pollinator behavior. Plants' visitation rates, the total number of flowers visited in each bout, type of insect visitor, gender-phase of each flower visited (on hermaphrodites), and the visitors' time spent handling individual flowers were calculated.

2.3.4 Seed fitness components

Total number of flowers on each plant, the number of flowers in each gender phase on hermaphrodite individuals, developing fruits, and old flowers with reproductive structures that had visibly wilted were counted every second day from day 2 to day 12 of the field experiment. Numbers of developing fruits were determined by gently squeezing the calyx; a noticeably enlarged gynoecium was recorded as a fruit. Some fruits were later determined to have aborted or otherwise failed to develop- nearly all of these were in the negative control group. At the end of the pollinator exposure, all remaining receptive flowers were removed to prevent further pollination and plants were moved to the Mountain Lake Biological Station (MLBS) deer-exclosure to mature their fruits. Fruits were collected upon maturation, after 25-35 days, just prior to capsule dehiscence. Up to three fruits were randomly selected from each plant for seed counts from those collected in the first 12 days, as the risk for uncontrolled pollination increased later in the

experiment when moths were found in two of the enclosures. Seeds were counted only if ovules were fully developed and bore a hard corpuscular seed coat.

2.3.5 Statistical analyses

ANOVAs were used for the analysis of pollinator visitation data and components of plant fitness, as detailed below. Planned contrasts were identified *a priori* and carried out for seed fitness values of a subset of the experimental groups, as noted in the results, using the ANOVA models and associated LSMeans estimates. Additionally, *post-hoc* multiple comparison using Tukey's honest significant difference (HSD) test are provided as a conservative means of comparing experimental groups and displayed in the figures for seed fitness measures.

We examined differences in plant visit durations, visitation rates, and bout length between pollinator treatments and constituent pollinator types, as well as between plant sex and individual flowers' gender-phase. Pollinator type was classified either according to the treatment groups (diurnal or nocturnal insects) or by the functional group (syrphid fly, *Bombus*, geometrid moth, or noctuid moth) and included in models with either plant sex (female or hermaphrodite), gender phase of the flower (male or female), or both plant sex and gender phase (female flower, hermaphrodite flower- female phase, or hermaphrodite flower- male phase). As some plants were used for multiple observation sessions, individual plant was included in models as a random effect.

Visitation rate, the numbers of pollinator visits per hour, was modeled as a Poisson process. A linear mixed effects model was constructed using the glmer function (package "lme4," R version 2.12.2, R Development Core Team, 2011) where the number of visits per hour was a function of plant sex, time of day, their interaction, total number of flowers, male-phase flowers, and female-phase flowers. This model was selected on the basis of lowest AIC. Effect significance was evaluated by parametric bootstrapping (Faraway, 2006), creating simulated distributions against which to evaluate the observed

data. `Boots.lrt` was used to compare the log likelihood of two models, one including a particular parameter and the other omitting it. In each case, 5000 simulated data sets were fit to pairs of models and the $-2 \times \log$ likelihood was calculated. *P*-values correspond to the fraction of simulated data sets with higher log-likelihoods than the observed data. This model comparison was done to determine the significance of each relevant factor including time of day, plant sex, and their interaction, as well as flower number and flower gender phase. The interaction between plant sex and time of day was examined first and determined to be highly significant, requiring data simulations and log-likelihood ratio tests to be performed separately for both sexes and treatments to avoid the confounding effects of the sex-by-time-of-day interaction term. Separate analyses were performed either including or ignoring the gender-phase of flowers on hermaphrodite plants in order to contrast the effects of overall display size with preference for pollen-bearing flowers.

Bout length, the number of flowers successively visited on a plant by an individual insect, was modeled as a geometric function of the time of day (morning or dusk), plant sex (hermaphrodite or female), and their interaction.

Fruit set was modeled using logistic regression, with a flower developing into a fruit categorized as a “success” and one not developing categorized as a “failure.” The probability of fruit set by day 12, the last census before termination of the experiment, was modeled as a function of plant sex, time of pollinator exposure, and their interaction. Replicate was included as a random effect. The model was implemented using the GLIMMIX procedure, SAS 9.2 (SAS Institute, 2009), assuming a binomial distribution for fruit set. Fruit set also was used to infer adequacy of pollinator visits. Plants with fruit set lower than positive controls were considered visit-limited.

Total number of fruits produced was modeled as a linear function of plant sex, time of exposure (treatment), their interaction, and a plant’s deviation in flower number. Females produced significantly more total flowers over the course of the experiment (μ_F

= 13.9, $\mu_H = 10.4$, F-ratio 4.69, $p < 0.034$), so sex and flower number were correlated. To properly partition the variance in fruit and seed production between the effects of sex and flower number, while accounting for variability in plant size, we included the deviation from mean flower number (DMF), rather than total flower number, as a covariate. DMF was calculated as the difference between a particular plant's flower number and the mean flower number for that sex, divided by the sex's standard deviation in flower number. There was no difference in flower number among treatment groups. Lastly, this model included replicate as a random effect.

Seeds per fruit was modeled as a linear function of plant sex and time of exposure with replicate and plant included as random effects using restricted maximum likelihood (REML) least-squares ANOVA in JMP 10 (SAS Institute, 2012). Flower number deviation from female and hermaphrodite means (see previous paragraph), was included as a covariate, along with all possible interaction terms, to determine if relative display size affected the number of seeds per fruit. Seeds-per-fruit was used to infer pollen limitation, on an individual flower basis. Plants with fewer seeds per fruit than positive controls of that sex were considered pollen limited at the flower level.

Estimated total numbers of seeds per plant were calculated as the product of average number of seeds per fruit for a particular plant multiplied by its total number of fruits on day 12 of the experiment. Seeds-per-plant was modeled as a linear function of plant sex, time of pollinator exposure, plants' flower number deviation, and all possible interaction terms, with replicate included as a random effect, using REML Least-squares ANOVA. Plants with fewer total seeds than positive controls were considered limited by the number and quality of pollinator visits.

2.4 Results

2.4.1 Pollinator behavior

Diurnal visitors to the experimental *Silene vulgaris* plants during the 12 one-hour observation periods consisted of syrphid flies (65% of daytime visits, $n=22$), small *Bombus* (32% of daytime visits, $n=11$), and one large *Bombus* (3% of daytime visits, $n=1$). Additional functional groups of diurnal arthropods, including diurnal lepidopterans, ants, and small beetles, were occasionally observed on flowers outside of the observation periods. Nocturnal visitors during 14 one-hour observation periods consisted almost entirely of noctuid moths (97% of nocturnal visits, $n=68$) and, rarely, small geometrid moths (3% of nocturnal visits, $n=2$).

Handling time, the time spent by insect visitors on individual flowers, depended strongly on the interaction of time of day and flower gender with diurnal visitors making disproportionately long visits to male phase flowers (Time x gender phase, $F_{2,103} = 5.34$, $p = 0.0063$, Table 2.1, Figure 2.1). Diurnal, pollen-collecting visitors (*Bombus*, syrphid flies) stayed longer on pollen-bearing flowers than on pistillate flowers (female flowers and hermaphrodites in female phase; LSMeans planned contrast, $F_{1,98}=14.4$, $p = 0.0003$, Figure 2.1). Nocturnal insects, however, did not significantly differ in their time spent on any flower type (LSMeans Tukey's HSD, $p > 0.1$). Handling time also varied by pollinator type (Table 2.4): syrphid flies remained on flowers for much longer than *Bombus*, especially male-phase hermaphrodite flowers (Table 2.4). Geometrid moths' visits were significantly longer than those of noctuid moths (Table 2.4).

Mean bout length, the number of flowers sequentially visited on a particular plant, was relatively short (average 2.20 flowers, Tables 2.2, 2.4). Most visits consisted of only 1-4 flowers visited and a minority of visitation bouts were longer. Bout length did not differ significantly between nocturnal and diurnal visitors, between females and hermaphrodites, or for the interaction of plant sex and visitor type (Tukey's HSD, p -value for all comparisons ≥ 0.5). No aspect of display size, including total number of flowers,

number of male-phase flowers, and the number of female flowers, significantly affected bout length on these experimental *S. vulgaris* (Tukey's HSD, p -values ≥ 0.5).

Visitation rates to hermaphrodites were higher than to females in both the day and night, but this difference was smaller during the night, reflecting less discrimination against females by nocturnal pollinators (Table 2.3, Figure 2.2a, Sex*Time of Day). Total number of open flowers (total display size) was significantly associated with higher visitation rates for all plants (Table 2.3). The number of male-phase flowers had a highly significant effect on visitation rates to hermaphrodites (LLR = 12.6, $p = 0.0028$). When visitation rates to only stigmatic flowers on hermaphrodites were compared with visitation to females, the differences between females and hermaphrodites were reduced, with no difference during the night (Table 2.4, Figure 2.2b).

2.4.2 Seed fitness

Overall, nocturnally exposed plants produced more fruits than diurnally exposed plants (LSMeans planned contrast, $F_{1,141} = 41.2$, $p < 0.0001$, Table 2.5, Figure 2.3). Females produced more fruits than hermaphrodites at night (LSMeans planned contrast, $F_{1,141} = 21.2$, $p < 0.0001$), and when comparing day and night females had a greater increase in fruit production than hermaphrodites, though this was not significant (Treatment*Sex, Table 2.5, Figure 2.3). Nocturnally exposed plants did not differ in fruit production from positive controls (Student's t -test, $t_{141} = 0.65$, $p > 0.5$) indicating that they were not visit limited. Generally, plants with more flowers (greater DMF) produced more fruits (Table 2.5). Large plants produced more fruit during the nocturnal and positive control treatments than during the diurnal exposure treatment (DMF*Treatment interaction term, Table 2.5).

Diurnally exposed females and hermaphrodites had similar fruit set (planned contrast, $X^2 = 0.0031$, $p > 0.9$, Table 2.6, Figure 2.4), but nocturnally exposed females produced significantly more fruits per flower than hermaphrodites (planned contrast, $X^2 =$

12.5, $p < 0.0004$, Table 2.6, Figure 2.4). Nocturnally-visited flowers were almost twice as likely to set fruit than diurnally-visited plants, and female fruit set was higher relative to hermaphrodites only when exposed to nocturnal pollinators (Treatment * Sex term, Table 2.6, Figure 2.4). Diurnally exposed plants had significantly higher fruit set than negative controls ($X^2 = 23.3$, $p < 0.0001$), while nocturnally exposed plants had fruit set similar to positive controls ($X^2 = 0.05$, $p > 0.8$).

Nocturnally-exposed plants produced significantly more seeds per fruit than diurnally-exposed plants (LSMeans planned contrast, $F_{1,83} = 40.0$, $p < 0.0001$) and females produced significantly more seeds than hermaphrodites (LSMeans planned contrast, $F_{1,93} = 7.2$, $p < 0.009$, Table 2.7, Figure 2.5). As with the other seed fitness components, females' increase in seed production per fruit between diurnally- and nocturnally-visited plants was larger than that of hermaphrodites, though this interaction term was not statistically significant (Table 2.7). Overall, the number of flowers on a plant (*total display size*) did not significantly affect number of seeds per fruit and omitting display size from the ANOVA models did not appreciably change the F-values or interpretations of other included variables. The effect of display size on seeds per fruit, however, did vary with pollinator treatment (Treat * #Flowers interaction term, Table 2.7), having significantly greater influence on diurnally exposed plants.

At the whole plant level, females produced significantly more seeds than hermaphrodites when exposed to nocturnal pollinators (LSMeans planned contrast: $F_{1,68} = 23.2$, $p < 0.0001$, Tukey's test of HSD, Table 2.8). When exposed to diurnal pollinators, however, females and hermaphrodites produced similar numbers of seeds (LSMeans planned contrast: $F_{1,68} = 0.31$, $p > 0.5$, Figure 2.6, Table 2.8). Plants with more flowers produced more total seeds (DMF term, Table 2.8). The relationship between total flower number and seed production varied significantly with time of day (Treatment*DMF interaction term, Table 2.8), strong at night for both sexes, weak for hermaphrodites during the day, and non-existent for diurnally visited females.

2.5 Discussion

2.5.1 Effects of pollinator community on female advantage and breeding system evolution

We observed female advantage in the form of elevated seed production relative to hermaphrodites in our experimental populations of *Silene vulgaris*. Female advantage increased female seed production to more than double that of hermaphrodites, but only when exposed to nocturnal pollinators. This relationship between female advantage and pollination context appears to be the result of differences in female and hermaphrodite interactions with their insect visitors. Nocturnal moths were abundant, frequent visitors, and visited both sexes for similar durations. Accordingly, when nocturnally exposed, neither sex of *S. vulgaris* was pollen-limited relative to the positive controls and females showed elevated seed production relative to hermaphrodites. Visitation by diurnal insects, however, was less frequent and visitors spent longer periods of time on hermaphrodite than female plants, especially when handling male-phase flowers. Thus, when exposed to diurnal pollinators, females were more visit- and pollen-limited than hermaphrodites, relative to the positive controls, and experienced lower relative fruit and seed production. The resulting two-fold change in female advantage with exposure to different pollinator communities makes clear that a change in pollination context can alter females' relative seed production.

Gynodioecy is facilitated when females' seed fitness exceeds hermaphrodites' (Lewis, 1941). A two-fold increase is required for females to spread when male sterility is under strict nuclear control (Lewis, 1941), whereas a smaller difference is needed when male sterility is under cyto-nuclear control. Given the seed production values measured in our study, it would be more difficult for females to be maintained if flowers were only open during the day, even if control of sex expression were strictly cytoplasmic (Lewis, 1941). In reality, *S. vulgaris* shows cytonuclear sex determination (Charlesworth and Laporte, 1998; Bailey and McCauley, 2005); nevertheless our study illustrates how sensitive female advantage can be to pollinator context. Nocturnal pollinators were the

main vectors favoring the spread of females because they showed no preference for pollen as a reward, foraging instead for nectar, which both sexes and gender phases provide. These results illustrate the potential importance of the pollinator community for the maintenance of gynodioecy. Attributes of *Silene vulgaris* that improve its attractiveness to nocturnal pollinators could also be viewed as attributes that favor the evolution and stability of gynodioecy in this system. Populations would likely have fewer females if their reproductive strategy were more generalized, attracting more diurnal pollen collectors at the expense of nocturnal visitors, given the local pollinator community from our experiment. We speculate that availability of pollinators that enhance female fitness may underlie some clines in sex ratio variation, such as those found in the Swiss alps where pollinators and females *S. vulgaris* are increasingly rare at high elevations (Landergrott et al., 2009) as well as variation among populations (McCauley et al., 2000; Štorchová and Olson, 2004; Glaetli et al., 2006; Caruso and Case 2007; Cuevas et al., 2008). Mapping breeding systems and pollinator communities onto phylogenies may provide an opportunity to explore this relationship further: Our results generate the prediction that transitions to nectar-collecting pollinators could facilitate transitions from hermaphroditism to gynodioecy. Similar pollinator-switch mechanisms have been suggested for the evolution of gynodioecy in *Nemophila menziesii* (Ganders, 1978) and *Wurmbea dioica* (Case and Barrett, 2001).

It is intriguing that, despite pollen limitation and diurnal pollinators' strong preference for hermaphrodites in both visitation rates and handling time, females in the diurnal exposure group were able to produce as many seeds as hermaphrodites. It appears that, in addition to avoiding inbreeding depression, females may compensate for reduced attractiveness to infrequent diurnal visitors with a longer period of stigmatic receptivity. This suggests that part of female advantage in this system is to avoid the opportunity cost associated with spending several days in male-phase, which limits the time available for hermaphrodites to import pollen. This cost is apparent when

comparing Figures 2.2a and 2.2b: Despite pollinators' preference for hermaphrodites (Figure 2.2a), visitation rates to stigmatic flowers on plants of either sex (Figure 2.2b) are similar. Hermaphrodites' displays divide their time between pollen export (male-phase) and pollen import (female-phase), resulting in stigmatic display size on hermaphroditic plants being only about half of total display size. These phenological aspects of female advantage may play an important and widespread role, especially in pollen-limited environments or in the context of hermaphrodite-biased pollinators, but are rarely noted (but see Ashman and Stanton, 1991). Additional studies of hermaphrodite and female floral longevity, the amount of time hermaphrodites allocate to each sex function, and how these values vary with level of pollen limitation or pollen to ovule ratio may prove a fruitful line of inquiry.

Whereas pollen limitation is often only reported for females, it is significant to note that both sexes were pollen limited under diurnal exposure in terms of seeds per fruit as well as visit limited in terms of fruit set, as indicated by comparison with the positive controls. Neither sex was limited under nocturnal exposure, in either seeds per fruit or fruit set. Hermaphrodite pollen limitation, as seen in these diurnally exposed plants, is rarely reported in the wild (although, Taylor and McCauley, 1999), possibly on the persistent presumption that hermaphrodites are not subject to pollen limitation (Lewis, 1941). Hermaphrodites are vulnerable to pollen and visit limitation unless they possess a delayed selfing mechanism (Lloyd and Schoen, 1992; Yuan-Wen et al., 2010), underscoring that any study of female advantage should examine these phenomena in both sexes.

Genetically determined sexual dimorphism between females and hermaphrodites, including display size and other aspects of compensation, likely plays a central role in female advantage (Pettersson, 1992; Morris and Doak, 1998; Asikainen and Mutikainen, 2003; Chang, 2006; but see Van Etten et al., 2008), but our study suggests that the efficacy of the female strategy is contingent upon favorable local ecology. The

interaction between plant sex and pollinator exposure time on total seed production underscores the ecological-sensitivity of fitness advantages associated with maintenance of gynodioecy. Female *S. vulgaris* flowers are likely metabolically less costly to produce because they lack developed anthers and produce less nectar (Jolls et al., 1994), but other aspects of floral dimorphism between the sexes, including display size, were minor and could not explain the differences in female advantage between nocturnally and diurnally exposed plants. We also note that increased display size was of little benefit in a visit-limited environment. We can thus conclude that, in addition to genetically determined compensation and increased display size, the quality and quantity of pollination services to both sexes are a critical aspect of female advantage with the potential to dramatically influence relative seed production.

An important aspect of seed production and female advantage that we did not directly measure is seed quality. As females are strict outcrossers, they are expected to produce higher quality seeds than hermaphrodites in populations where inbreeding depression is a factor (Darwin, 1877; Lewis, 1941; Emery and McCauley, 2002; Dufay and Billard, 2012). Effects of early-acting inbreeding depression include abortion of embryos, so it is possible that variation in levels of inbreeding is, in part, responsible for the difference in female advantage that we observed. Females, too, can show substantial biparental inbreeding depression within gynodioecious populations (Chang, 2007). Our design likely resulted in low to intermediate levels of inbreeding depression because hermaphrodite selfing rates are measurable in *S. vulgaris* when experimental population sex ratios are 50:50 (Miyake and Olson, 2009).

Previous studies have shown that pollinator communities are particularly influential on gynodioecious populations (Eckhart, 1992; Ashman and Diefendorfer, 2001; Alonso, 2005; Ashman and King, 2005; Orellana et al., 2005; Case and Ashman, 2009), yet many do not explicitly examine the relationship between pollinator variation and sex ratio or breeding system variation. Pollination processes influence the seed

fitness of females and hermaphrodites in pollen and visit limitation (Ashman, 2000; Yuan-Wen et al., 2010), selfing rate (Ganders, 1978; Sun and Ganders, 1986; Taylor et al., 1999; Brunet and Sweet, 2006; Miyake and Olson, 2009), to mating patterns (Taylor et al., 1999; Barthelmess et al., 2006), and should therefore be central in any study of dimorphic plants. At the level of breeding system, gynodioecy, like androdioecy, is a polymorphism where the unisexual morph is more reliant on pollen vectors than hermaphrodites. This asymmetry in ecological sensitivity underscores the importance of ecological context for the evolution of gynodioecy, variation in gynodioecious sex ratios, and the transitions between hermaphroditism and separate sexes. From pollination contexts' influence on female advantage, demonstrated herein, to its potential role in sex ratio, mating, and breeding system evolution, it is necessary to more deeply examine the role of variation in pollinator context in the evolutionary ecology of plant reproductive strategies.

2.5.2 Summary

Our experiment demonstrated that a simple change in the pollination context has the potential to greatly alter the selective pressures responsible for the maintenance of females in a gynodioecious population. It is unlikely that females could persist in *S. vulgaris* populations in an environment with only scarce pollen collecting insects, as in our diurnal treatment group. Conversely, females would likely be more common in the presence of abundant pollinators that do not seek pollen rewards, as in our nocturnal treatment group. Our experiment suggests that variation in pollinator communities across species' ranges could contribute to variation in population sex ratios. Gynodioecious sex ratio variation is jointly the result of the selective and non-selective evolutionary dynamics between male sterility genes and their associated restorers in finite populations (Van Damme, 1983; Koelewijn and Van Damme, 1995a; 1995b; Charlesworth and Laporte, 1998). We highlight the strong potential for pollination context to influence the

selective pressures responsible for female maintenance and variation in their frequency. Because of the complex functional relationships contributing to female advantage, including resource compensation, inbreeding depression, and pollination processes, it is not sufficient to examine any one of these interconnected processes without considering the broader genetic and ecological context. Additional detailed studies that control, manipulate, and quantify both genetic and ecological contributions are necessary to fully understand female advantage and its role in breeding system evolution.

2.6 References

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2.7 Tables

Table 2.1: ANOVA table for insects' visit durations on single flowers. We modeled flower visit duration as a linear function of the time of day (morning or dusk), flower type (male-phase hermaphrodite flowers, female-phase hermaphrodite flowers, or female plants' flowers), and their interaction. N = 104 visitations during 26 hours of observation.

Source	Nparm	DF	SSE	F Ratio	Prob > F
Time of Day	1	1	42809	5.2	0.025
Flower Type	2	2	77250	4.7	0.011
Time of Day * Flower Type	2	2	88018	5.3	0.006

Table 2.2: ANOVA table for bout lengths on experimental plants. The number of flowers successively visited on a single plant was modeled as a geometric function of the time of day (morning or dusk), plant sex (hermaphrodite or female), and their interaction. N = 53 unique foraging bouts during 26 hours of observation.

Source	Nparm	DF	SSE	F Ratio	Prob > F
Time of Day	1	1	32.03	1.3	0.260
Sex	1	1	86.24	3.6	0.071
Time of Day * Sex	1	1	5.58	0.2	0.634

Table 2.3: ANOVA table for visitation rates per hour of observation. The total number of visits to plant per one-hour observation period was modeled as a Poisson variable dependent on time of day, sex of plant, their interaction, and display size (total number of open flowers) during 26 hours of observation. P values were determined by simulating test distributions (Poisson for number of visits, normal for all other) based on 5000 bootstrapped data sets. Significance values were estimated using a likelihood ratio test, comparing the simulated and observed data sets. Replicate and individual were included in the model as random effects.

Source	Log-likelihood Ratio	Prob > Distribution
Time of Day * Sex	10.26	0.0032
Number of Open Flowers (All Plants)	5.36	0.0448
Number of Open Flowers (F plants)	0.79	0.431
Number of Open Flowers (H plants)	15.53	0.010
Sex (Diurnal visitation)	2.33	0.224
Sex (Nocturnal visitation)	0.51	0.558
Time of Day (F Plants)	8.64	0.007
Time of Day (H Plants)	3.78	0.113

Table 2.4: Summary of pollinator behaviors for principle visitors of *S. vulgaris*. F denotes female while H denotes hermaphrodite. Numbers given are LS Means estimates, with standard errors in parentheses, NV indicates no visits.

Behavior	Syrphid Fly	Bees	Noctuid Moth
Handling Time H, M-phase	227 (± 32)	2.8 (± 42)	14 (± 15)
Handling Time H, F-phase	15 (± 38)	4.3 (± 34)	24 (± 23)
Handling Time F	35 (± 27)	1.3 (± 60)	13 (± 17)
Bout Length H	1.9 (± 0.52)	2.5 (± 0.74)	2.2 (± 0.30)
Bout Length F	2.0 (± 0.66)	2.0 (± 1.5)	2.5 (± 0.44)
Visits/hr, M-phase flr/hr, H	0.67 (± 0.59)	0.2 (± 0.2)	0.57 (± 0.29)
Visits to F-phase flr/hr, H	0.82 (± 0.45)	NV	0.24 (± 0.12)
Visits/hr, F	0.078 (± 0.036)	0.017 (± 0.017)	0.38 (± 0.16)

Table 2.5: ANOVA table for total number of fruits produced by day 12 of exposure. The number of fruits per plant was modeled as a function of time of exposure to pollinators (Treatment), plant gender (Sex), deviation from female and hermaphrodite mean number of flowers (DMF), and their interactions. Replicate was included as a random effect. N=160 plants.

Source	Nparm	Den. DF	DF Residual	F Ratio	Prob > F
Treatment	3	3	141	34	<.0001
Sex	1	1	141	29	<.0001
Treatment * Sex	3	3	141	1.8	0.148
DMF	1	1	142	238	<.0001
Sex * DMF	1	1	142	0.80	0.372
Treat * DMF	3	3	142	11	<.0001
Sex * Treat * DMF	3	3	142	2.6	0.052

Table 2.6: ANOVA table for fruit set on day 12 of exposure. Fruit set was modeled as a function of plant's time of exposure to insect visitors (Treatment), plant gender (Sex), and their interaction. Fruit set, the likelihood that a flower becomes a fruit was modeled using logistic regression. Replicate was included in the model as a random effect to account for variation between replicates. Replicates' fruit sets did not significantly differ, N=80 plants.

Source	F Ratio	Prob > F
Treatment	69.2	<0.0001
Sex	7.3	0.0086
Treatment * Sex	5.3	0.0240

Table 2.7: ANOVA table for seeds per fruit for experimental plants. Seeds per fruit were modeled as a linear response to time of pollinator exposure (Treatment), plants' gender (Sex), plant's display size, and all interactions. Replicate and individual were factors included in the model as random effects. N = 120 individuals (0-3 fruits per plant, 233 total fruits).

Source	Nparm	DF	Den DF	F Ratio	Prob > F
Treatment	2	2	80.1	24	<0.0001
Sex	1	1	93.3	7.2	0.008
Treat * Sex	2	2	93.1	0.25	0.783
#Flowers	1	1	142	2.8	0.096
Sex * #Flowers	1	1	157	0.11	0.742
Treat * #Flowers	2	2	141	3.9	0.023
Sex * Treat * # Flrs	2	2	150	0.84	0.434

Table 2.8: ANOVA table for approximate total seeds per plant. An estimate of the total seeds produced by a plant was calculated as the average seeds per fruit for a particular plant multiplied by the number of fruits produced by that plant by day 12) and modeled as a function of time of pollinator exposure (Treatment), plant gender (Sex), the plant's deviation from mean hermaphrodite or female flower number (DMF), and all interactions. Replicate was included as a random effect. N = 80 individuals.

Source	Nparm	DF	Den DF	F Ratio	Prob > F
Treatment	1	1	68.7	45	<0.0001
Sex	1	1	68.4	14	0.0003
Treatment * Sex	1	1	68.5	9.1	0.0036
DMF	1	1	67.7	63	<0.0001
Sex * DMF	1	1	32.1	0.013	0.909
Treat * DMF	1	1	71.5	36	<0.0001
Sex * Treat * DMF	1	1	71.2	0.35	0.558

2.8 Figures

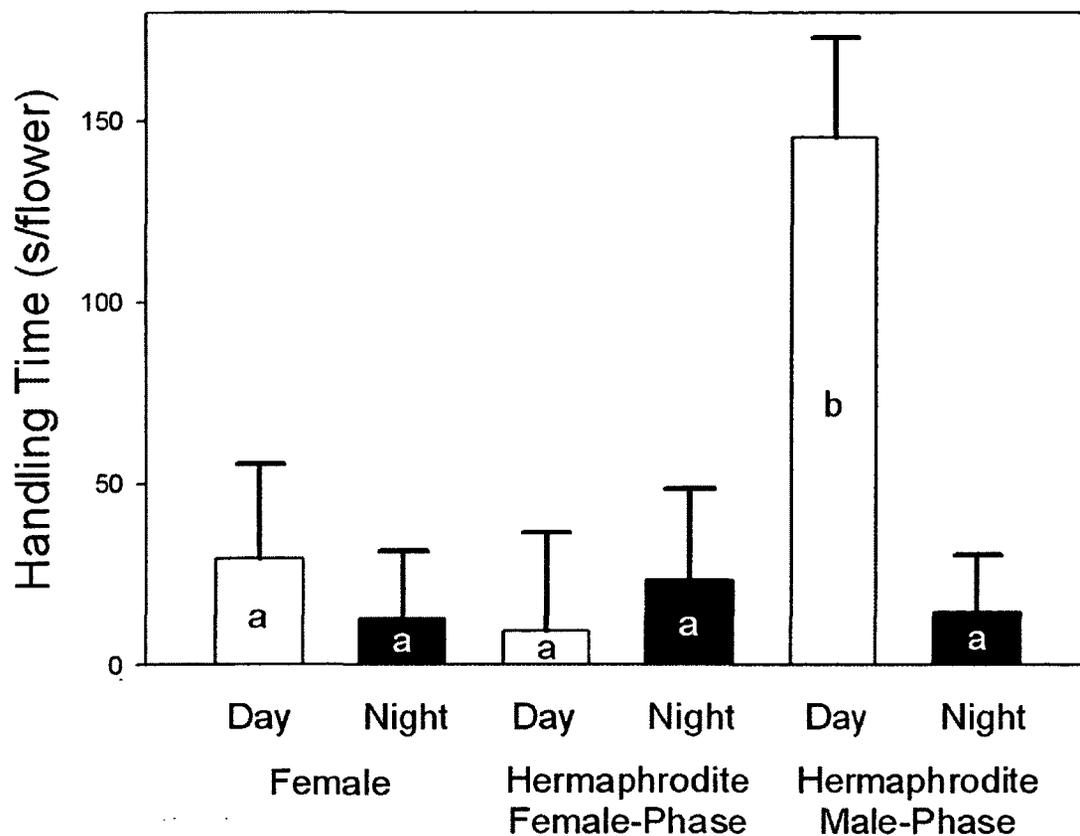


Figure 2.1: Handling times of insects on female and hermaphrodite *S. vulgaris* flowers. Flower visits were categorized according to diurnal (Day) and nocturnal (Night) insects as well as by flower gender phase. Bar heights indicate least squares estimates of the means and error bars represent one standard error of the mean. Groups not sharing the same letter significantly differ.

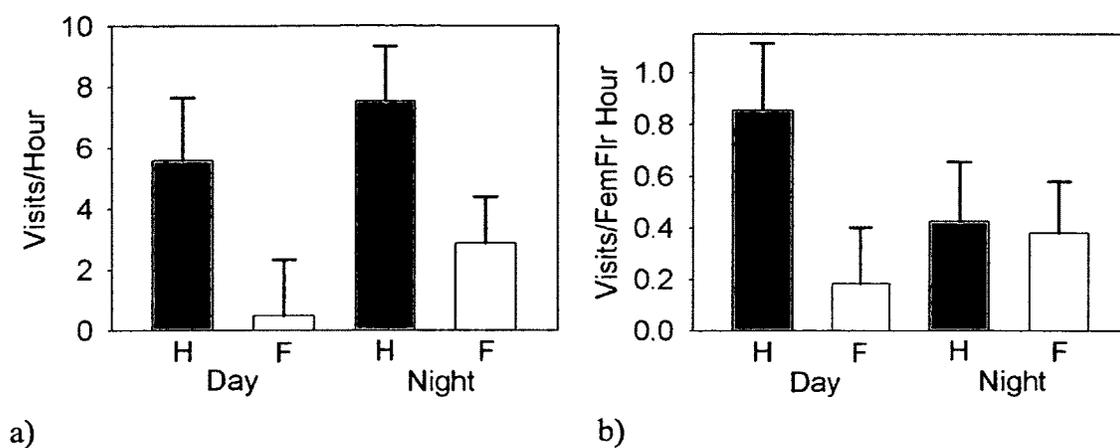


Figure 2.2: Visitation rates to experimental *S. vulgaris*. a) Visits per one-hour observation period to hermaphrodite (H) and female (F) plants, exposed to either diurnal (Day) or nocturnal (Night) insect visitors. Bar heights indicate least squares estimates of the means while error bars denote one standard error of the mean. b) Visits per flower per hour to stigmatic (female-phase) flowers on hermaphrodite and female plants under diurnal and nocturnal pollinator exposure. No pairwise comparisons were significant.

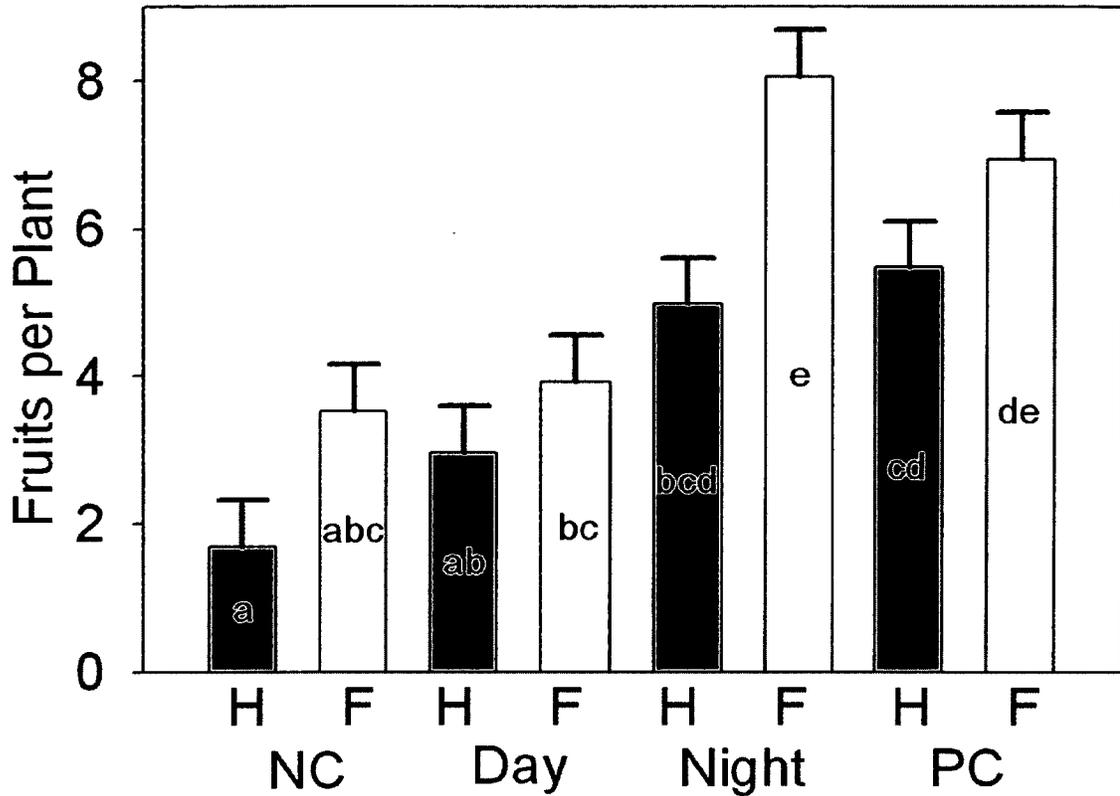


Figure 2.3: Total number of fruits produced by day twelve of experiment. The total number of fruits produced by the end of the 12 day exposure period, grouped by plant gender and pollinator exposure (negative controls - NC, diurnal exposure, nocturnal exposure, and positive controls - PC). H and F indicate hermaphrodite and female, respectively. Bar heights indicate least squares estimates of the means based on a model including plant sex, exposure treatment, their interaction, and plants' deviation in flower number (see Table 5). Error bars denote one standard error of the mean. Groups not sharing the same letter significantly differ.

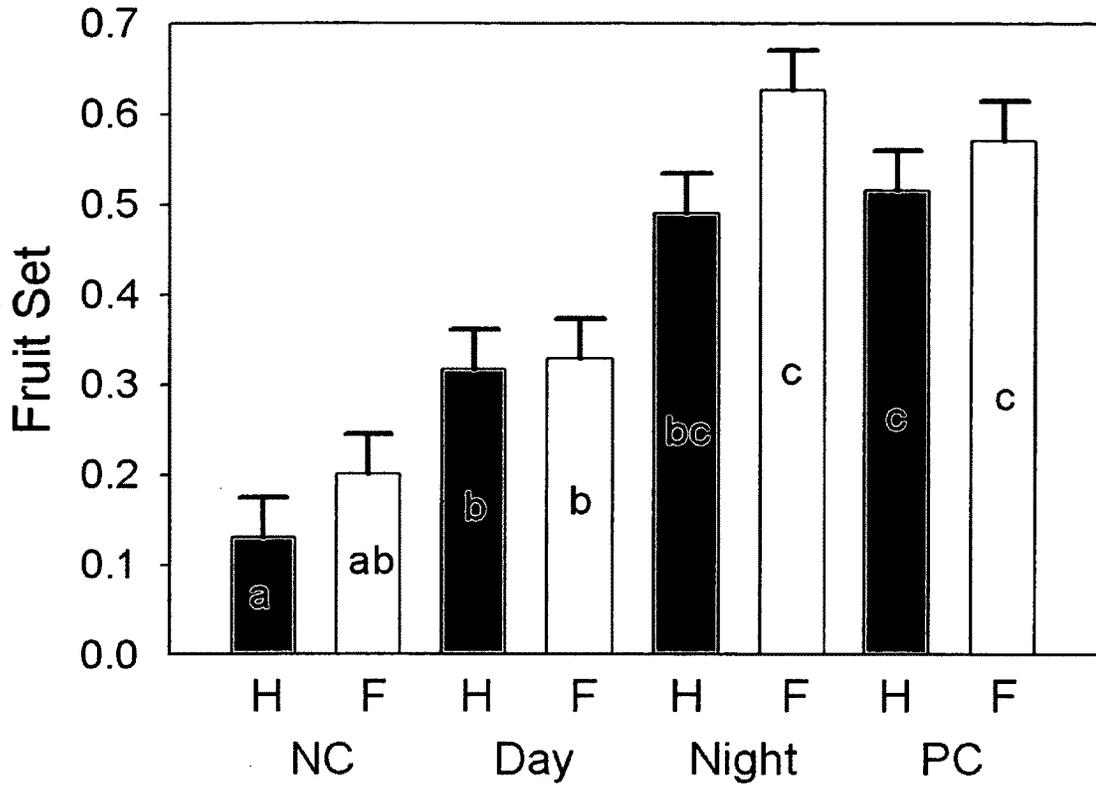


Figure 2.4: Fruit set by day twelve of experiment. The fraction of flowers on a plant that became fruits is given for hermaphrodites (H) and females (F), by pollinator exposure treatment (negative controls, diurnal exposure, nocturnal exposure, and positive controls). Bar heights indicate least squares estimates of the means based on a linear model including plant sex, exposure treatment, and their interaction (see Table 6). Error bars denote one standard error of the mean. Groups not sharing the same letter significantly differ.

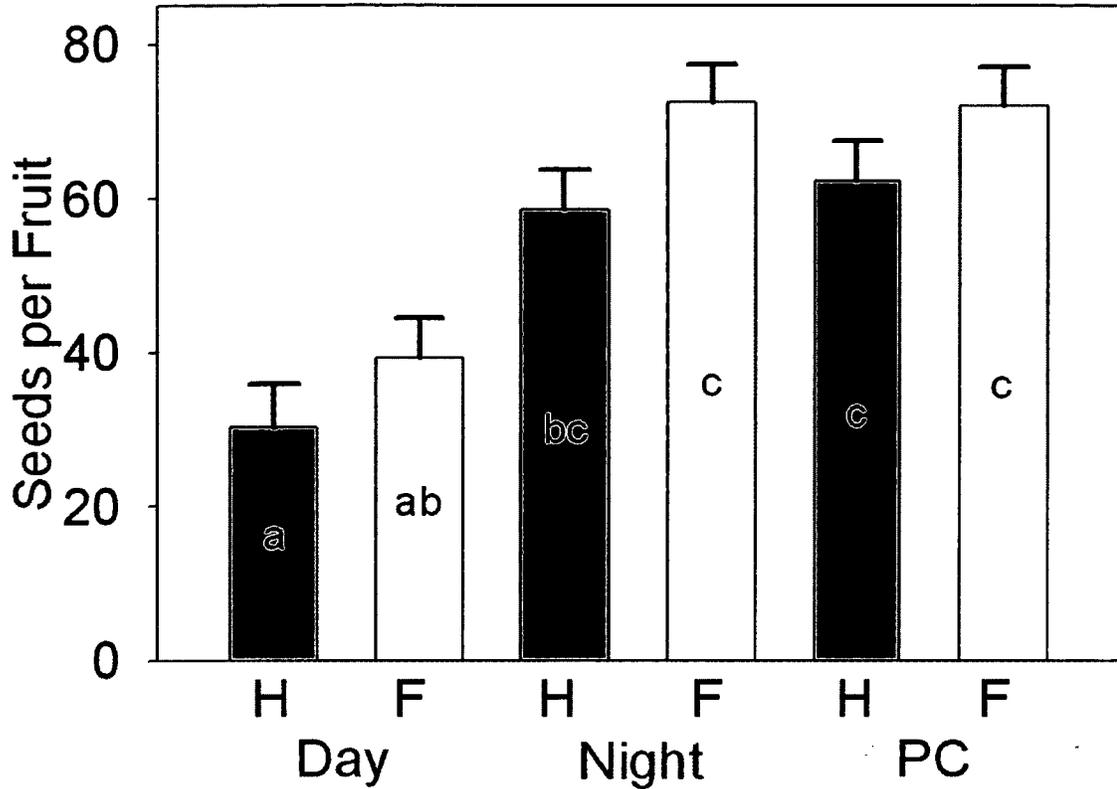


Figure 2.5: Seeds per fruit for experimental *S. vulgaris*. The number of seeds per fruit is given for females (F) and hermaphrodites (H) exposed to insect visitors during the day (Diurnal) or at night (Nocturnal). Bar heights indicate least squares estimates of the means based on a model including plant sex, exposure treatment, total flower number, and their interactions (see Table 7), while error bars denote one standard error of the mean. Groups not sharing the same letter significantly differ.

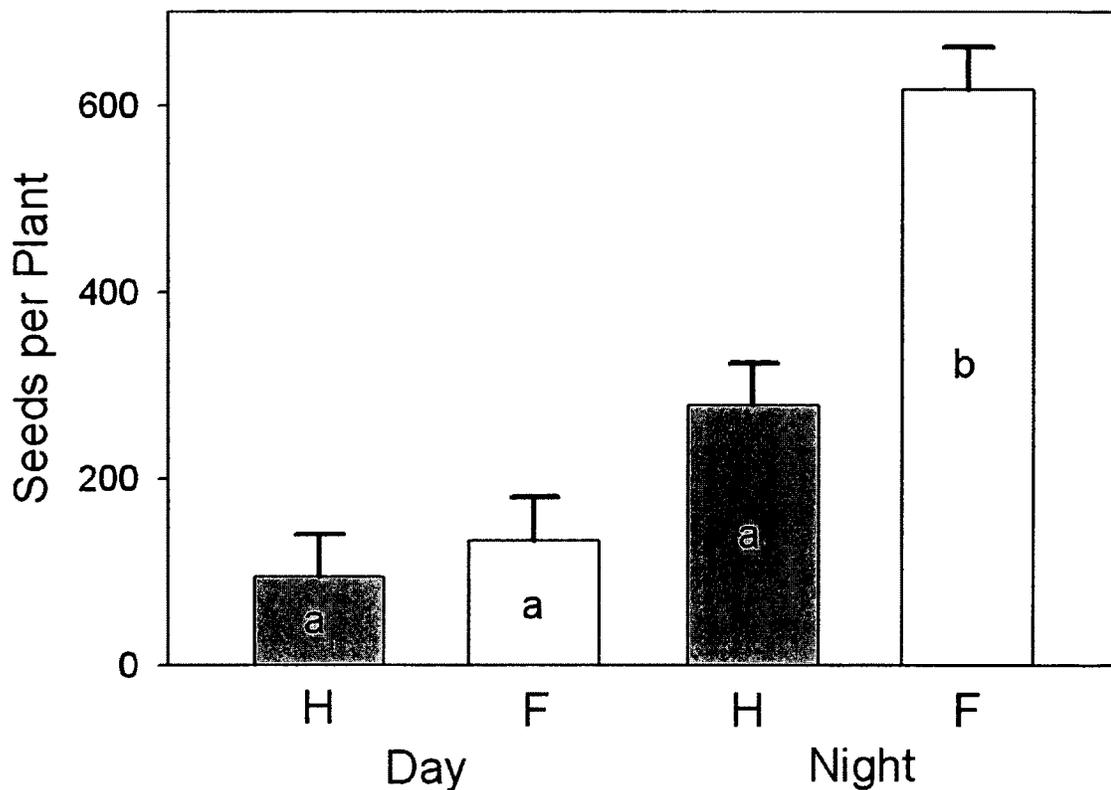


Figure 2.6: Approximate total seeds per plant by day twelve of exposure. The total number of seeds were estimated as the product of the average number of seeds per fruit for each plant times the number of fruits produced by that plant by day 12 of exposure for female (F) and hermaphrodite (H) plants exposed to pollinators during the day (Day) or at night (Night). Bar heights are least squares estimates of the means based on a model including plant sex, exposure-treatment, deviation from mean flower number, and their interactions. Error bars denote one standard error of the mean. Groups not sharing the same letter significantly differ.

Chapter III

No female advantage in artificial populations of Czech *Silene vulgaris* under multiple pollinator contexts ¹

3.1 Abstract

Females in gynodioecious populations are typically capable of producing more seeds than hermaphrodites. This “female advantage” is due to a combination of genetic and ecological factors. We tested the influence of pollination context on female advantage by exposing replicate populations of *Silene vulgaris* to either diurnal or nocturnal pollinators within its native range. Plant fitness depended strongly on pollinator exposure treatment, with nocturnally exposed plants having higher fitness than diurnally exposed, though females showed no significant seed production advantage under either pollination treatment. This was contrary to our prediction that females would be pollen-limited under exposure to diurnal pollen-collectors that prefer hermaphrodites and would show measureable female advantage in the context of nocturnal nectar collectors. Both sexes doubled their total seed production in the night with neither showing a seed production advantage. That there was no difference in female advantage between pollen collectors and nectar collectors is due in part to the absence of measureable resource compensation in females. Our results highlight pollination contexts and population characteristics that influence female advantage, a phenomenon of central importance to gynodioecious sex ratio and breeding system evolution.

¹ Stone, J.D., H. Štorchová, and M.S. Olson. Czech artificial populations of *Silene vulgaris* lack female advantage under multiple pollinator contexts. Prepared for submission to *The American Journal of Botany*.

3.2 Introduction

Adequate pollinator services are required for the maintenance or expansion of plant populations (Darwin, 1859; Allen-Wardell et al., 1998; Morgan et al., 2005; Vamosi et al., 2006; Moeller et al., 2012) and for the stability of plant reproductive strategies (Lloyd, 1974; Ashman, 2006; Dufay and Billard, 2012; Chapter 2). There is growing recognition of the importance of spatial and temporal variation in pollinator communities and the services they provide (Ashman et al., 2004; Knight et al., 2005a; Knight et al., 2005b; 2006; Ashman, 2006; Eckert et al., 2009; Harder and Aizen, 2010). For instance, pollen limitation increases at the edges of species ranges (Totland, 2001; Moeller et al., 2012), varies over time (Molina-Freaner et al., 2003; Waites and Agren, 2004; Case and Ashman, 2009), and changes with pollinator abundance (Franzén and Larsson, 2009). Pollen limitation can also result from habitat fragmentation (Hadley and Betts, 2012) and pollen availability gradients caused by population structure (McCauley and Taylor, 1997; Taylor et al., 1999), underscoring the potential for pollination context to alter plant fitness at nearly any scale. This suggests that pollination context, which includes the composition, abundance, and behavior of local pollinator communities, may limit where particular reproductive strategies can arise and persist.

Dimorphic breeding systems such as gynodioecy reveal additional connections between pollination context and plant populations. Even where pollination is sufficient for population viability, the pollinator services may vary for the two sexes, influencing breeding system evolution. For example, Chapter 2 illustrates that diurnal pollinator communities with primarily pollen-collecting insects may strongly favor visitation to hermaphrodite flowers in gynodioecious *S. vulgaris*, whereas nocturnal pollinators readily visit both sexes. These differences in pollinator behavior were associated with large differences in total seed production, as well as significant change in female seed fitness relative to that of hermaphrodites. Hermaphrodites had their highest relative fitness upon exposure to pollen collectors and in the presence of pollen limitation,

whereas females showed a more than two-fold seed production advantage in the context of nectar collecting pollinators that showed less of a preference for hermaphrodites. It is unclear, however, whether pollinator conditions that favor high female seed fitness, like those in Chapter 2, are a prerequisite for the evolution of gynodioecy and are found wherever this breeding system occurs. Although multiple studies suggest a link between pollinators and breeding system evolution over deep timescales (Renner and Ricklefs, 1995; Barrett, 1992; Wolf and Takebayashi, 2004), proximate data are lacking.

At generational timescales, selfing rate and female advantage have been identified as important for the maintenance of gynodioecy (Charlesworth and Charlesworth, 1978; Chapter 4), and both are influenced by pollinator context (McCauley and Taylor, 1997; Ashman et al., 2000; Ashman and King, 2005; Brunet and Sweet, 2006; Chapter 2). Pollinators with a very strong preference for hermaphrodites, for example, might cause the loss of the genes underlying a female phenotype. Accordingly, pollinator context has been noted as a contributing factor in the relative fitness of gender morphs (Ganders, 1978; Weller and Sakai, 1990; Delph, 1990), and hermaphrodite-biased pollinator visitation has been recorded in several gynodioecious systems (Ashman and Stanton, 1991; Delph and Lively, 1992; Williams et al., 2000; Cuevas et al., 2008; Chapter 2). Additionally, several studies have reported variable levels of female advantage (reviewed in Shykoff et al., 2003; Spigler and Ashman, 2012; Dufay and Billard, 2012), but few have quantified pollinators' role by directly measuring female advantage in multiple pollination contexts (Ashman and King, 2005; Chapter 2). This underscores the need to measure female and hermaphrodite fitness in gynodioecious populations under varied pollination contexts, while controlling for factors that may also affect seed fitness.

In this study we directly test whether pollination context alters female advantage by selectively exposing artificial populations of gynodioecious *Silene vulgaris* to different segments of its natural pollinator guild. In Chapter 2, we described the results of an experiment comparing the influences of diurnal and nocturnal pollinators on the

maintenance of gynodioecy in the introduced range of *S. vulgaris*, in North America. Here we present the results of a complementary study that was conducted in the native range of *S. vulgaris*, in Europe, to determine whether diurnal and nocturnal pollinators have consistent effects in different regions of the species' range. This North American-European comparison provides several contrasts. First, European populations of *S. vulgaris* provide a different genetic background: European populations display a high degree of endemism, with several subspecies and ecotypes across its range (Marsden-Jones and Turrill, 1957; Aeschimann and Bocquet, 1980), whereas North American populations are a recombinant mosaic of that diversity, and increased vigor (Taylor and Keller, 2007; Keller et al., 2009). Second, the *Silene* pollinator communities show key differences between the North America and Europe (Kephart et al., 2006). *Hadena spp.* moths are co-evolved pollinators and seed predators that lay eggs in the developing fruits of *S. vulgaris*. These moths are important pollinators in Europe but are absent in North America. Lastly, the artificial populations used in this study were set up in the context of a large natural population of *S. vulgaris*, increasing the availability of pollinators and pollen donors compared to our US study.

3.3 Methods

3.3.1 Study system

Silene vulgaris (Caryophyllaceae) is a perennial herb native to Eurasia that occurs in meadows and along human-disturbed sites such as roads and fields. Populations may consist of large, long-established, continuous groups of more than one hundred individuals, as in the natural population surrounding our experimental population at Kovary Meadows, Czech Republic, or may be patchily distributed, as in some regions of North America (McCauley et al., 2000; Olson et al., 2005; Olson et al., 2006). *S. vulgaris* has a gynodioecious breeding system and sex expression is controlled by a cytonuclear polymorphism with multiple *CMS* types and several pairwise-compatible

nuclear restorers of male fertility, *Rf* (Charlesworth and Laporte, 1998; Bailey and McCauley, 2005). The sex ratio of populations varies widely in European populations, from 10-100% hermaphrodites (Štorchová and Olson, 2004; Glaettli et al., 2006). Hermaphroditic plants are self-compatible (Taylor et al., 1999; Miyake and Olson, 2009) and self-fertilization (selfing) occurs primarily through geitonogamy as flowers are strongly protandrous. Individual plants in the wild vary in size, many having 10-50 flowers open at a time. *S. vulgaris* shows traits consistent with nocturnal specialization but compatibility with diurnal bees and flies (Pettersson, 1991a; 1991b; Jürgens et al., 2002; Jürgens, 2006), with white, scented flowers that open at dusk and stay open for up to 5 days. The corolla opening is large enough to admit halictid bees, syrphid flies, small geometrid moths, large *Bombus*, and noctuid moths. All diurnal visitors in this study were observed handling hermaphrodites' anthers, whereas nocturnal visitors' contact with the stigmas and anthers appeared incidental. The timing of flower opening also exhibits nocturnal specialization (detailed in Chapter 2).

3.3.2 Experimental populations

Eighty female and eighty hermaphrodite plants were grown from seed in the Institute of Experimental Botany (IEB) of the Czech Academy of Sciences greenhouse. Open flowers were removed at the beginning of the experiment to ensure all pollination occurred during the experiment. At the end of June, coinciding with flowering of local populations of *S. vulgaris*, artificial populations were established in Koverý Meadows, 18 km NW of Prague (50.185°N, 14.252°E), within a large natural population. Each plant was randomly assigned to one of four replicates within Koverý Meadows, with replicates separated by 80-100 m. Plants within each replicate were assigned to one of four pollinator exposure treatment groups: 1) diurnal pollinators only (exposed to pollinators from 0800 to 2100), 2) nocturnal pollinators only (exposed to pollinators from 2100 to 0800), 3) both diurnal and nocturnal pollinators (always exposed to pollinators), and 4)

neither diurnal nor nocturnal pollinators (never exposed to pollinators). We constructed a nylon mesh enclosure (2 m by 3 m floor and 1.5 m high, enclosed in bridal veil with <5 mm mesh size) for each replicate to prevent flying insects from pollinating flowers. We also constructed a nylon mesh shade tent to control for shade effects while permitting insect access. All plants were moved daily between enclosures and shade tents according to their treatments (1-2) or within the shade tent or enclosure (3 and 4, respectively) to control for handling and spatial effects. Our artificial populations comprised of greenhouse-grown plants allowed us to reduce variation in seed production resulting from plant age, size, genetic relatedness, microhabitat, and spatial effects. At the end of the experiment, all unopened flower buds and exposed stigmas were removed to halt further pollination, and plants were transferred to the IEB greenhouse patio. Fruits were matured for approximately 4-6 weeks and collected just before capsule dehiscence to prevent seed loss.

3.3.3 Pollinator observations

In order to better understand the relationship between insect visitors and the pollination services received by female and hermaphrodite plants, we monitored nocturnal and diurnal insect visitation patterns using video recorders. Up to four plants were individually monitored at a time using cameras placed 50 to 150 cm from a focal plant. Video recordings were made just after dusk from 21:30-22:30 with near-infrared illumination (λ roughly 800-1000nm) and from 08:00-09:00 with visible light, times that coincided with maximum pollinator activity. Very cool, very hot, rainy, or windy conditions were expected to reduce insect foraging activity and were avoided as observation periods. Every open flower on a focal plant was identified at the beginning of the recording. If the focal individual was a hermaphrodite, the gender-phase each individual flower also was noted. Video recordings were later reviewed to collect data on pollinator visitation rates, the total number of flowers visited in each bout, the type of

insect visitor, the gender-phase of each flower visited (on hermaphrodites), and the visitors' time spent handling individual flowers.

3.3.4 Seed fitness components

During the field experiment plants were censused every two days, from day 2 through day 12. For each plant the total numbers of flowers, the flowers in each gender phase on hermaphrodite individuals, the developing fruits, the number of fruits damaged by seed-predating larvae, and the old flowers with reproductive structures that had visibly wilted were each recorded each census day. Numbers of developing fruits were determined by gently squeezing the calyx; a noticeably enlarged gynoecium was recorded as a fruit. After maturing, a haphazard subsample of up to six fruits from each plant was dissected and the mature seeds counted. Seeds were categorized as mature only if ovules were fully developed and bore a hard corpuscular seed coat.

3.3.5 Statistical analyses

Pollinator visitation data were analyzed using ANOVA as in Chapter 2, except that plant ID was not included because no plant was used for more than one observation period. Measures of plant fitness including the number of fruits, fruit set, seeds per fruit, and total seeds, were analyzed using ANOVA. All analyses were conducted using JMP 10 (SAS Institute, 2012). For analyses of fruit production and fruit set, positive and negative controls are included. For seeds per fruit, negative controls were omitted because, despite setting some fruit, these fruits rarely contained developed seeds, resulting in a very small number of negative control individuals that could be included, violating the assumption of approximately equal sample sizes. For the analysis of total seed production, only diurnally and nocturnally exposed plants are included; the controls were not fully comparable as they included gender-shifting plants deemed unsuitable for the diurnal and nocturnal treatment groups. All analyses of plant fitness components

included display size as a covariate, except for fruit set where it served as the number of binomial trials. Total flower number was used as the display size covariate as flower number did not differ by sex in this study. Planned contrasts were identified *a priori* to compare subpopulations of particular interest. *Post hoc* pairwise comparisons were conducted for the majority of analyses using Tukey's test for honest significant differences (HSD) and Bonferroni correction for the fruit set analysis. Statistical methods are presented in greater detail in Chapter 2.

We also compared the results from this study in the Czech Republic with results from Virginia (Chapter 2) using an ANOVA that included pollination treatment, sex, and deviation from mean flower number, the display size covariate used in Chapter 2, as well as the factor "location" corresponding to Virginia or Czech, all interaction terms, and replicate as a random effect. Female advantage, here the ratio of female to hermaphrodite seed production, was also calculated for diurnally and nocturnally exposed plants from both experiments.

3.4 Results

3.4.1 Pollinator behavior

Diurnal visitors to the experimental *Silene vulgaris* plants during observation periods consisted of syrphid flies (49% of daytime visits, n=38), halictid bees (49% of daytime visits, n=38), a single large *Bombus* (1% of daytime visits, n=1), and a single butterfly (1% of daytime visits, n=1). Additional functional groups of diurnal arthropods including diurnal lepidopterans, ants, and small beetles were occasionally noticed on flowers outside of the observation periods. Nocturnal visitors consisted entirely of noctuid moths (*Noctuidae*, 100% of nocturnal visits, n=26), primarily *Autographa gamma*. Rarely, small moths (*Geometridae*) were viewed on plants outside of the observation periods.

Handling time (time spent on individual flowers) was approximately three times longer for diurnal insects than for nocturnal insects (Table 3.1, Figure 3.1a) and did not significantly vary by flower sex. Syrphid flies' handling times, however, were three times longer on hermaphrodite flowers than on female flowers (Figure 3.1b), though this difference was only marginally significant (LSMeans Planned Contrast, $F_{1,212} = 3.05$, $p = 0.082$, Table 3.4). There were no other significant differences among flower types or pollinators for handling times (Tables 3.1, 3.4, Figures 3.1a, b).

Hermaphrodites experienced a significantly higher visitation rate than females overall (Table 3.2, Figure 3.2a). Visitation rate also was significantly associated with display size (total number of open flowers; Table 3.2). Visitation rates were several times higher during the day than at night for both sexes and there was no significant interaction between plant sex and time of day on visitation rate (Table 3.2, Figure 3.2a). When only considering stigmatic (female-phase) flowers, diurnal visitation rates were significantly higher than nocturnal ones (Table 3.2, Figure 3.2b). Although stigmatic flower visitation rates did not differ between the sexes overall, they were significantly higher for hermaphroditic plants during the day (Table 3.2, Figure 3.2b, interaction term).

Mean bout length, the number of flowers sequentially visited on a particular plant, was relatively short (average 2.17 flowers, $N=104$ unique bouts). Most visits consisted of only 1-4 flowers visited, whereas a few visitation bouts were longer, up to 11 flowers. Bout lengths were slightly longer on larger plants, but did not significantly differ between nocturnal and diurnal visitors, between females and hermaphrodites, or for the interaction of plant sex and visitor type (Table 3.3, Figure 3.3). Average bout lengths varied little among types of floral visitors (Figure 3.3).

3.4.2 *Plant fitness*

Nocturnally exposed plants produced significantly more fruits than diurnally exposed plants (LSMeans Planned Contrast, $F_{1,137} = 10.3$, $p = 0.0016$, Figure 3.4), and

hermaphrodites produced more fruits than females when negative controls were excluded (LSMeans Planned Contrast, $F_{1,102} = 8.98$, $p = 0.0034$, Figure 3.4), with no significant interaction between sex and pollinator treatment (Table 3.5). Display size strongly affected fruit production for both sexes, with more flowers resulting in significantly more fruits (Table 3.5). Positive control plants produced fewer fruits than nocturnally exposed ones, though the difference was not significant (Tukey's HSD). Flowers on nocturnally-visited plants were about 25% more likely to set fruit than those on diurnally-visited plants (Planned contrast: LR $\chi^2 = 18.6$, $p < 0.001$, Figure 3.5) and hermaphrodites had higher fruit set overall, when negative controls were excluded (Planned contrast: LR $\chi^2 = 21.7$, $p < 0.001$, Figure 3.5).

Nocturnally-exposed plants produced significantly more seeds per fruit than diurnally-exposed plants (LSMeans Planned Contrast: $F_{1,76} = 14.1$, $p = 0.0003$, Table 3.7, Figure 3.6) but neither group differed significantly from the positive controls (Tukey's HSD). Females and hermaphrodites did not significantly differ in their seed production (Table 3.7, Figure 3.6). The total number of flowers on a plant (*cumulative* display size) did not significantly affect number of seeds per fruit ($F_{1,77} = 0.188$, $p > 0.6$). A weak, but statistically significant, three-way interaction term indicated that larger floral displays increased female seed production among diurnal plants more than it did among nocturnally exposed and hermaphroditic plants (Table 3.7).

At the whole-plant level, individuals produced significantly more total seeds when exposed to nocturnal pollinators than to diurnal ones (Table 3.8, Figure 3.7), but females and hermaphrodites produced similar numbers of seeds, regardless of pollination treatment (Figure 3.7, Table 3.8). The estimated total number of seeds per plant depended strongly on display size and whether a plant had more or fewer flowers than average (Table 3.8). Seed quality, or the fraction of fully formed seeds, did not differ for any combination of sex or treatment (ANOVA, binomial distribution, $p > 0.2$ for all terms).

Seed predation was low for all plants and only marginally significantly greater than zero for nocturnally exposed females (0.32 fruits eaten per plant, $p = 0.09$, t -ratio = 1.75, $DF_{Den} = 145$, Figure 3.8, Table 3.9). Seed predation did not significantly differ with sex, display size, or time of exposure, though rates were too low to reliably measure.

Comparing data from these experiments in the Czech Republic to data from a parallel experiment conducted one year earlier in Virginia, USA (Chapter 2), revealed both similarities and differences. In both studies seed production was higher when plants were exposed to nocturnal pollinators than when plants were exposed to diurnal pollinators (Figure 3.9, Table 3.10). Female seed production was higher than that of hermaphrodites in Virginia, but only at night (Tukey's HSD, Figure 3.9); this pattern was not seen in the Czech Republic, where relative seed production of female and hermaphrodites did not differ across treatments (Figure 3.9, Table 3.10, treatment * sex * location interaction term).

3.5 Discussion

Despite the importance of female advantage in gynodioecy theory and its prevalence in empirical studies (Couvét et al., 1990; Shykoff et al., 2003; Spigler and Ashman, 2012), as well as abundant pollinators and access to many hermaphrodite pollen donors, we did not detect a significant female advantage in our Czech Republic (CR) artificial populations across multiple pollination contexts. This is in contrast to our study on Virginia (US) *S. vulgaris* (Chapter 2); where a female advantage was evident in nocturnally pollinated plants. Conversely, CR hermaphrodites did not exhibit significant reductions in seed fitness, despite having to allocate resources to male reproduction and having a shorter period of stigmatic receptivity time than females. These results indicate that pollination processes in the CR population, specifically sex-specific pollen and visit limitation, were not sufficient to explain the maintenance of females.

Our study provides the opportunity to examine how female advantage arises and why it is or is not realized. The first factor contributing to the absence of female advantage and to its insensitivity to pollinator context is the apparent lack of resource compensation among our CR plants. Resource compensation (Darwin, 1877), the reallocation of resources away from male function to higher seed fitness on females, is found in the majority of gynodioecious species (Couvét et al., 1990; Shykoff et al., 2003; Spigler and Ashman, 2012). Resource compensation is expected to elevate females' seed fitness in the absence of pollen limitation (Lewis, 1941; Lloyd, 1974). Female CR plants, however, did not produce more flowers than hermaphrodites as females did in our parallel US study (Chapter 2). Moreover, CR females did not show elevated seed production under any pollination context, despite visitation rates several times higher and the greater availability of pollen donors in the surrounding natural *S. vulgaris* population compared to females in our US study. Female advantage was strong in our US study, with nearly identical potting medium, fertilization levels, and experimental protocol. Thus, it is plausible that differences in resource compensation contribute to the differences contribute to differences in female advantage between the genotypes used in the Virginia (US) and Kovary (CR) experiments.

The second factor that may contribute to the absence of female advantage and insensitivity to pollinator context is the behavior and abundance of local pollinators. The CR diurnal pollination context featured visitation rates more than three times greater than any other we studied. For females, diurnal visitation rates were ten times greater than those in our US study, presumably owing to the natural meadow context in the CR. Accordingly, diurnal pollination in CR was sufficient for both sexes to achieve high fruit set. Neither sex produced as many seeds per fruit with diurnal pollination in the CR as at night, but there were no significant differences based on sex. The abundance of diurnal visitors in the CR, as well as females' period of stigmatic exposure, nearly twice as long as hermaphrodites', likely eliminated the negative effects of pollinators preferentially

visiting hermaphrodites. Nocturnal pollinators delivered more pollen in fewer visits, providing higher fitness to both sexes of nocturnally specialized *S. vulgaris*, but again, neither sex showed measurable fitness advantage. The absence of resource compensation in females would preclude female advantage even in the context of abundant, unbiased pollinators as in the CR nocturnal pollinator treatment.

Lastly, population context may affect the level of female advantage and a population's sensitivity to pollination context. The placement of the artificial CR population within a large, natural population of *S. vulgaris*, for example, increased the availability of pollen donors and vectors. Having numerous hermaphrodites is expected to reduce pollen limitation and facilitate outcrossing (Miyake and Olson, 2009). The presence of hundreds of wild hermaphrodites providing pollen and attracting pollinators accounts for both sexes' high fruit set under both CR pollination contexts- higher than in the US. The number of seeds per fruit, however, was lower among CR plants than US plants, owing to smaller flowers with fewer ovules (data not shown). The US experiment was conducted in an area devoid of other *S. vulgaris* for at least 2 km, meaning pollinators had to be recruited and no outside pollen was available. Accordingly, diurnal visitation rates were low in the US and pollen limitation was very strong. In the CR experiment, however, the abundance of local pollen sources and vectors reduced the potential costs associated with hermaphrodite-preferring diurnal pollinators.

It is worth noting that, despite both sexes experiencing their highest visitation rates and longest handling times during the day, plants had higher seed fitness with nocturnal exposure. *S. vulgaris*' morphology and phenology fit a moth pollination syndrome (Pettersson, 1991b; Jürgens, 2002; Jürgens et al., 2006), so this is not unexpected, but does highlight the greater effectiveness of the coevolved pollinators. Similar elevated seed production with nocturnal pollination has been noted in *Silene alba* (Young, 2002) and *S. vulgaris* (Chapter 2).

We expected to observe a larger impact of seed predation on plant fitness in the CR because of the presence of *Hadena* pollinators, which oviposit eggs near the plant ovaries after pollinating *S. vulgaris* (Pettersson, 1992). We suspect that *Hadena* oviposition may occur earlier in the season in Kovary or vary from year to year as *Hadena spp.* were not observed during surveys which indicated that the predominant nocturnal pollinators during the CR experiment were *Autographa spp.* Additionally, exposure to seed predators for a period longer than our 12-day experiment may be required to exhibit an effect. Moreover, wild plants are often larger than those used in our experiment, which may have made our experimental plants less attractive to seed predators (Dötterl et al., 2005; Muhlemann et al., 2006; Giménez-Benavides et al., 2007). Additional work on populations over the entire flowering period is necessary to determine if seed predation is sex-biased and/or directed towards whichever group has a seed production advantage.

Gynodioecy without female advantage, as we saw for *S. vulgaris* in our CR plants, is uncommon, but not unique. Gynodioecy in the absence of measureable female advantage may highlight additional factors responsible for the maintenance of gynodioecy. Females are common in Kovary meadows, so our experimental plants were either unrepresentative in terms of female advantage, or other factors maintain gynodioecy in Kovary. Out of 54 gynodioecious and subdioecious populations reviewed in Couvet et al. (1990) and Spigler and Ashman (2012), more than 80% showed female seed production advantage. The remaining minority represents cases where female advantage is variable or absent. This may be due to pollinator bias and pollen limitation as with the US diurnally exposed plants (Chapter 2). Alternatively, female maintenance without female advantage may indicate that gynodioecy is maintained by nonselective processes as in Belhassen et al. (1989). Indeed, *CMS-rf* dynamics, demographic history, and stochastic processes are all expected to be disproportionately influential in

cytonuclear species such as *S. vulgaris* (Bailey and Delph, 2007; McCauley and Bailey, 2009; Barrett, 2010).

The absence of female advantage in our CR population provides a stark contrast to our US experiment and evidence that pollinators have greater potential to influence female advantage if the females display resource compensation. A comparison of our US and CR experiments provides evidence that female advantage will depend strongly on the local pollinator community if the plants show resource compensation. A similar conclusion is reached by Sato (2002) and Johnston et al. (2009), using gain curves as well as Chapter 4, using pollinator behavioral models, illustrating the interaction between resource compensation and pollinator behavior on female fitness and gynodioecious sex ratios. Accordingly, we bridge a gap in our understanding of pollinators influence on gynodioecy by providing two empirical examples that illustrate both the strong, direct role of pollinators on female relative fitness as well as variation in pollinator sensitivity depending on a population's broader context and genetic composition.

3.6 References

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3.7 Tables

Table 3.1: ANOVA table for the handling times on CR *S. vulgaris*. The average duration of a single flower was modeled as a linear function of the time of day (morning or dusk), flower type (male-phase hermaphrodite flowers, female-phase hermaphrodite flowers, or female plants' flowers), their interaction, comparing visits on female vs hermaphrodite flowers, or female and male phase flowers on both sexes. N = 226 visitations during 10 hours of observation.

Comparison	Source	Nparm	DF	SSE	F Ratio	Prob > F
F/H Plants	Time of Day	1	1	10530	6.755	0.010
F/H Plants	Flower Type	2	2	11.98	0.996	0.996
F/H Plants	T.o.D. * FlowerType	2	2	1108	0.356	0.701
F/M Flowers	Time of Day	1	1	10800	6.945	0.009
F/M Flowers	Flower Type	1	1	23.55	0.015	0.902
F/M Flowers	T.o.D. * FlowerType	1	1	2.357	0.002	0.969

Table 3.2: ANOVA table for visitation rates to CR *S. vulgaris*. The total number of visits to plant per 1-hour observation period was modeled as a Poisson variable dependent on time of day, sex of plant, their interaction, and display size (total number of open flowers), for either all flowers (a), or only female-phase (stigmatic) flowers (b). N=10 hours of observation. Degrees of freedom were insufficient to include all possible interaction terms.

Flowers	Source	DF	Likelihood Ratio χ^2	Prob > χ^2
All Flowers	Sex	1	4.886	0.0271
All Flowers	Time of Day	1	7.288	0.0069
All Flowers	Sex * Time of Day	1	0.180	0.6718
All Flowers	# Open Flowers	1	10.26	0.0014
F-Phase Only	Sex	1	0.0118	0.913
F-Phase Only	Time of Day	1	46.70	<.0001
F-Phase Only	Sex * Time of Day	1	8.886	0.0029
F-Phase Only	# Open Female Flrs.	1	5.545	0.0185

Table 3.3: ANOVA table for bout lengths on CR *S. vulgaris*. The total number of flowers probed by a visitor was modeled as a function of time of day, sex of plant, their interaction, and display size (total number of open flowers), N=102 unique foraging bouts over 10 hours of observation. Two bouts, one by a large *Bombus* and another by diurnal lepidopteran, were excluded as the lone visits by each type. Degrees of freedom were insufficient to include all possible interaction terms.

Source	Nparm	DF	DF Den.	F Ratio	Prob > F
Sex	1	1	0.262	0.076	0.783
Time of Day	1	1	0.672	0.195	0.660
Time of Day * Sex	1	1	0.764	0.222	0.639
Flower #	1	1	14.21	4.128	0.045

Table 3.4: Summary of pollinator behaviors for principle visitors of *S. vulgaris* in CR. F denotes female, M denotes male, and H denotes hermaphrodite. Numbers given are LSMeans estimates, with standard errors given in parentheses:

Source	Syrphid Fly	Halictid Bee	Noctuid Moth
Handling Time H, M-phase	21.2 (± 7.5)	34.2 (± 9.7)	8.5 (± 6.9)
Handling Time H, F-phase	19.6 (± 6.3)	46.1 (± 7.3)	5.2 (± 12.5)
Handling Time F	7.2 (± 5.8)	48.0 (± 7.3)	12.4 (± 10.0)
Bout Length H	2.5 (± 0.38)	1.9 (± 0.39)	1.9 (± 0.41)
Bout Length F	3.1 (± 0.49)	1.6 (± 0.46)	2.3 (± 0.75)
Visits/hr, M-phase flr/hr, H	1.66 (± 1.1)	0.78 (± 0.50)	1.34 (± 1.0)
Visits to F-phase flr/hr, H	1.17 (± 0.75)	0.87 (± 0.62)	0.39 (± 0.19)
Visits/hr, F	1.04 (± 0.73)	0.53 (± 0.34)	0.49 (± 0.32)

Table 3.5: ANOVA table for fruit production by CR *S. vulgaris*. The total number of fruits produced by day 12 of exposure was modeled as a function time of exposure to pollinators (Treatment), plant gender (Sex), total flower number (Flr#), and their interactions. Replicate and Family were included as random effects, N=160 plants.

Source	Nparm	DF	DF Den.	F Ratio	Prob > F
Treatment	3	3	138	30.1	<.0001
Sex	1	1	121	1.88	0.172
Flr #	1	1	143	254	<.0001
Sex * Flr #	1	1	144	3.69	0.057
Treat * Sex	3	3	138	1.91	0.131
Treat * Flr #	3	3	141	13.2	<.0001
Treat * Sex * Flr #	3	3	141	1.96	0.123

Table 3.6: ANOVA table for fruit set among CR *S. vulgaris*. Fruit set, the binomial probability of a flower becoming a fruit, was modeled as linear response to the plant's time of pollinator exposure (Treatment: day, night, and positive controls), plant gender (Sex: female or hermaphrodite), and their interaction. N=160 plants.

Source	DF	Likelihood Ratio χ^2	Prob > χ^2
Treatment	3	234	<0.0001
Sex	1	7.7	0.1810
Treatment * Sex	3	20	0.0032
Replicate	3	9.5	0.0234

Table 3.7: ANOVA table for seeds per fruit for CR *S. vulgaris*. Seeds per fruit were modeled as a linear response to time of pollinator exposure (Treatment, Treat), plants' gender (Sex), total flower number (Flr #), and their interaction terms. Treatment includes day, night, and positive control plants. Replicate and individual were included in the model as random effects. N = 105 individuals, 460 fruits.

Source	Nparm	DF	Den DF	F Ratio	Prob > F
Treatment	2	2	74	7.2	0.001
Sex	1	1	74	1.3	0.266
Flr #	1	1	77	0.19	0.666
Sex * Flr #	1	1	77	0.041	0.840
Treat * Sex	2	2	74	0.34	0.715
Treat * Flr #	2	2	75	0.10	0.901
Treat * Sex * Flr #	2	2	76	3.5	0.035

Table 3.8: ANOVA table for approximate total seeds per individual CR *S. vulgaris*. The total number of seeds per plant was calculated as the product of the average number of seeds per fruit times the number of fruits produced by that plant by day 12. This response variable was modeled as a function of time of pollinator exposure (Treatment, Treat), plant gender (Sex), a plant's total flower number (Fl #), and their interactions. Treatment includes diurnally and nocturnally exposed plants. Replicate and family were included as random effects. N = 80 individuals.

Source	Nparm	DF	Den DF	F Ratio	Prob > F
Treatment	1	1	65	25	<.0001
Sex	1	1	49	0.11	0.744
Flower #	1	1	72	48	<.0001
Sex * Fl #	1	1	68	0.07	0.798
Treat * Sex	1	1	64	0.09	0.766
Treat * Fl #	1	1	66	5.2	0.026
Treat * Sex * Fl #	1	1	65	0.11	0.742

Table 3.9: ANOVA for fruits damaged by seed predators in CR *S. vulgaris*. The number of damaged fruits was modeled as a function of pollinator exposure treatment (Treat), plant sex (Sex), total display size (Flr #), and their interactions. Replicate and Family were included as random effects. N=160 plants.

Source	Nparm	DF	DF Den.	F Ratio	Prob > F
Treatment	3	3	140	0.350	0.790
Sex	1	1	67	0.885	0.350
Flr #	1	1	109	0.891	0.347
Sex * Flr #	1	1	78	0.082	0.775
Treat * Sex	3	3	139	0.688	0.561
Treat * Flr #	3	3	141	1.006	0.392
Treat * Sex * Flr #	3	3	136	0.117	0.950

Table 3.10: ANOVA for seed production by CR and US populations of *S. vulgaris*.

Estimated total seed production is calculated as the product of fruits per plant and seeds per fruit, then modeled as a linear function of pollinator treatment, plant sex, deviation from mean flower number, experiment location, and all interactions. Replicate was included as a random effect. Control plants were excluded. Deviation from mean flower number (DMF) was calculated separately for each sex and location. Location refers to the experiment in Virginia (Chapter 2) and the experiment in the Czech Republic (this study). N=160 plants.

Source	Nparm	DF	Den DF	F Ratio	Prob > F
Treatment	1	1	137	63.5	<.0001
Sex	1	1	137	13.3	0.0004
DMF	1	1	121	105	<.0001
Location	1	1	4.32	48.8	0.0016
Treat * Sex	1	1	137	8.76	0.0036
Treat * DMF	1	1	144	42.0	<.0001
Treat * Location	1	1	137	22.0	<.0001
Sex * DMF	1	1	122	0.987	0.3226
Sex * Location	1	1	137	12.1	0.0007
DMF * Location	1	1	121	19.9	<.0001
Treat * Sex * DMF	1	1	143	0.948	0.3318
Treat * Sex * Location	1	1	137	7.08	0.0087
Treat * DMF * Location	1	1	144	23.3	<.0001
Sex * DMF * Location	1	1	122	0.192	0.6618
Treat * Sex * DMF * Location	1	1	143	1.66	0.1999

3.8 Figures

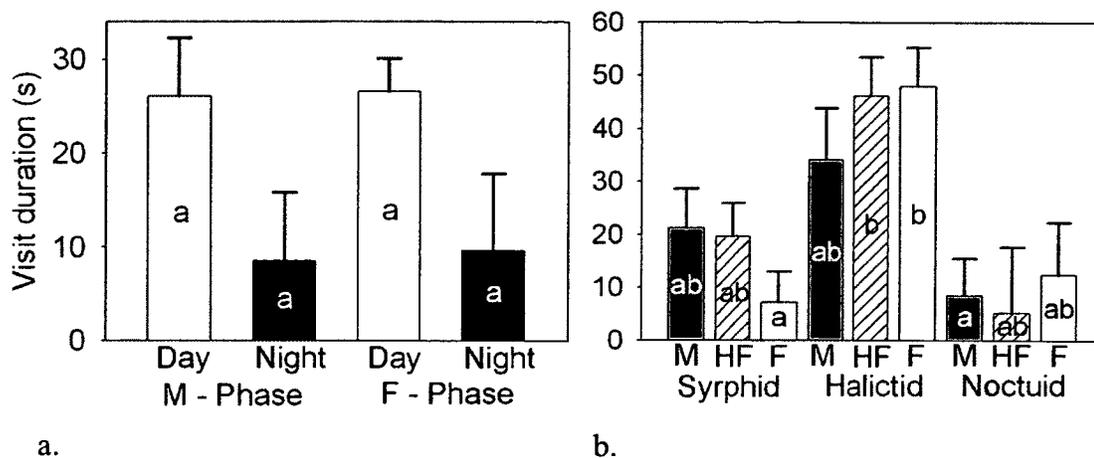


Figure 3.1: Handling times by the principle floral visitors of *S. vulgaris* in the CR. a) Handling time, the duration of individual flower visits, is organized by time of exposure: Day (flies and bees) and Night (moths), and by flower gender (female-phase hermaphrodite flowers are lumped together with female flowers). b) Handling times are given for each of the principal floral visitors: pollen-collecting, diurnal syrphid flies and halictid bees and nectar-collecting, nocturnal noctuid moths. Visit durations are organized by flower type: pollen-bearing male-phase hermaphrodite (M) flowers as well as female-phase hermaphrodite (HF) and female (F) flowers. Bar heights indicate least squares estimates of the means while error bars equal 1 standard error of the mean. Groups not sharing the same letter significantly differ (Tukey's HSD). Note different scales in each plot.

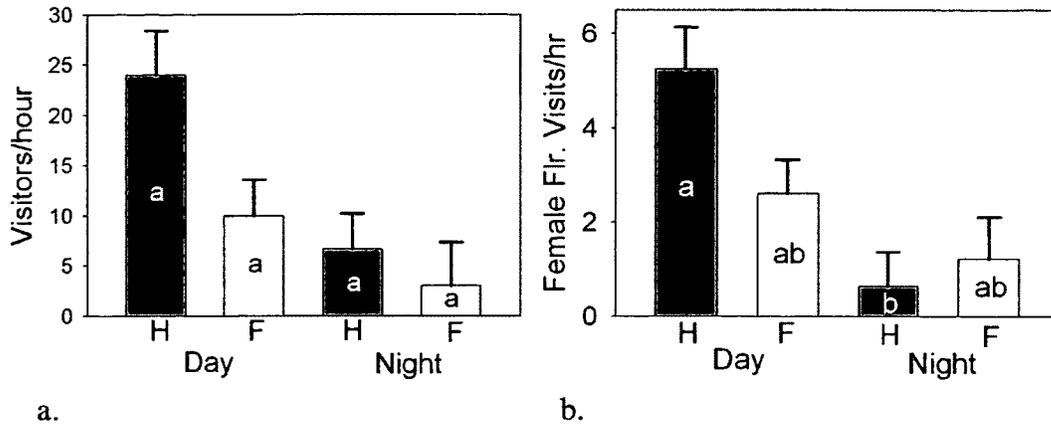


Figure 3.2: Visitation rates to CR *S. vulgaris*. a) Visits per one-hour observation period to hermaphrodite (H) and female (F) plants, exposed either during the day (Day) or at night (Night) to insect visitors. b) Visitation rates to stigmatic (female-phase) flowers on both hermaphrodites and females, exposed either during the day or at night. Visitation rates in a) are to an entire plant and b) are on a per-female flower basis (number of visits to female flowers divided by open female flowers). Bar heights indicate least squares estimates of the means while error bars denote one standard error of the mean. Groups not sharing the same letter significantly differ (Tukey's HSD). Note different scales in each plot.

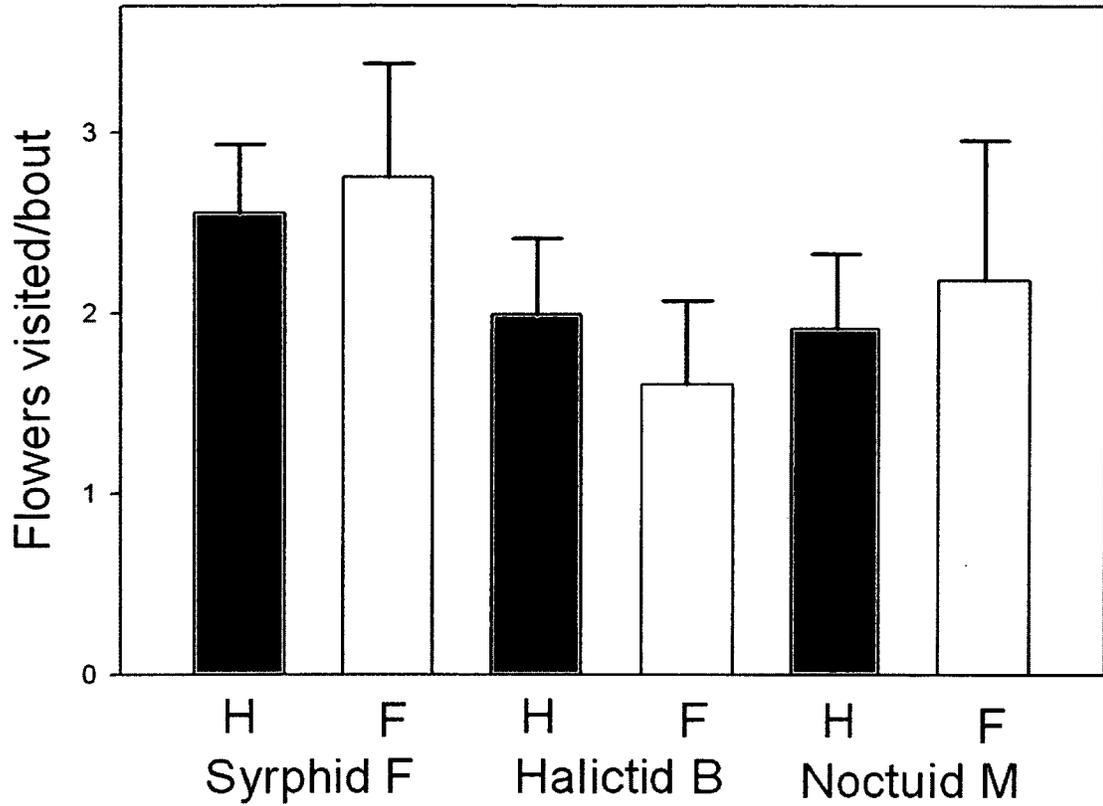


Figure 3.3: Bout lengths for primary floral visitors to CR *S. vulgaris*. The number of flowers visited in a single foraging bout is given for each of the main categories of floral visitor to hermaphrodite and female plants. Bar heights indicate least squares estimates of the means based on a model including plant sex, visitor type, and their interaction. Error bars denote one standard error of the mean. No pairwise comparisons were statistically significant.

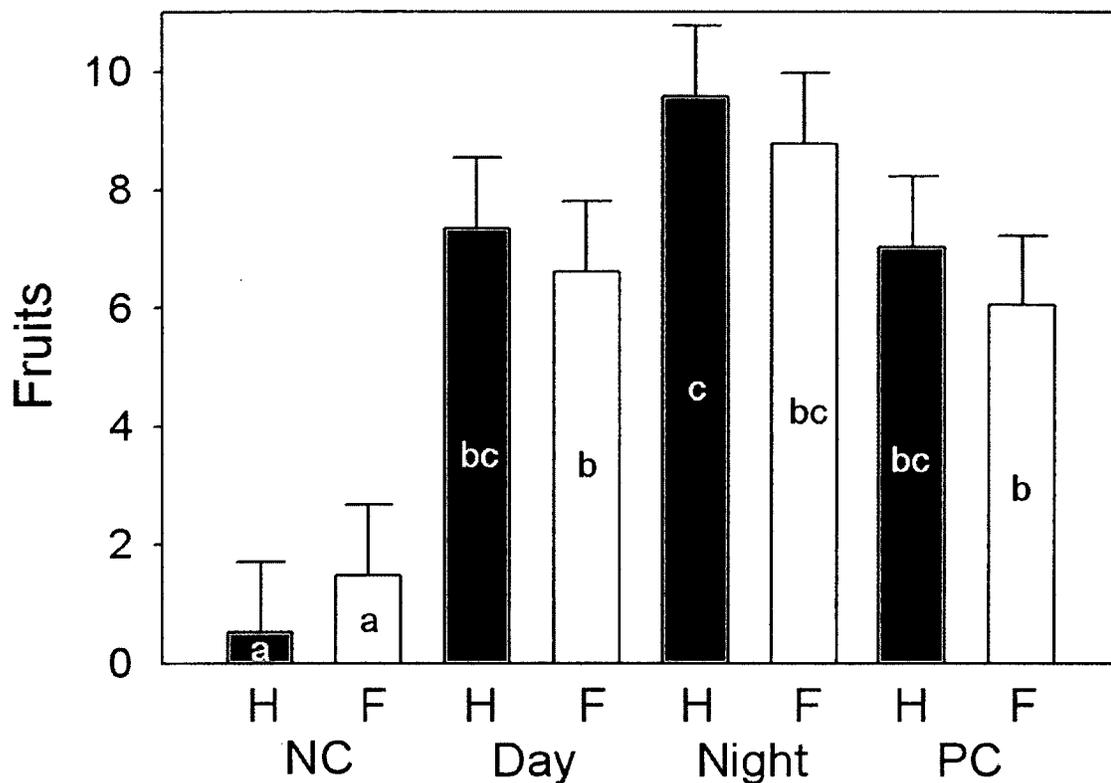


Figure 3.4: Total number of fruits produced by CR *S. vulgaris*. The total number of fruits produced by a plant during the 12 day exposure period are shown, grouped by plant gender and time of pollinator exposure: Negative Control, Day, Night, and Positive Control, respectively. H and F indicate hermaphrodite and female, respectively. Bar heights indicate least squares estimates of the means based on a model including plant sex, exposure treatment, and their interaction (see Table 3.5) while error bars denote one standard error of the mean. Groups not sharing the same letter significantly differ (Tukey's HSD).

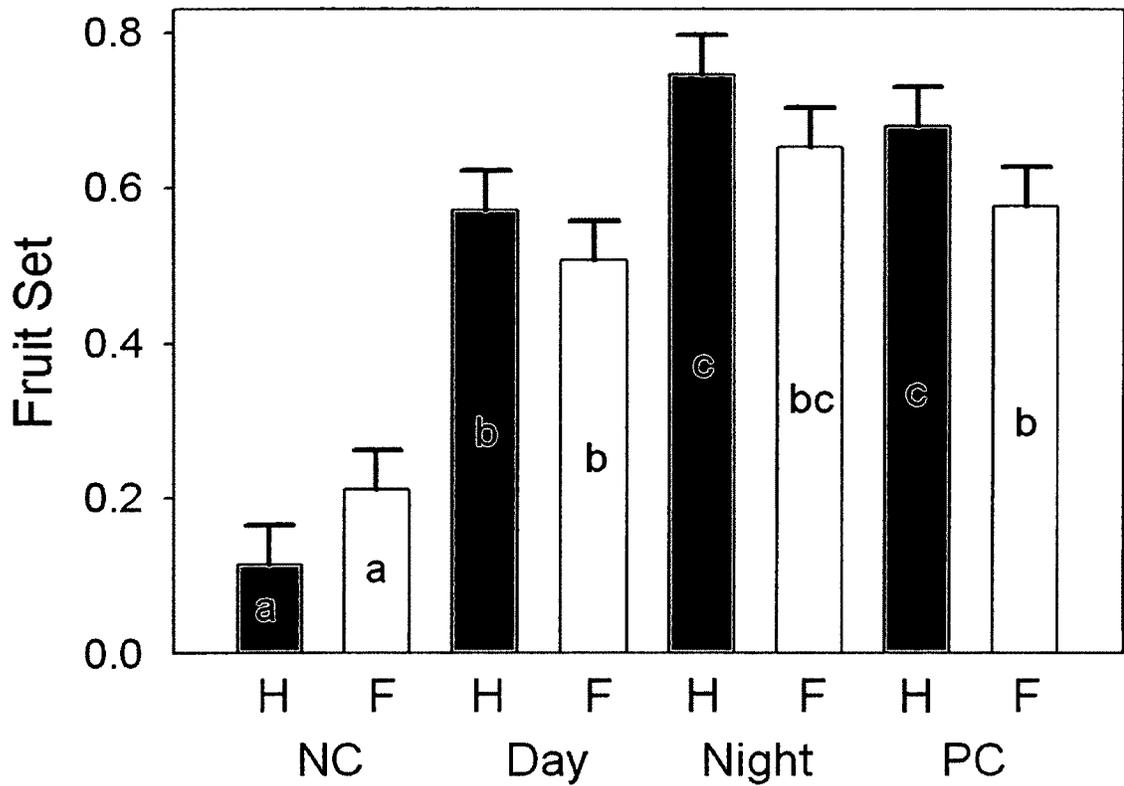


Figure 3.5: Fruit Set for CR *S. vulgaris*. The probability that a flower on a plant becomes a fruit (fruit set) is shown for experimental *S. vulgaris*, grouped by sex (H=hermaphrodite, F= Female) and time of pollinator exposure (NC = negative control, Day, Night, and PC = positive control). Bar heights indicate least squares estimates of the means based on a linear model including plant sex, exposure treatment, and their interaction (see Table 3.6) while error bars denote one standard error of the mean. Groups not sharing a letter differ significantly.

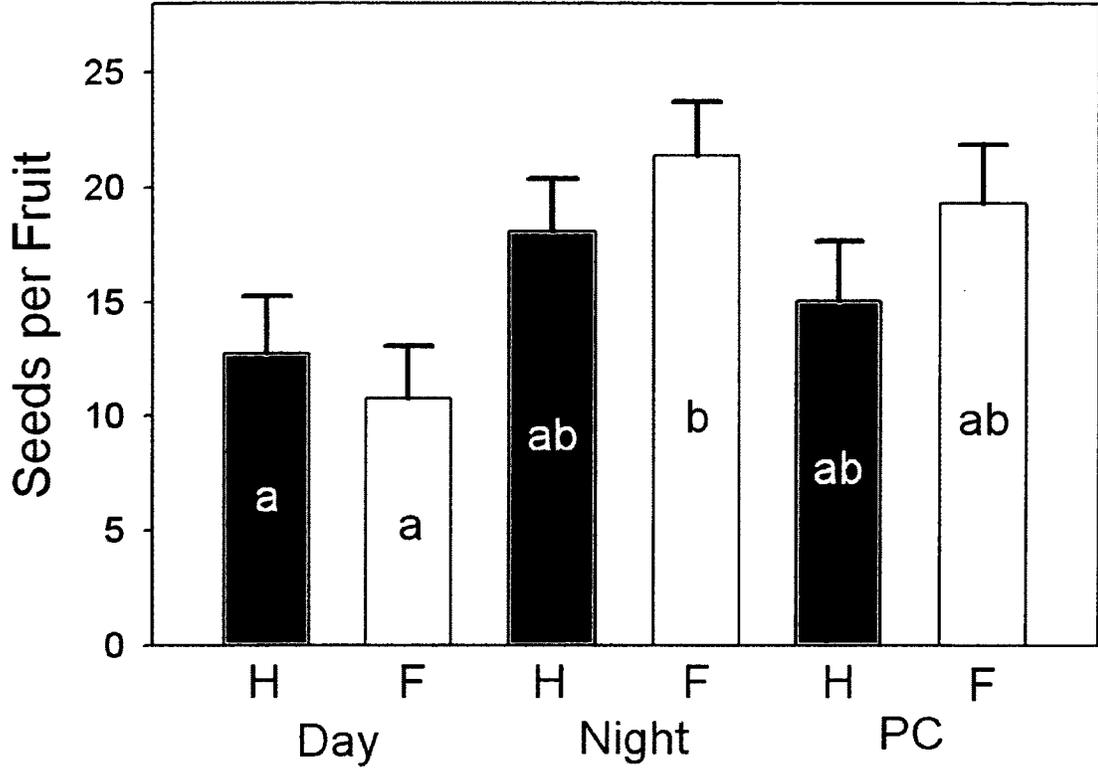


Figure 3.6: Seeds per fruit for CR *S. vulgaris*. The average number of seeds per fruit is given for females (F) and hermaphrodites (H) exposed to insect visitors during the day (Day), at night (Night), or both (PC). Bar heights indicate least squares estimates of the means based on a model including plant sex, exposure treatment, their interaction, and total flower number (see Table 3.7) while error bars denote one standard error of the mean. Groups not sharing the same letter significantly differ (Tukey's HSD).

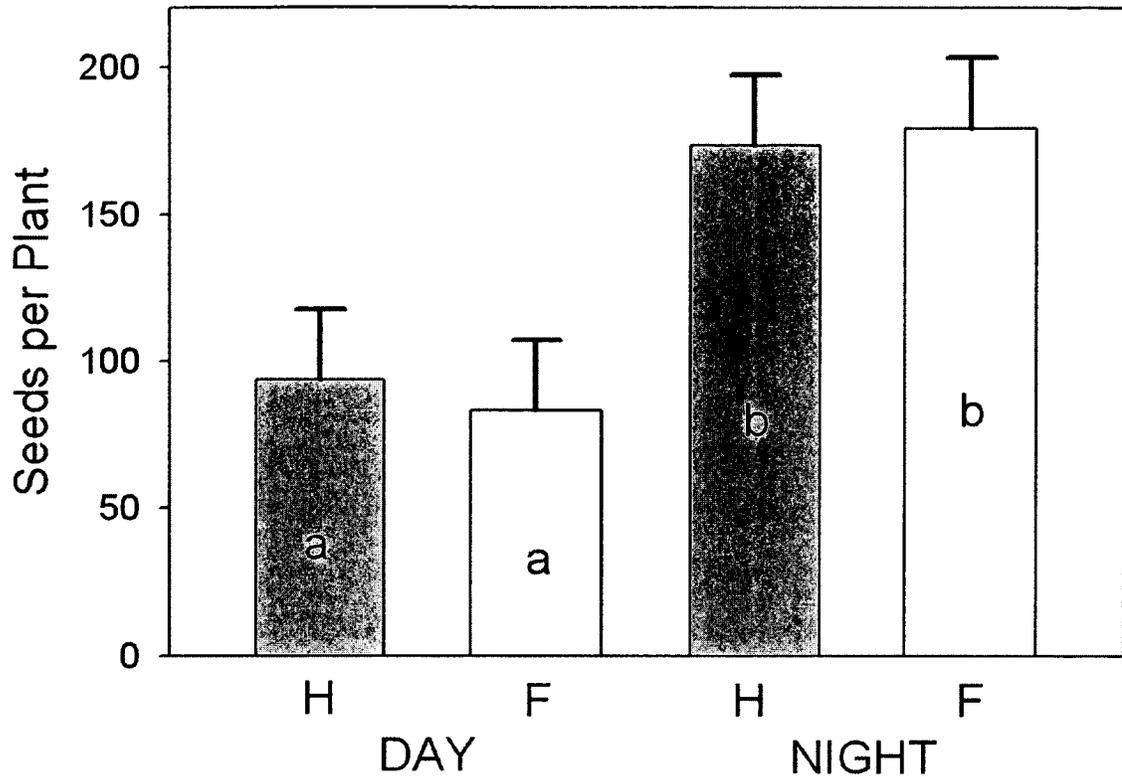


Figure 3.7: Approximate total seeds per plant for CR *S. vulgaris*. The average number of seeds per plant was calculated as the product of the average number of seeds per fruit times the number of fruits produced by that plant for female (F) and hermaphrodite (H) plants exposed to pollinators during the day (Day) or at night (Night). Bar heights indicate least squares estimates of the means based on a model including plant sex, exposure-treatment, their interaction, and total flower number (see Table 3.8) and error bars denote one standard error of the mean. Groups not sharing the same letter significantly differ (Tukey's HSD).

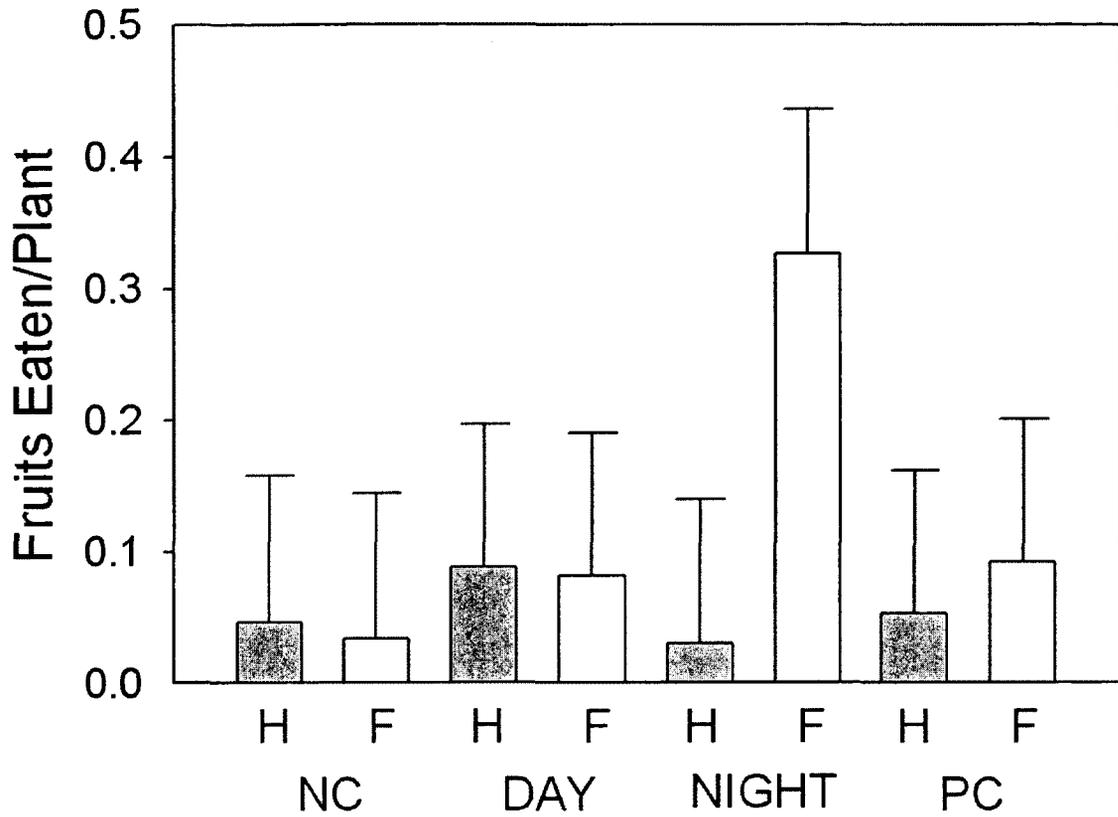


Figure 3.8: Seed predation experienced by CR *S. vulgaris*. The number of fruits eaten is shown for female and hermaphrodite plants under negative control (NC), diurnal (Day), nocturnal (Night), and positive control (PC) pollinator exposure treatments. Bar heights correspond to least squares estimates of the means for a model including plant sex, treatment, sex * treatment, display size (total flowers), and replicate (as a random effect). Error bars denote one standard error of the mean. No pairwise comparisons were statistically significant.

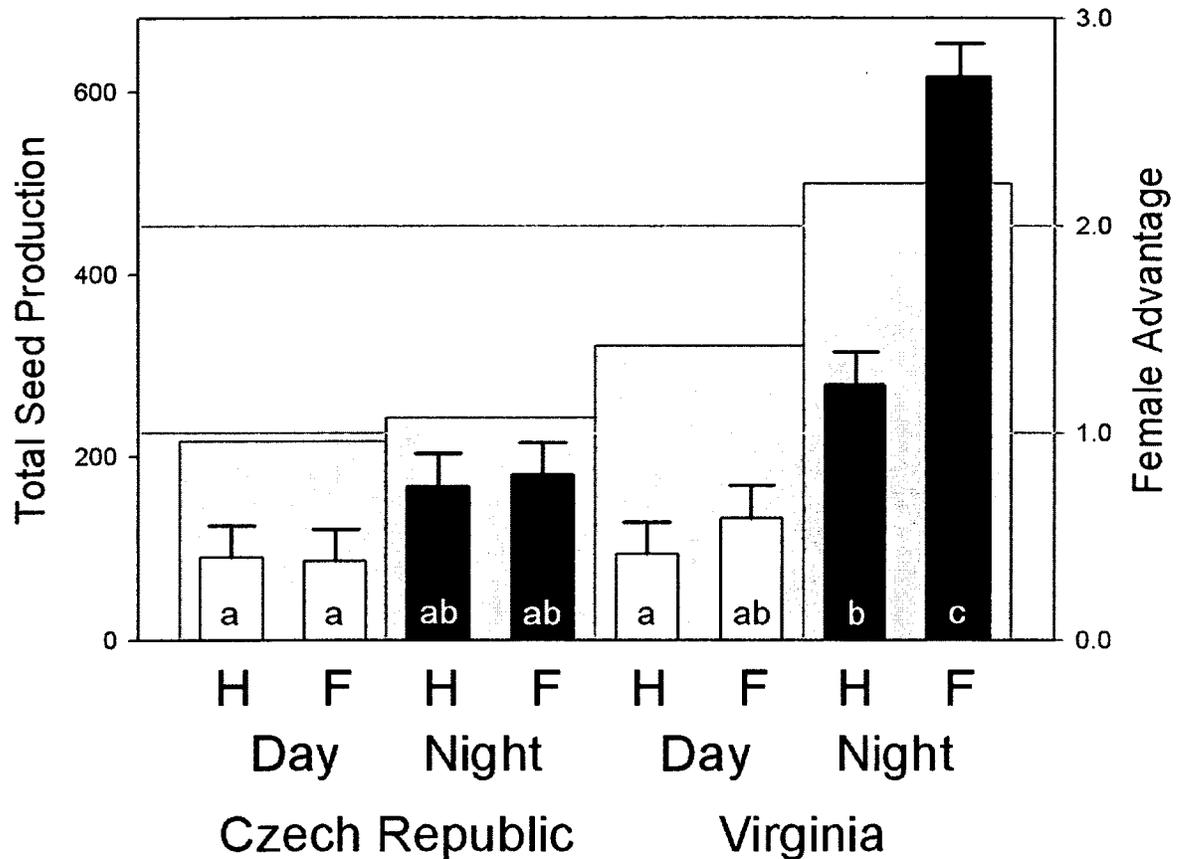


Figure 3.9: Comparison of total seed production for CR and US *S. vulgaris*. LSMeans estimates of total seed production are given for female (F) and hermaphrodite (H) plants exposed to diurnal (Day) or nocturnal (Night) pollinators, in both the Czech Republic and Virginia. Czech Republic data are from the present study while Virginia data are from Stone and Olson Chapter 2. Foreground bar heights represent LSMeans of estimated total seed production. Error bars denote one standard error of the mean. Average female seed production, relative to hermaphrodites, is shown in grey bars in the background and the corresponding Y-axis (right hand side). Grey lines at female advantage of one and two are included, corresponding to the levels of female relative fitness required for evolution of cytoplasmic and nuclear gynodioecy, respectively. Groups not sharing the same letter significantly differ (Tukey's HSD).

Chapter IV

Modeling the effect of pollinators on plant fitness and sex ratio evolution in gynodioecious populations¹

4.1 Abstract

Gynodioecious sex ratios vary dramatically, from 0-100 % female, and current models do not adequately explain that variation. Pollinator behavior is a potentially pervasive contributor to sex ratio evolution, yet pollinators' influence on sex-specific fitness variation is largely unmodeled. To address this gap, we present a model connecting the seed fitness of female and hermaphrodite individuals with several aspects of pollinator behavior. We incorporate this into a single-locus nuclear model of gynodioecious sex ratios to analyze the effect of changes in pollinator behavior on equilibrium female frequencies. We find that pollination ecology can restrict gynodioecy and reduce female frequency for biologically realistic parameter values. The extent of pollinator influence depends on the pollination processes included in the model. We find sensitive regions of parameter space where small changes in pollinator behavior have a large, threshold-like effect on female frequency as well as conditions where the effect of pollinator behavior is minimal. Our analyses reveal that pollinator behavior can play a decisive role in gynodioecious sex ratio evolution and that differences in pollination can contribute to variation in female frequencies.

¹ Stone, J.D., Y. Iwasa, and N. Takebayashi. Modeling the effect of pollinators on plant fitness and sex ratio evolution in gynodioecious populations. Prepared for submission to *The American Naturalist*.

4.2 Introduction

Despite more than a century of research on gynodioecy (Darwin, 1877), gaps remain in our understanding of the ecological conditions required for its maintainance (Ashman, 2006; Spigler and Ashman, 2012). Gynodioecy, where females and hermaphrodites coexist, is the most common dimorphic breeding system (Yampolsky and Yampolsky, 1922; Richards, 1997). A major unresolved issue is that we cannot fully account for the remarkable diversity in gynodioecious sex ratios observed in natural populations (Couvét et al., 1990; Delph, 1990; Laporte et al., 2001; Shykoff et al., 2003). In gynodioecious *Silene vulgaris*, for example, females may range from 0-90% of a population over relatively small geographic scales (McCauley et al., 2000; Štorchová and Olson, 2004). One possible explanation, which is frequently suggested without theoretical investigation, is that the inter-population differences in pollination ecology are a source of variation in gynodioecious plant fitness and sex ratios. Pollinators vary in their distribution, abundance, behavior, and effectiveness (Pettersson, 1991; Morris et al., 1995; Alonso et al., 2007; Cosacov et al., 2008; Case and Ashman, 2009). This may result in pollen limitation (Jennersten, 1988; Wilson et al., 1994; Ashman et al., 2004) and variation in the fitness of both sexes (Chapters 2 and 3; Young, 2002; Barthelmess et al., 2006), altering the selective pressures on gynodioecious females (Chapter 2). Thus, variation in pollination processes offers unexplored explanatory potential for gynodioecy and gynodioecious sex ratios (Ashman, 2006).

In order to connect variation in pollinator behavior with variation in females' relative fitness and sex ratio, we developed models incorporating the phenomena of pollen limitation, visit limitation, hermaphrodite selfing rate, and assortative mating. These processes are all pollinator-mediated (Brunet and Sweet, 2006; Alonso, 2005; Giménez-Benavides et al., 2007; Cuevas et al., 2008) and therefore functionally connected through pollination processes. Nevertheless, existing gynodioecious models handle these processes independently and only pollen limitation has been explicitly

modeled in terms of pollinator behavior. Modeling the pollination processes underlying these phenomena allows us to examine how they interact to influence gynodioecious plants' fitness. Given pollinators' key role in several reproductive processes, we work towards a more integrated and comprehensive examination of their influence on plant fitness, allowing us to quantify their contribution to sex ratio variation and the maintenance of gynodioecy.

Our current understanding of sex ratio evolution in gynodioecious populations is based on more than a century of empirical and theoretical work; here we briefly outline the development of the theoretical side, with an emphasis on models including pollinator influence. Lewis (1941) established the cornerstones of our theoretical understanding of gynodioecious sex ratio evolution with nuclear and cytoplasmic models. Lewis showed that, assuming no viability selection, females must produce more seeds than hermaphrodites to be maintained, now an axiom of most models of gynodioecy. Lewis parameterized females' relative fitness with a simple expression,

$$f(1-p)^x \quad (1)$$

where f is the maximum female fertility, adjusted by sex ratio, where p is female frequency. The parameter x , which determines the strength of the relationship between hermaphrodite frequency and female fitness, may account for variation in pollinator behavior and abundance, but does not offer a biological mechanism for pollen limitation. Nevertheless, Lewis' models demonstrated that female frequency is limited by frequency dependent selection. Lloyd (1974) offered an explicitly pollinator-mediated form of pollen limitation by incorporating the number of pollinator visits a female plant receives. Females' fertility is multiplied by $(1 - p^V)$, the probability of fertilization, where p refers to female frequency and V refers to the number of pollinator visits a female flower. Thus, Lloyd (1974) assumed that pollinators only deposit pollen on females immediately following a visit to a hermaphrodite. Lloyd and Lewis both assumed that hermaphrodites are never pollen limited, and self-fertilization and inbreeding depression may only be

incorporated by altering the relative fecundity parameter (Lewis, 1941; Lloyd, 1974). Delannay et al. (1981) modified Lloyd's parameterization to allow pollen limitation to both sexes, but retained Lloyd's assumption that pollinator visits to female plants only result in pollination if the previous visit was to a hermaphrodite. This assumption improved tractability but becomes increasingly unrealistic as female frequency and pollen carryover increase female-to-female transits that result in pollination. In both models, pollen limitation strongly influenced female frequency and the conditions permitting gynodioecy. Our basic understanding of visitation rates notwithstanding, we do not yet have a model accounting for the effects of *most* aspects of pollinator behavior on gynodioecious populations, including foraging bout lengths, pollen carryover rates, and pollinator preference for a particular sex, all of which have the potential to influence the fitness of females and hermaphrodites.

Several additional models have made significant progress in defining the conditions favoring gynodioecy, although they do not incorporate pollinator behavior directly. For example, Charlesworth and Charlesworth (1978) defined the relative levels of selfing, inbreeding depression, and resource compensation permitting the existence of females as well as providing expressions of their equilibrium frequencies. Their model allows us to better disentangle these important components of plant fitness that were subsumed in the female fertility parameter, f , in Lewis' model, and fertility and survival parameters in Lloyd's. Their model does not, however, incorporate pollen limitation or visit limitation; thereby assuming both sexes always receive sufficient pollination for full seed set.

Very few models related to breeding system evolution have incorporated multiple aspects of pollinator-mediated plant fitness. Maurice and Fleming (1995), provide one such model, allowing correlation between "realized selfing rate" and pollen limitation, both of which are influenced by pollinator behavior. Of the few models incorporating aspects of pollinator behavior, including pollen limitation and selfing rates, most do not

explicitly model pollination processes. Furthermore, they treat each process independently (Schultz, 1994; Hodgins et al., 2009; Johnston et al., 2009), preventing analysis of pollinators' simultaneous contributions through multiple processes. These typically portray male and female fitness as gain curves between reproductive investment and payoff, trading increased flexibility for reduced insight regarding specific biological mechanisms (Sato, 2002; Ehlers and Bataillon, 2007; and Johnston et al., 2009).

This review of models of pollinator-mediated processes in gynodioecious populations highlights the need to integrate the multiple phenomena influencing female and hermaphroditic fitness, root them in pollination processes, and allow them to vary as sex ratios evolve and pollinator conditions change. We present a model that incorporates pollen limitation, visit limitation, pollen carryover, and selfing rate, connecting them through pollination processes. This model is used to define the relationships among selfing rate, pollen limitation, and plant fitness in terms of pollinator behavior, plant size, resource compensation, inbreeding depression, and population sex ratio. We then incorporate these definitions of gynodioecious plant fitness into a single locus, two-allele model of nuclear gynodioecy (Charlesworth and Charlesworth, 1978). We use this model to describe numerically how changes in pollinators' abundance, preference, bout length, and plant size alter equilibrium female frequency.

4.3 A model of pollen limitation and selfing rate in gynodioecious populations

4.3.1 Bout lengths, visitation rates, and pollinator preferences

Reduction of fruit and seed set through inadequate pollen receipt is the result of two related processes: pollen limitation and visit limitation. We use the term pollen limitation to refer to inadequate pollen to achieve full seed set on visited flowers. Visit limitation, also referred to as pollinator limitation or pollination failure, occurs when a fraction of an inflorescence goes unvisited, and therefore receives no pollen. Both pollen and visit limitation may be experienced to varying degrees by both sexes, and are

considered in our model. Hermaphrodites are typically the more attractive sex to pollinators, offering pollen rewards, increased nectar, showier displays, and increased chemoattractants (Assouad et al., 1978; Stevens, 1988; Willson and Ågren, 1989; Jolls et al., 1994). We include a parameter, α , describing pollinator preference for hermaphrodites and apply it to visitation rates, bout lengths, or both, depending on model variant. When α is greater than one, hermaphrodites are preferred, when it is less than one, females are preferred. When α equals one, pollinators treat both sexes equally in terms of visitation rates and/or bout lengths. Furthermore, we assume hermaphrodites are subject to visit limitation (no autogamous selfing) and are self-compatible through geitonogamous selfing. We do not address pollen discounting (Holsinger, 1993; Iwasa et al., 1995), assuming that pollen available for outcrossing depends only on carryover rate and pollinator movement patterns. We also assume that a visit to a hermaphrodite is sufficient to replenish pollen loads to a level capable of providing full seed set and that pollen loads diminish geometrically during visitation bouts according to the pollen carryover parameter. We assume that hermaphrodites are simultaneous hermaphrodites with perfect flowers, later discussing the implications of strategies including monoecy, protandry, and delayed selfing.

We first develop expressions describing components of hermaphrodite and female plant pollination processes, using the parameters in Table 4.1, and combine them into a phenotypic model of gynodioecious plant fitness. We discuss each process as it is presented and then, after the full model is assembled, examine how these processes interact. We define the probability that hermaphrodite flowers remain unvisited, U_H , given the number of flowers probed per visit, B , the display size (number of flowers), F , and number of pollinator visits, V , received:

$$U_H = \left(1 - \frac{B}{F}\right)^V \quad (2)$$

where B is $\leq F$, F is ≥ 1 , and V is ≥ 0 . Thus, flowers are more likely to be visited when bout length is a larger fraction of display size and when the numbers of visits are high.

In order to account for the widespread observation that pollinators prefer visiting hermaphrodites, we include the parameter, α , describing pollinators' tendency to visit females less frequently. Accordingly, females' probability of going unvisited, U_F , is:

$$U_F = \left(1 - \frac{B}{F}\right)^{\frac{1}{\alpha}} \quad (3)$$

Pollinator preference for hermaphrodites ($\alpha > 1$) decreases the probability that females will be visited. Intermediate visitation rates and bout lengths maximize disparity in pollination failure between females and hermaphrodites, $U_H - U_F$ (Figure 4.1). The disparity in female and hermaphrodite pollinator failure reaches the maximum value of $U_H \alpha (1 - \alpha)^{\frac{\alpha}{1-\alpha}}$ when bout lengths are related to visitation rates according to the expression, $B = (1 - \alpha)^{\frac{\alpha}{1-\alpha}}$, when $\alpha \neq 1$. Roughly speaking, the greatest disparity between females and hermaphrodites in pollination failure occurs when bout lengths are inversely proportional to the number of visits a plant receives, when one sex achieves full visitation and the other remains visit limited, hence the shape of the ridge in Figure 4.1. Any additional visitation or longer bout lengths reduce disparity, as the preferred sex is visit-saturated, while the less preferred sex becomes decreasingly visit-limited.

4.3.2 Pollen carryover and selfing rate

We assume the amount of available pollen follows a simple geometric decay as a pollinator visits subsequent female flowers; pollen load is fully replenished once another hermaphrodite plant is visited (Bateman, 1947). This is a long-standing parameterization of pollen carryover that remains popular for its simplicity, though more complicated models have been proposed (Morris et al., 1994). In the basic carryover model, the m^{th} flower in a visitation bout receives c^{m-1} pollen, where c is the amount of outcross pollen retained after visiting a flower, as a fraction of the amount required for full seed set. If c is 1, all visited flowers achieve full seed set. As c decreases, flowers probed later in a visitation bout receive less outcross pollen. The average outcross pollen imported by a flower in a single pollinator visit, after visiting a hermaphrodite, can now be written as:

$$\frac{1}{B} \sum_{m=1}^B c^{m-1} \quad (4)$$

We use the discrete geometric distribution to facilitate simultaneously calculating pollen received as well as pollen composition, and later generalize it to an exponential form.

Geitonogamous self-fertilization is incorporated by assuming that, after the first flower on a hermaphrodite is visited, self-pollen constitutes an increasing fraction of the available pollen load with each subsequent flower visited on the same plant. Provided the previous plant visited was a hermaphrodite, the average self-pollen transported to the stigmas of visited flowers on a hermaphrodite, as a fraction of their possible seed set, is:

$$\frac{1}{B} \sum_{m=1}^B 1 - c^{m-1} \quad (5)$$

Outcross- and self- pollination rates must also be adjusted to account for the possibility that a pollinator has visited one or more females since it replenished its pollen load. Higher female frequencies result in a lower probability that the pollinator arrives from a hermaphrodite plant, reduces outcross pollen imported to hermaphrodites, and increases hermaphrodite selfing rates. A weighted sex ratio, $v_{WSR} = \frac{\alpha v_H}{\alpha v_H + v_F}$, is used to describe the probability that the previous plant visited was a hermaphrodite, where v_H and v_F are hermaphrodite and female frequencies, respectively. The probability that j females have been visited since the last hermaphrodite is $v_{WSR} (1 - v_{WSR})^j$ from a geometric distribution. Therefore, the conditional probability that a particular ovule on a visited flower receives pollen imported from another hermaphrodite is:

$$O_H = \frac{v_{WSR}}{B} \sum_{j=0}^{\infty} (1 - v_{WSR})^j \sum_{m=j*B}^{(j+1)B-1} c^m \quad (6a)$$

this can be rewritten as:

$$O_H = \frac{\alpha v_H (1 - c^B)}{B(1 - c)[\alpha v_H + v_F (1 - c^B)]} \quad (6b)$$

Thus, longer bout lengths and female-biased sex ratios both decrease the probability of outcrossing. Accordingly, larger display sizes create the potential for

longer visitation bouts and increased geitonogamous selfing (de Jong et al., 1992, de Jong et al., 1993). On the other hand, greater pollen carryover and pollinator preference for hermaphrodites increase outcrossing. We assume that ovules of *visited* flowers not receiving outcross pollen are geitonogamously self-fertilized. This corresponds to the situation where outcross pollen has precedence. Therefore, the proportion of self-fertilized ovules in hermaphrodites is:

$$S_H = 1 - (U_H + O_H) \quad (7)$$

In the case where females are subject to visit limitation but not pollen limitation, the proportion of female ovules fertilized by outcross pollen is:

$$O_F = 1 - U_F \quad (8)$$

Incorporating pollen carryover rate as the basis for female pollen limitation, and assuming that the amount of outcross pollen imported by visited flowers on females equals that on hermaphrodites (6a), the probability of female ovules being fertilized by outcross pollen equals that of hermaphrodites, so $O_F = O_H$. Female plants may receive shorter foraging bouts than hermaphrodites, especially when they offer fewer rewards. To capture this, we modify bout length on females according to pollinator preference by dividing B by α . Expression 8b is necessary when pollinator preference, α , alters both visitation rates and the number of flowers visited according to plant sex. We change the indexing of the second summation to account for the change in foraging bout lengths on females:

$$O_F = \frac{\alpha v_{WSR}}{B} \sum_{j=0}^{\infty} (1 - v_{WSR})^j \sum_{m=j\frac{B}{\alpha}}^{(j+1)\frac{B}{\alpha}-1} c^m \quad (8b)$$

For the discrete form of these expressions, adjusted bout lengths are only biologically realistic when bout length is divisible by α without a remainder. The exponential form of these expressions, however, is appropriate for all positive values of α . For simplicity, we present only the discrete case for most of the expressions given.

Next, we consider the case when pollinator preference, α , affects both visitation rates and bout lengths on females. Accordingly, the probability of outcrossing by

hermaphrodites must be adjusted to account for pollen dissipation on females and the indexing for the second summation is modified to account for female bout lengths:

$$O_H = \frac{v_{WSR}}{B} \sum_{j=0}^{\infty} (1 - v_{WSR})^j \sum_{m=j\frac{B}{\alpha}}^{j\frac{B}{\alpha}+B-1} c^m \quad (6c)$$

Selfing rate, the ratio of selfed to total fertilized ovules, can be given in terms of pollinator preference for hermaphrodites, carryover, and bout length, with B limited by display size:

$$\frac{1 - (U_H + O_H)}{1 - U_H} \quad (9)$$

$$= \frac{Bv_F(1-c)(1-c^B) + \alpha v_H[B(1-c) - (1-c^B)]}{B(1-c)[\alpha v_H + v_F(1-c^B)]} \quad (10)$$

From the summations, it can be determined that increases in bout length and the frequency of females both lead to an asymptotic increase in the hermaphrodite selfing rate. Increased pollinator preference for hermaphrodites, however, reduces hermaphrodite selfing rate.

We now have a set of terms describing several aspects of the pollination process that may be modified to handle additional pollinator scenarios, as described in Table 4.2. The remainder of our analyses is built around the expressions in equations (2-8). We begin with a model, M2 (Table 4.2) that assumes all the ovules of *visited* flowers are fertilized (no pollen limitation) corresponding to pollinators whose pollen load diminishes negligibly while foraging. M2 also allows for visit limitation, meaning that unvisited flowers receive no pollen. M3 adds pollen limitation to females, such that flowers on female plants visited later in a foraging bout receive some pollen, but not enough to fertilize all ovules. Under both M2 and M3, hermaphrodites experience geitonogamous selfing, and all ovules on visited hermaphroditic flowers are fertilized, although they are increasingly self-pollinated on flowers later in a foraging bout. The models also differ in that the parameter for pollinator preference for hermaphrodites, α ,

influences only visitation rates in M2, whereas it influences both visitation rates and bout lengths in M3. Finally, we calculate equilibrium female frequencies for these models and compare them to a classical model, M1, by Charlesworth and Charlesworth (1978; Table 4.2), noting that the framework could be expanded to other scenarios.

4.3.3 Incorporating pollinator behavior into a model of sex ratio evolution

We define fitness for females and hermaphrodites in terms of the probabilities of selfing, outcrossing, and unvisited ovules as described in expressions (2-8). Following Charlesworth and Charlesworth (1978), seed production by hermaphrodites and females, respectively, are:

$$\omega_H = (1 - U_H)[S_H(1 - \delta) + O_H] \quad (11)$$

$$\omega_F = (1 - U_F)O_F(1 + k) \quad (12)$$

where δ represents inbreeding depression, a fitness cost associated with selfed offspring, and k represents resource compensation, a seed fitness advantage common in gynodioecious females.

We implement these expressions into a one-locus, two-allele model of gynodioecious sex ratios. We assume male fertility of hermaphrodites is dominant, with genotypes RR and Rr hermaphroditic, and genotype rr genotype female. Charlesworth and Charlesworth (1978) note that this is a relatively rare type of gynodioecy and that females will spread slowly when recessive. Nevertheless, this simple and well-studied single locus nuclear model is a logical starting point for describing the influence of pollinator behavior on female frequencies. After incorporating the pollinator behaviors described above, we obtain the following recursive equations of genotypic frequencies:

$$v'_{RR} = \frac{1}{\bar{w}}(1 - U_H)[S_H(1 - \delta)(v_{RR} + \frac{1}{4}v_{Rr}) + O_H(v_{RR} + \frac{1}{2}v_{Rr})P_R] \quad (13)$$

$$v'_{Rr} = \frac{1}{\bar{w}} \{ (1 - U_H) [\frac{1}{2} S_H (1 - \delta) v_{Rr} + O_H (v_{RR} p_r + \frac{1}{2} v_{Rr} p_R)] + (1 - U_F) O_F (1 + k) v_{rr} p_R \} \quad (14)$$

$$v'_{rr} = \frac{1}{\bar{w}} \{ (1 - U_H) [\frac{1}{4} S_H (1 - \delta) v_{Rr} + \frac{1}{2} O_H v_{Rr} p_r] + (1 - U_F) O_F (1 + k) v_{rr} p_r \} \quad (15)$$

where v_{RR} , v_{Rr} , and v_{rr} are the genotypic frequencies, the prime indicates the frequency at the next generation, p_R and p_r denote the allele frequencies in the pollen pool, and \bar{w} corresponds to the number of zygotes produced by the entire population, as defined below:

$$p_R = \frac{(v_{RR} + \frac{1}{2} v_{Rr})}{v_{RR} + v_{Rr}} \quad (16)$$

$$p_r = \frac{\frac{1}{2} v_{Rr}}{v_{RR} + v_{Rr}} \quad (17)$$

$$\bar{w} = (1 - U_H) [S_H (1 - \delta) + O_H] (v_{RR} + v_{Rr}) + (1 - U_F) O_F (1 + k) v_{rr} \quad (18)$$

These recursion equations allow us to numerically analyze how changes in pollinator preference, bout length, visit number, and plant size affect equilibrium sex ratios using a program written in C, for any model variant (Table 4.2).

4.4 Results:

Model M2 results in reduced female frequency as hermaphrodites are increasingly preferred, regardless of all other parameters (Figure 4.2). However, even with high pollinator preference for hermaphrodites, α , resource compensation could maintain females (Figure 4.2). For instance, high levels of resource compensation or inbreeding depression reduce the maximum preference for hermaphrodites permitting females and increase the equilibrium female frequency. A population's sensitivity to pollinator preference, as inferred from the slopes of curves in Figure 4.2, varies from high to negligible, depending on the level of female compensation and inbreeding depression.

This suggests that slight changes in pollinator fauna or behavior could dramatically influence the equilibrium female frequency in one species, but another may not respond, depending on the plants' levels of k , δ , and pollinators' bias.

Pollination failure (visit limitation) in females does not depend on sex ratio or resource compensation, but increases with pollinator preference, α , once hermaphrodites are preferred. Hermaphrodite selfing rate increases with both female frequency and pollinator preference for females. Different threshold values of α for pollination failure and selfing rate explain the shape of the equilibrium female frequency curves, where two regions with rapid change in female frequency are observed. Mechanistically, the first steep slope region corresponds to a rapid decrease in the selfing rate and the other region corresponds to the rapid increase in pollination failure (Figure 4.3). The relative importance of each phenomenon on sex ratio depends on the levels of inbreeding depression and female compensation. It is apparent in Figure 4.3 that selfing has a larger effect for these parameter values, when $k = 0.5$, whereas pollination failure has a larger effect when $k = 1.0$.

Lastly, we consider a model with more pervasive pollinator influence, M3 (Figures 4.4 and 4.5). This model includes female pollen limitation, pollinator-mediated selfing, biased visitation rates, and biased bout lengths. Pollinator preference now leads to more severe pollen limitation and larger changes in selfing rate than in M2. Accordingly, higher levels of resource compensation are required for female maintenance. Under this model, pollinator preference for females can reduce their frequency (Figure 4.4), to an extent depending on visit limitation and the level of pollen carryover (Figure 4.5). Comparing the results of models with (M3) and without (M2) female pollen limitation (Figure 4.3) illustrates that the inclusion of pollen limitation results in a fitness cost associated with very strong preference for females. While slight preferences for females increase their frequency, strong preferences lead to long foraging bouts on females, causing pollen limitation beyond what resource compensation can

make up for. Increasing inbreeding depression and carryover displaces female frequency curves to the left, as greater preference for females is needed before female pollen limitation outweighs hermaphrodites' inbreeding depression (data not shown, though similar to the displacement in threshold values of alpha permitting females when comparing Figures 4.2a, b, and c). If females are strongly preferred, hermaphrodites retain a fitness advantage through geitonogamous selfing, despite being severely visit limited. Female biased visitation is rare and its detrimental effects offer a new perspective on why females are consistently the less-rewarding sex.

4.4.1 Comparison to classic model without pollinator influence

Charlesworth and Charlesworth (1978) noted that a rare female would spread only if:

$$1 + k > 2(1 - S_H \delta) \quad (19)$$

Thus a male sterility allele increases in frequency only when females produce more than twice hermaphrodites' seed ($k > 1$), unless selfing and inbreeding depression reduce hermaphrodite fitness. Accordingly, Charlesworth and Charlesworth (1978) conclude that hermaphrodite selfing and inbreeding depression facilitate the evolution of gynodioecy. Similarly, we include the contributions of pollination failure and a pollinator behavioral parameterization of selfing to get:

$$(1 + k)(1 - U_F)O_F > 2(1 - U_H)[O_H + S_H(1 - \delta)] \quad (20a)$$

rearranged in terms of k :

$$1 + k > 2(1 - \delta S_H) \frac{1 - U_H}{O_F(1 - U_F)} \quad (20b)$$

When we consider the biologically realistic case where hermaphrodites are more likely to be visited than females, $(1 - U_H)/(1 - U_F) > 1$, the right hand side of inequality (20b) becomes larger than that of (19) (note that $0 \leq O_F \leq 1$). Therefore, higher female compensation is required for the invasion of females in models with pollinator behavior.

Female-biased visitation can, however, make satisfying (20b) easier than in the model without pollinator behavior (19), but female-biased visitation is rarely observed (Ashman 2000).

We numerically compare equilibrium female frequency of several models presented in this paper with the model of Charlesworth and Charlesworth (1978) in Figure 4.6. In order to compare the Charlesworth and Charlesworth model (M1), where selfing rate is a static parameter, to models including pollinator preference (M2 and M3) we used the selfing rate as calculated in (10) for each level of α . Essentially, this makes the selfing rate used for comparison with Charlesworth and Charlesworth (1978) a function of sex ratio and pollinator preference, but does not include the influences of pollen or visit limitation. For realistic values of pollinator preference where hermaphrodites are preferred, female frequency is always reduced, regardless of model, as noted in (20b) and consistent with the comparison of the invasion conditions shown above. Female frequency is increased if pollinators prefer females, but only in models that include hermaphrodite-biased visit limitation, M2. A model including female pollen limitation as well as visit limitation, M3, reduces female frequency compared with M1, regardless of inbreeding depression and pollen carryover rate, unless pollinators have longer foraging bouts on females than hermaphrodites or the mechanism of selfing is changed, as considered in the discussion. M2 can be made loosely equivalent to M1 if $\alpha = 1$ as sex bias no longer alters female and hermaphrodite fitness, the only pollinator influence that remains is on selfing rate, as depicted in Figure 4.6. M3 can also be made equivalent to M1 unless both α and c are set to 1, removing all pollinator-mediated processes except for selfing.

4.5 Discussion

The addition of pollinator behavior parameters to a nuclear model of gynodioecy generally makes the invasion condition for females more stringent and reduces female

frequencies for realistic values of pollinator preference (Figure 4.6). Whereas a slight preference for hermaphrodites leads to sharp reductions in equilibrium female frequency, a stronger preference for females is required to similarly increase female frequency beyond that predicted by a model without pollination processes. No matter how strongly females are preferred, they only receive pollen after a pollinator visits hermaphrodites, they have no pollinator-mediated fitness assurances under hermaphrodite-biased visitation, and may even face a pollen limitation cost despite female-biased visitation (M3, Figures 4.4 and 4.5). Hermaphrodites, conversely, gain fitness via male function as well as seed fitness through geitonogamous selfing, even if pollinators show strong preference for females. Exceptions to this pattern occur when inbreeding depression is severe, when populations show self-incompatibility, or when visitation bouts are very short, such that any preference for females leads to strong visit limitation and pollen limitation in hermaphrodites. This sex asymmetry in plant-pollinator interactions, specifically hermaphrodites' accelerating benefits associated with pollinator preference differing from female's diminishing returns or costs associated with pollinator preference, is compatible with Bateman's principle (1948) and the prediction that pollinator attraction is more important for functional males, i.e., hermaphrodites in gynodioecy, than females (Bell, 1985).

We are newly intrigued by how common gynodioecy is in light of our finding that pollinator preference for hermaphrodites generally restricts females. Similarly, we have a deeper appreciation for the comparative rarity of nuclear gynodioecy. Our findings suggest that nuclear gynodioecy is predicted to be even more rare than estimated by classical models (Lewis, 1941; Charlesworth and Charlesworth, 1978). Pollinators generally prefer to visit hermaphrodites over females, as in Chapters 2 and 3, because they offer both pollen and larger nectar rewards (Willson and Ågren, 1989; Ashman and Stanton, 1991; Delph and Lively, 1992; Eckhart, 1992; Case and Ashman, 2009; but see Ashman, 2000). The maintenance of females, despite the apparent hurdle imposed by

biased pollinator visitations, becomes a more remarkable and challenging phenomenon to explain than the simpler case without pollinator influence.

Pollinators' ability to restrict females may, however, help solve another evolutionary question: how to stop the spread of females in the case of cytoplasmic male sterility, *CMS*, which favors the spread of females due to maternal transmission advantage (Lewis, 1941). Indeed, *CMS* so greatly facilitates the spread of females that the prevention of female fixation, not the existence of cytonuclear gynodioecy, requires explanation (Houliston and Olson, 2006). From our nuclear models it is clear that pollination processes can limit the spread of females, indicating their potential as a stabilizing factor in cytonuclear gynodioecy. Although we do not directly model cytonuclear gynodioecy, our analyses suggest that pollinator behavior may stabilize cytonuclear gynodioecy by introducing sex biased pollen and visit limitation and influencing selfing rate. Several studies have noted the potential for pollen limitation to reduce the spread of females (Lewis, 1941; Lloyd, 1974; Maurice and Fleming, 1995; McCauley and Taylor, 1997), but we offer a new perspective on the mechanisms of pollinator-mediated restriction of gynodioecious females.

Our models' assumptions require discussion to better understand their applicability to natural systems. We only consider the first visit to a flower for simplicity, when in reality a single flower may receive multiple visits unless strongly visit-limited. Multiple visits are likely to benefit females for the following two reasons. First, multiple visits per flower would generally reduce pollen limitation to both sexes, as multiple visits per plant reduced female-hermaphrodite disparity in pollination failure in this study. Second, multiple visits per flower can increase female relative fitness by creating the potential for ovule discounting, the interference of self-pollen with future outcrossing potential (Lloyd, 1992; Harder and Barrett, 1995). Additionally, we assume a specific mode of geitonogamy in order to make the selfing rate a function of pollinator behavior, whereas a more complex mode of geitonogamy may occur involving

outcrossing mechanisms such as protandry (Buide and Guitián, 2002), herkogamy (Webb and Lloyd, 1986; Takebayashi et al., 2006), and inflorescence architecture (Pyke, 1978; Lloyd and Schoen, 1992). Regardless, hermaphrodites remain subject to geitonogamous self-fertilization according to pollinator movement patterns unless self-incompatible (Lloyd and Schoen, 1992; Buide and Guitián, 2002), making our theoretical framework a sound starting point for further theoretical investigations of pollinators' role in gynodioecious populations.

Few empirical studies have directly examined the impacts of pollinator behavior on gynodioecious populations, and those that have offer mixed results, preventing generalizations about pollinators' role in gynodioecious populations. In some cases, pollinators have strong influences on female relative fitness through sex-biased visitation, as predicted in this study (Case and Ashman, 2009; Delph et al., 2011; Chapter 2), whereas other studies find pollinators to have negligible effects (Asikainen and Mutikainen, 2005; Ashman, 2006; and references therein, Chapter 3). The models presented in this study offer perspective on why pollinator variation may matter in some contexts, but not others. For example, changes in pollinator behavior are expected to have little effect on sex ratio if increases in female pollen limitation are balanced by increased hermaphrodite selfing, as in the level regions on the left sides of Figures 4.2 and 4.3. Furthermore, pollinator behavior will not be influential, regardless of preference for a particular sex, if both sexes reach pollinator saturation when visit number is very high, as in the valley of Figure 4.1. Other parameter values, however, reveal a large change in female fitness and equilibrium sex ratios due to a small change in pollinator behavior or resource compensation. This corresponds to a value of pollinator preference where the benefit of selfing or cost of pollination failure outweighs female advantage, as in the steeper, threshold-like regions of Figures 4.2-4.5, or critical values in expression 20b. As such, small ecological changes in the plant-pollinator relationship, such as a

departure from a co-evolved pollinator, or a change in pollinator abundance, may have a large effect on sex ratios and breeding systems.

Our analyses suggest that a plant's phenotypic characteristics related to pollination syndrome (Ollerton et al., 2009) may predispose it towards a particular breeding system. Likewise, a pollinator's functional characteristics such as size and foraging preferences may make it better suited to maintain dimorphic systems such as gynodioecy. In all cases, plants must attract adequate pollination services to females for gynodioecy to be maintained: namely a combination of high visitation rates, low pollinator bias, and high pollen carryover rates. Similarly, pollinators providing high pollen carryover, unbiased visitation, and a moderate preference for females facilitate gynodioecy. Accordingly, specialization towards unbiased pollinators may create opportunities for gynodioecy, whereas biased pollinators may leave one gender's attributes maladaptive (Thompson, 2001) and select for hermaphroditism.

Taxonomically broad studies comparing the pollination syndromes and prevalence of gynodioecy would permit us to test for associations between pollinator attributes, degree of specialization, and pollination syndromes with breeding system diversity.

Understanding how females manage to elevate their fitness over hermaphrodites is fundamental to understanding gynodioecy. It has been clear since Lewis (1942) that gynodioecy could not simply be an outcrossing mechanism and that pollen limitation plays a decisive role. Integrating the genetic and ecological phenomena in theoretical and empirical studies improves our understanding of the evolution and maintenance of gynodioecy as well as sex ratio variation in natural populations. We add a practical new layer to our understanding of how sex ratios evolve in gynodioecious populations. Pollinators serve as a unifying factor, underlying several sources of variation in female relative fitness, gynodioecious sex ratios, and breeding system stability. Given the complex and interconnected ecology and genetics of gynodioecy, a pollinator-behavioral perspective on sex ratio and breeding system evolution is valuable.

4.6 Tables

Table 4.1: Parameters used in models with descriptions.

<i>Parameter</i>	<i>Description</i>
F	Number of flowers on a plant
V	Number of pollinator visits each hermaphrodite receives
B	Bout length: the number of flowers sequentially visited on a plant
S_H	Probability of hermaphrodite selfing
$O_{F,H}$	Probability of outcrossing for females or hermaphrodites
$U_{F,H}$	Probability of pollination failure for females or hermaphrodites
k	Female compensation
δ	Inbreeding depression
c	Pollinator's carryover rate as a fraction of pollen needed for full seed set
α	Pollinator preference for hermaphrodites; $\alpha=1$ indicates no preference
ν_H	Frequency of hermaphrodites in population
ν_F	Frequency of females in population ($= 1-\nu_H$)
ν_{WSR}	Weighted sex ratio = $\frac{\alpha\nu_H}{\alpha\nu_H + \nu_F}$

Table 4.2: Gynodioecious sex ratio model variants used in this study. The pollination processes included in each model and references to their expressions are given. Parenthetical numbers correspond to expressions in text; parameters correspond to those in Table 1.

Model	¹ Pollinator influenced processes	² Sex bias	³ U_H	³ U_F	⁴ Female pollen limitation	⁵ Selfing
⁶ M1	Selfing (see text)	None	0	0	None	S_H
M2	Selfing, visit limitation	V	(2)	(3)	None	(6a, 7)
M3	Selfing, visit, & pollen limitation	V, B	(2)	(3)	(8b)	(6c, 7)

¹ Indicates which pollinator-mediated phenomena are modeled

² Indicates what pollinator parameter is sex-biased, according to α . In model variants 2 and 3, α also modifies mating frequencies and expected genotypic frequencies.

³ Probabilities of hermaphrodite and female plants going unvisited, respectively.

⁴ Expression for female pollen limitation used in model

⁵ Expression for hermaphrodite selfing rate used in model

⁶ Equivalent to gynodioecious model of Charlesworth and Charlesworth (1978)

4.7 Figures

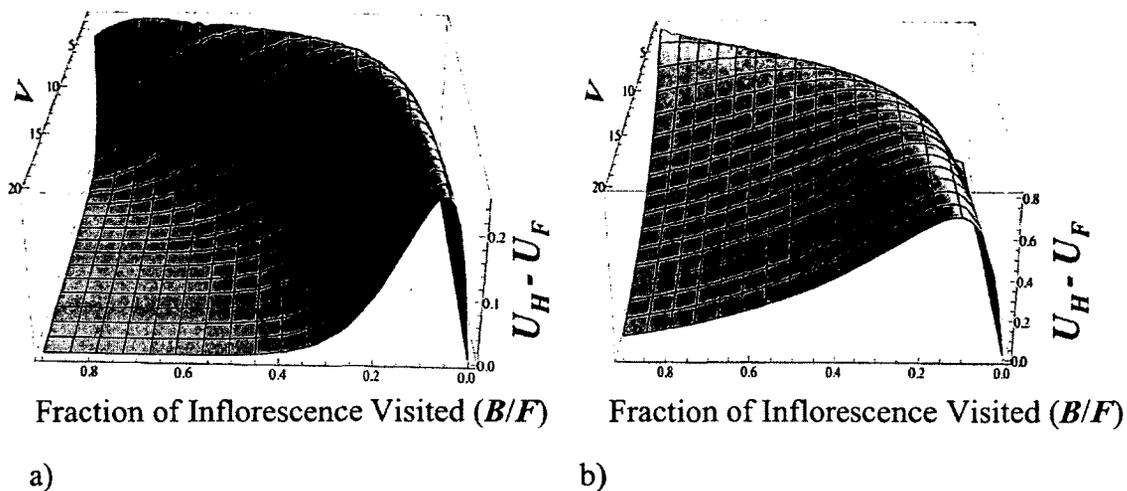


Figure 4.1: Differences in hermaphrodite and female visitation rates due to pollinator bias. Discrepancy in visitation rates, $(U_F - U_H)$, are shown for two types of pollinator preference. Disparity in pollination failure is on the vertical axis, fraction of flowers visited in each visitation bout is on the horizontal axis (B/F), and number of visits to a hermaphrodite is on the z-axis. Figure a) corresponds to the case where females receive half as many visits as hermaphrodites ($\alpha = 2$) and b) corresponds to the case where females receive one third as many visits and one third as many flowers are visited in each visit ($\alpha = 3$).

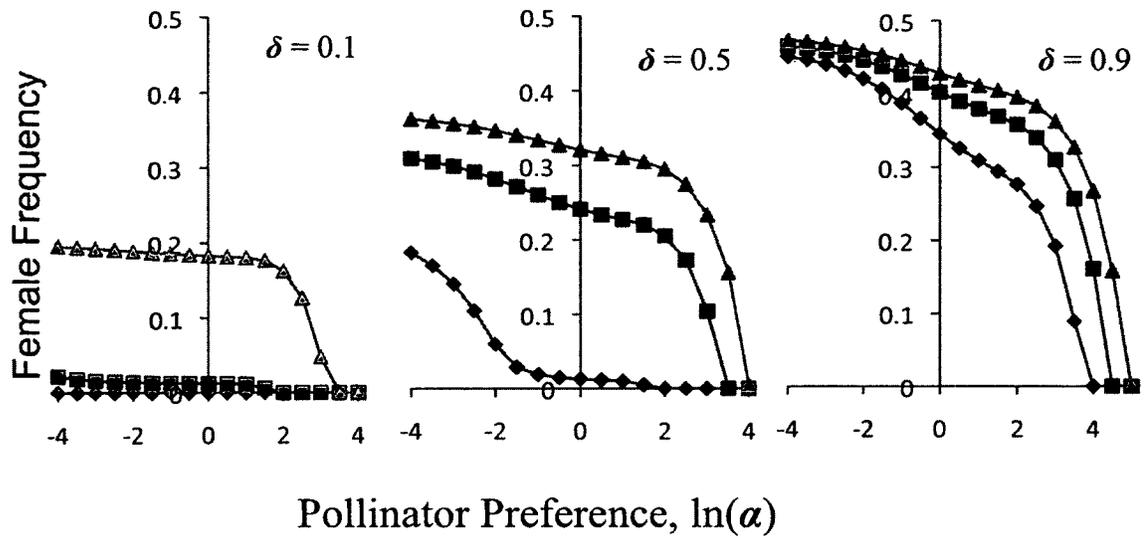


Figure 4.2: Gynodioecious sex ratio vs. pollinator preference, female compensation, and inbreeding depression. Equilibrium female frequencies under M2 are given for a range of values for pollinator preference for hermaphrodites, α , for increasing levels of female compensation (diamonds $k = 0.4$; squares $k = 0.9$; and triangles $k = 1.4$), and inbreeding depression, δ , as noted in each panel.

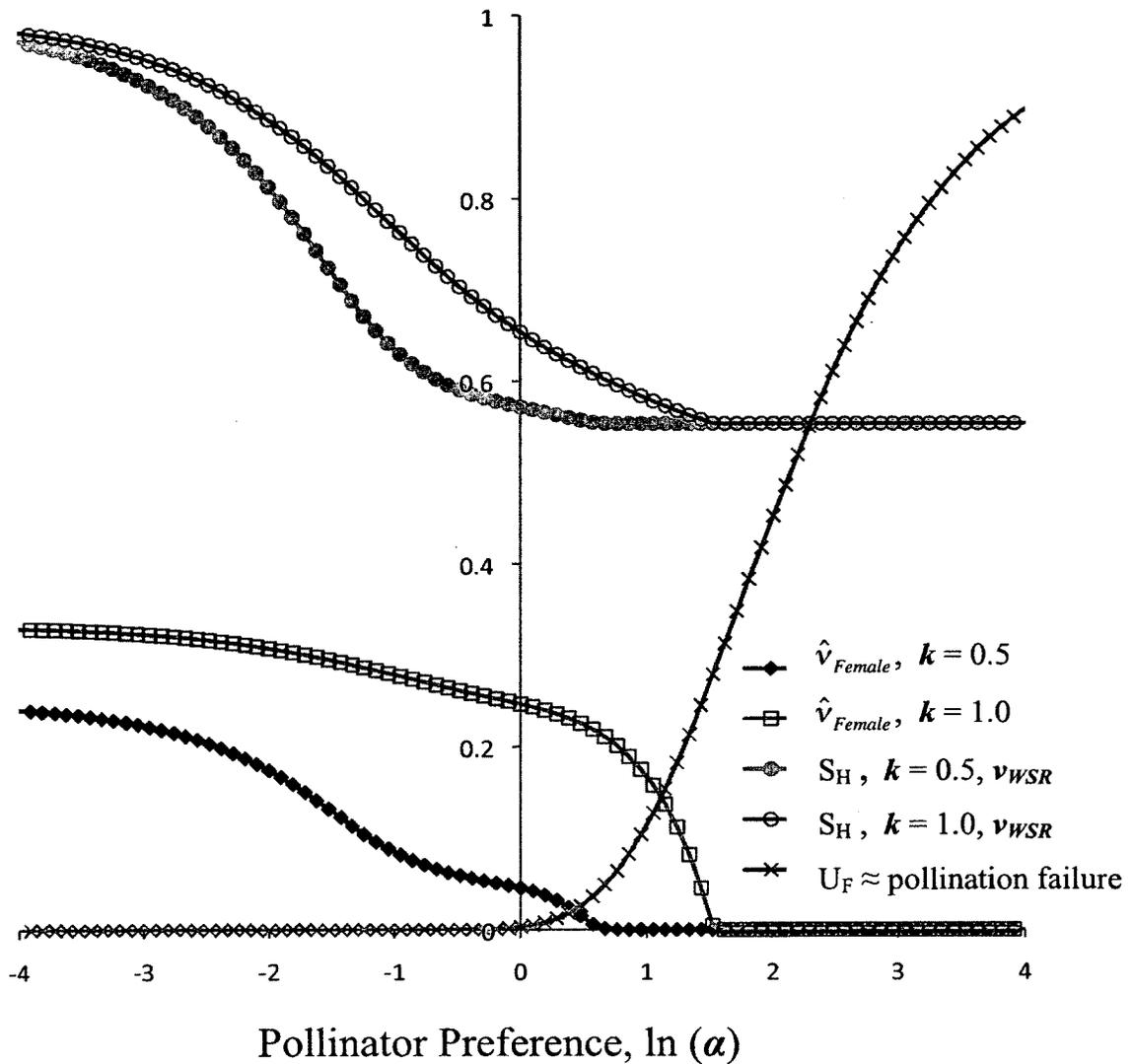


Figure 4.3: Sex ratio, selfing rate, and pollination failure vs. pollinator preference. Note, y-axis indicates sex ratio, selfing rate, *and* pollination failure, see legend. Equilibrium female frequency (\hat{v}_{Female}) is shown for M2 with two levels of female resource compensation, $k = 0.5$ (filled diamonds) and $k = 1.0$ (empty squares), across a range of pollinator preference, α . Corresponding levels of hermaphrodite selfing (S_H) are shown for $k = 0.5$ (filled circles) and $k = 1.0$ (empty circles) as well as levels of pollination failure (U_F) for females (hatches), applicable to any value of k . Additional parameters are $B = 10$, $F = 16$, $V = 6$, $c = 0.8$, $\delta = 0.5$.

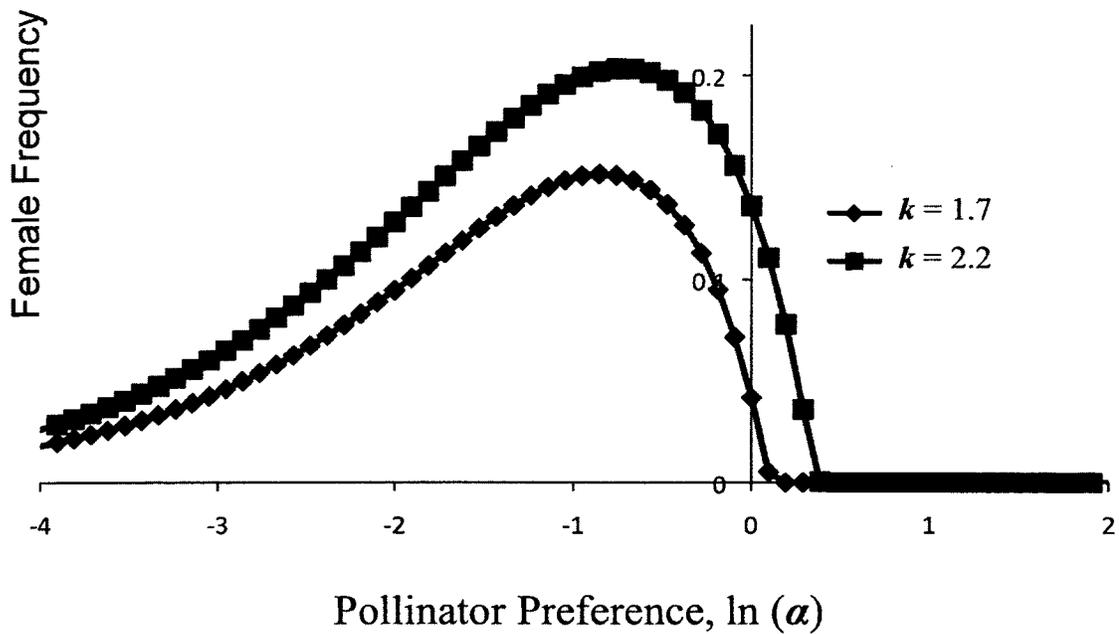


Figure 4.4: Gynodioecious sex ratio vs. pollinator preference for a model including visit and pollen limitation. Equilibrium female frequencies are given for a range of levels of pollinator preference for hermaphrodites, α , for two values of resource compensation, $k = 1.7$ (diamonds), and $k = 2.2$ (squares), using model M3 with pollinator preference affecting both sexes' outcrossing rate, hermaphrodite selfing rates, and female visitation rate. Additional parameters are $B = 10$, $F = 16$, $V = 6$, $c = 0.9$, $\delta = 0.5$.

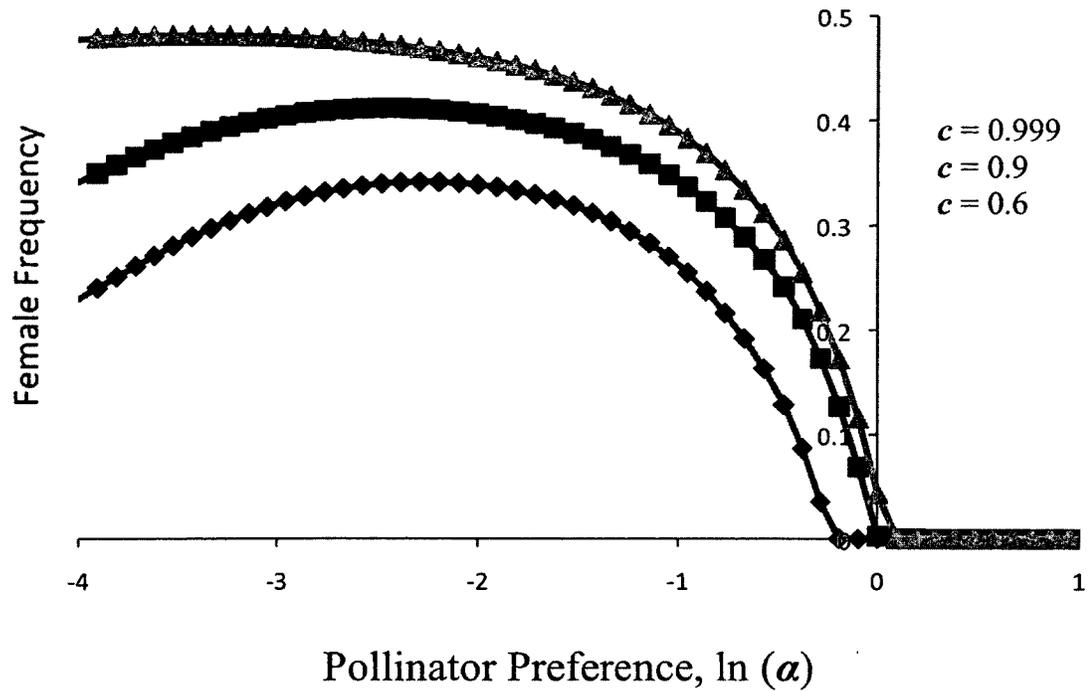


Figure 4.5: Gynodioecious sex ratio vs. pollinator preference with visit limitation and variable pollen carryover. Equilibrium female frequency and pollinator preference for hermaphrodites are shown for three values of pollen carryover, $c = 0.999$ (triangles), and $c = 0.9$ (squares), and $c = 0.6$ (diamonds), for a model where pollinator preference affects both sexes' outcrossing rate, hermaphrodite selfing rates, and female visitation rate, M3. Additional parameters are $B = 5$, $F = 16$, $V = 0.1$, $\delta = 0.9$, $k = 1.1$. Visitation rate is very low, emphasizing the potential for pollen limitation in females, despite being preferred.

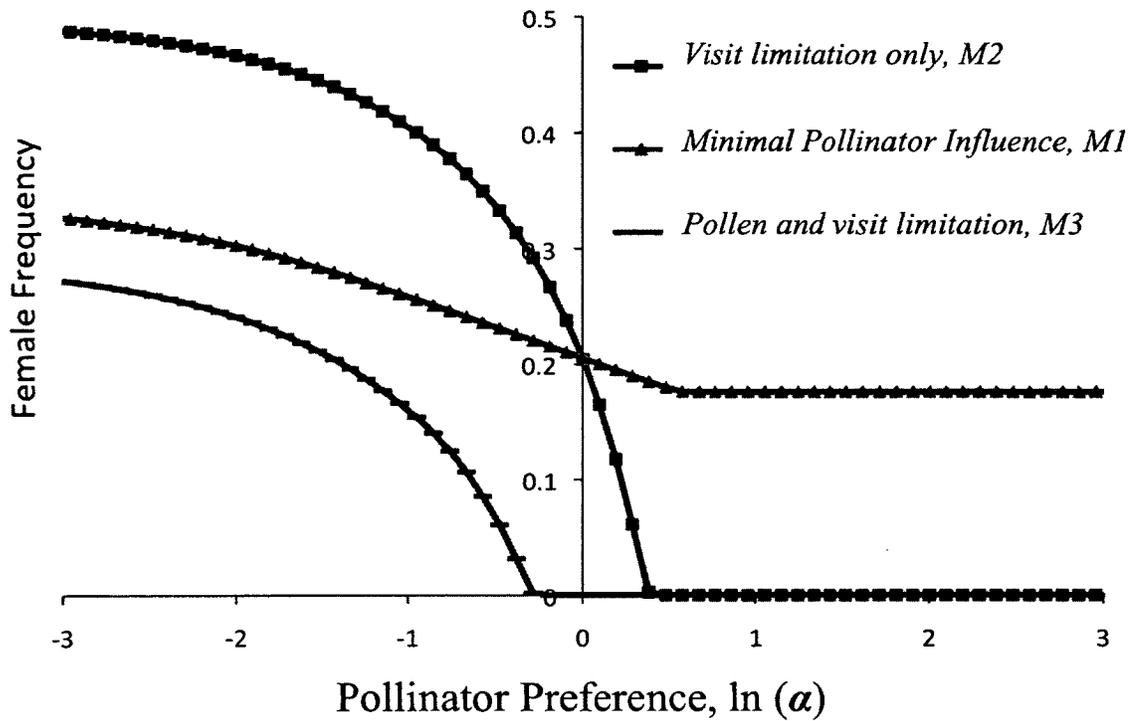


Figure 4.6: Equilibrium female frequency and pollinator preference for hermaphrodites under several models of gynodioecious sex ratio evolution. M1 (triangles): the model of Charlesworth and Charlesworth (1978), which lacks direct pollinator influence. M2 (squares): our model that includes visit limitation to both sexes. M3 (dashes): our model that includes both visit and pollen limitation. For all models $k = 1.1$, $B = 10$, $F = 16$, $V = 2$, $c = 0.9$, $\delta = 0.5$.

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Chapter V

General conclusions

In Chapter 2, I found that our North American artificial populations of *Silene vulgaris* were sensitive to pollination context, had a high potential for female advantage, and that female advantage depended on pollination context. Females were capable of producing more than twice as many seeds as hermaphrodites, sufficient to explain the existence of females under nuclear or cytonuclear gynodioecy (Lewis, 1941). This advantage was only realized, however, in the context of nocturnal moths that provided female plants with adequate pollination to achieve elevated seed production. On the other hand, visitation only by diurnal pollinators reduced the seed production of both sexes, and had a disproportional negative impact on females. This significant reduction in female relative fitness was associated with a strong visitation bias against females by diurnal pollen collecting insects. We found that both sexes were pollen and visit limited when exposed to diurnal visitors, but that females managed to produce as many seeds as hermaphrodites despite biased pollinator visitation. Females appear able to compensate for their reduced attractiveness to pollinators through increased floral longevity- this phenological aspect of female seed production advantage is likely a factor maintaining gynodioecy in many systems. With only diurnal pollinators, females were able to match the pollinator-favored hermaphrodites in seed set, but only with nocturnal pollination could females produce significantly more seeds.

In Chapter 3 we were surprised to find no female seed production advantage among our Czech artificial populations in either pollination context, despite high visitation rates to both sexes. Both females and hermaphrodites were less pollen- and visit-limited in the context of diurnal visitors than in the North American populations (Chapter 2). Pollinator visitation data account for this outcome: diurnally exposed plants received five times as many visits in the Czech populations as they did in the North America populations. The absence of female advantage among nocturnally exposed

plants, despite the presence of many pollen donors and frequent visitation by presumably effective pollen vectors, suggests that the genetically determined enhancement of female fecundity is minimal in this population. Indeed, Czech females produced no more flowers than hermaphrodites, unlike the plants in North America. Furthermore, Czech population females were somewhat inconsistent in their sex expression, with some having both female and hermaphrodite flowers while others were true females, blurring the lines between female and hermaphrodite. Large-scale studies (Keller and Taylor, 2010) on European and North American plants reveal striking differences in their genomic composition and elevated vigor in North American *S. vulgaris* resulting from novel recombinations of European endemic ecotypes. This may contribute to higher potential for resource compensation in North American females. Czech populations may also be the product of local selective pressures for generalist pollination in *S. vulgaris*, reducing the effects of our experimental change in pollination context and helping explain why female relative fitness was constant regardless of pollinator treatment. As Czech plants can achieve significant seed fitness during the day, it benefits them to remain open and take advantage of diurnal vectors, rather than close during the day to reduce water loss and exposure to pollenivorous animals. Once the fixed costs associated with producing a flower are expended, it is generally advantageous to utilize the flower to import or export as much pollen as is possible. Nectar and olfactory cues may provide some cost to remaining open during the day, but these could be minimal as small-bodied pollen and nectar collectors require less of these inducements than large bodied, nectivorous moths. Elevated inbreeding depression is also a risk associated with diurnal pollination (Barthelmess et al., 2006; Brunet and Sweet, 2006), though that is reduced in populations such as Kovary with high hermaphrodite density (Miyake and Olson, 2009). Thus, it appears that reduced resource compensation and consistent pollination by abundant pollen vectors partly explain the absence of female advantage and its insensitivity to our pollination treatments.

In Chapter 4, our modeling efforts produced several general conclusions. As expected, conditions that increase selfing rate, such as longer visitation bouts and reduced pollen carryover, can increase female frequency. Generally though, the inclusion of pollination processes in models of gynodioecy reduces female frequencies relative to a model omitting them. This is true when pollinators preferentially visit hermaphrodites, as is typical (Bell, 1985; Eckhart, 1992; Ashman, 2000; Case and Ashman, 2009). Female-biased visitation can increase female frequencies, but only when hermaphrodites are subject to visit limitation and there is high pollen carryover to minimize female pollen limitation. Hermaphrodite-biased pollen limitation was never substantial given our assumption of geitonogamous selfing, though it could be in the doubly atypical case of a self-incompatible gynodioecious system where females were preferentially visited. My conclusion, that pollination processes generally restrict females' fitness rather than increasing it, is not new. Lewis (1941) noted that pollen limitation would restrict females, though it was a straightforward outcome of a model where hermaphrodites faced no pollen limitation. I show that pollen limitation is still expected to restrict gynodioecy even when hermaphrodites are subject to visit limitation. I further clarify the direct impacts of several aspects of pollinator behavior on mating system parameters, female relative fitness, and gynodioecious sex ratios. I note that the relationship between pollinator context and gynodioecy depends strongly on which aspects of plant fitness are influenced by pollinators. Lastly, I offer a framework for modifying our model to realistically account for pollinators' role in a particular gynodioecious system.

Together, Chapters 2 and 3 provide case studies as to what mix of conditions make a gynodioecious population sensitive to changes in pollinator context. Sensitivity to pollinator context in terms of realized female advantage appears to be increased by both genetically-based resource compensation and a change from effective pollinators to ones creating inter-sexual variation in pollen limitation. In Virginia, both conditions were substantially supported, resulting in strong female advantage and significant

sensitivity to pollination context. In our Czech study, however, resource compensation appeared minimal and both pollinator contexts had high visitation rates to both sexes, resulting in no realized female advantage and little sensitivity to pollination context.

A reciprocal transplant study could be used to verify the conclusions from my empirical research. I speculate that if we raised plants with high potential for female advantage (Virginia plants, for example) in a pollination context with consistently high visitation rates and low pollinator bias (as in both treatments of the Czech experiment), females would show elevated seed production with both diurnal and nocturnal pollinators. Female relative fitness would not be expected to change significantly as pollinator bias did not differ between treatments. Conversely, if we raised plants with reduced capacity for female advantage (Czech plants) in the strongly differing pollination contexts found in our Virginia study, I predict that females would show negative female advantage during the day and seed production parity at night. The strong differences in preference for hermaphrodites and effectiveness of pollinators meant the switch from Virginia diurnal visitors to nocturnal ones led to a change in female advantage as well as a change in overall fitness.

Chapters 2, 3, and 4 offer the opportunity to qualitatively compare theoretical expectations with empirical outcomes. Indeed, a model with parameter values based on the Virginia experiment draws the same conclusion: that high levels of resource compensation make a population sensitive to changes in pollinator abundance and effectiveness. Furthermore, parameter values taken from the Czech study would uphold our findings that, in the absence of significant resource compensation and in response to minor changes in pollination services, female relative fitness is not expected to change.

It is apparent that there are characteristics of universal benefit to females in a gynodioecious population: resource compensation, increased floral longevity, and floral traits that cater to visitors not interested solely in pollen collection. Similarly, there are pollinator attributes as well as characteristics of a pollination context that favor

gynodioecy, including: visiting both sexes without significant bias, high visitation rates, and high pollen carryover. As such there may be a “gynodioecy syndrome” or set of pollinator ecological traits that serve as exaptations for gynodioecy or for a transition to dioecy. Studies of correlations between floral characteristics and gynodioecy are limited (Bawa, 1980; Kephart, 2006) and it remains to be determined if there are floral traits associated with particular breeding systems.

Mapping breeding systems and pollinator shifts onto phylogenies could be used to test the idea that gynodioecy and dioecy evolve more readily in groups with particular floral characteristics. Conversely, it may be that breeding system transitions are associated with or driven by changes in pollinator context (Ganders, 1978) and that pollinators, rather than floral characteristics of a clade, will either promote or restrict breeding system transitions. Pollination context could, therefore, be either a symptom or a cause of breeding system, though it is likely that they co-evolve. Chapter 4 suggests that gynodioecy and dioecy have pollination context requirements, but does not consider how plant-pollinator interactions may change along as females spread or as a population transitions towards separate sexes.

In the case of *S. vulgaris* it appears that generalization to diurnal insects helps hermaphrodites more than females, while specialization on moths disproportionately increases female fitness. These divergent sets of pollinators could, thus, be imposing antagonistic selection on sex determining *CMS* and restorer genes in gynodioecious populations with generalist pollination attributes. This process would be similar to selection on sex-linked genes that increase either female or male fitness, as has been documented in *Mimulus* (Fenster and Carr, 1997), and in *Fragaria* (Ashman, 1999). These gene-by-environment interactions should factor into explanations of breeding system transitions.

Additionally, Chapters 2 and 3 highlight features of *S. vulgaris*' natural history that likely maintain females. Females' longer period of stigmatic receptivity, by avoiding

male-phase entirely, likely helped females in both experiments to cope with reduced pollinator attractiveness. This is a potentially important aspect of female advantage only rarely noted (Stevens, 1988; and Ashman, 1991). Ecological mechanisms such as this plant phenology-pollinator bias interaction may vary from system to system, but highlight the importance of developing a rich ecological understanding of the conditions favoring gynodioecy.

Cytonuclear gynodioecy greatly relaxes the conditions necessary for gynodioecy (Lewis, 1941), as in *S. vulgaris*, the cytonuclear gynodioecious system used in Chapters 2 and 3. Why females do not spread to fixation becomes the more challenging question (Lewis, 1941). It appears that pollination ecology is at least part of the answer to that question (Chapters 2 and 4), as well as genetic factors including a cost of restoration to cytoplasmic male sterility (Dufay et al., 2007). Our results support the conclusion that pollination ecology may stabilize gynodioecious populations by restricting females' seed fitness advantages. It would be useful to build similar pollination-focused models for subdioecy, androdioecy, etc. to examine the stability of these systems in light of pollinator contributions. I suspect that, as in gynodioecy, pollinators' role may vary from facilitation to prevention, and from stabilizing to transition inducing.

Lastly I advocate for studying pollinators' role in natural sex ratio and female advantage variation. There is a great deal of variation in gynodioecious sex ratios and female relative fitness (McCauley et al., 2000; Štorchová and Olson, 2004; Alonso, 2005; Dufay and Billard, 2012; Spigler and Ashman 2012), some of it unexplained, and the influence of pollination context infrequently considered, and rarely quantified. The need for greater attention directed to ecology in studies of gynodioecious sex ratios, including pollination ecology, has been acknowledged (Ashman, 2006), though direct studies of pollinators' influence remain lacking. We are left to infer pollinators' influence from reviews of gynodioecious studies measuring variation in female fitness or frequencies (Shykoff et al., 2003; Dufay and Billiard, 2012). There are likely cases where pollinator

variation explains sex ratio and female fitness variation as well as where it does not, depending on the magnitude of female advantage and variability in pollination context. Both of these outcomes are compatible with the understanding generated by our studies, and significant work remains in measuring variation in pollinator services in natural populations before we can more broadly evaluate its influence on variation in female relative fitness. Reanalysis of existing studies on gynodioecious sex ratio variation and female relative fitness (reviewed in Dufay and Billard, 2012) will shed some light on this topic, but additional studies are required where alternative sources of female fitness variation are controlled. Additional modeling efforts will improve our understanding, as gaps remain concerning the effects of stochasticity in pollination processes (Burd et al., 2009) and spatial effects of pollination processes (Taylor et al., 1999). A wealth of additional insight stands to be gained from synthetic studies that tie together empirical and theoretical approaches to understanding the genetics and ecology of gynodioecy and sex ratio evolution, as has been done with the process of cost of restoration (Caruso and Case, 2012), and as I have attempted in this dissertation. Additional, complementary field and theoretical studies of pollinators' numerous influences on plant populations promises a richer understanding, not just of how gynodioecy evolves and is maintained, but of the staggering variation in plant reproductive strategies that has intrigued us since Linnaeus and Darwin first described them, centuries ago.

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