POPULATION SEX RATIO AND SIZE AFFECT POLLINATION, REPRODUCTIVE SUCCESS, AND SEED GERMINATION IN GYNODIOECIOUS LOBELIA SIPHILITICA: EVIDENCE USING EXPERIMENTAL POPULATIONS AND MICROSATELLITE GENOTYPES

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in partial fulfillment of the requirements
for the degree of Masters of Science

By
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TABLE OF CONTENTS

LIST OF FIGURES………………………………………………………………iv
LIST OF TABLES………………………………………………………………v
ACKNOWLEDGEMENTS……………………………………………………vi

CHAPTER 1: INTRODUCTION…………………………………………………………1

CHAPTER 2: EFFECTS OF VARIATION IN ARRAY SEX RATIO AND SIZE ON
POLLINATOR VISITATION AND PARENTAL FECUNDITY IN
GYNODIOECIOUS LOBELIA SIPHILITICA………………………………………9
  Abstract……………………………………………………………………9
  Introduction……………………………………………………………9
  Methods………………………………………………………………13
    Study species…………………………………………………………13
    Study site…………………………………………………………14
    Plant culture………………………………………………………14
    Timing of the experiment…………………………………………15
    Experimental design………………………………………………15
    Pollinator visitation observations………………………………17
    Analysis of maternal reproductive success……………………….19
    Estimating male fitness with microsatellites…………………….20
    Estimating hermaphrodite total fecundity: seed production plus
    paternity……………………………………………………………23
  Results……………………………………………………………………24
    Pollinator visitation is affected by array size, sex ratio, and individual
    plant sex………………………………………………………………24
    Female fecundity affected by array sex ratio………………………25
    Assessment of male fitness…………………………………………26
    Relative fitness of the sexes: total fitness of hermaphrodites vs. females
    ………………………………………………………………………27
  Discussion………………………………………………………………28

CHAPTER 3: POPULATION SIZE, AND NOT POPULATION SEX RATIO OR
LEVELS OF INBREEDING AFFECT GERMINATION IN NATURAL AND
EXPERIMENTAL POPULATIONS OF LOBELIA SIPHILITICA………42
  Abstract…………………………………………………………………42
  Introduction……………………………………………………………43
  Methods………………………………………………………………47
Study species: Lobelia siphilitica

Germination of seeds collected from natural populations

Effects varying levels of inbreeding on fecundity, germination, and sex ratio

Effects of experimental population sex ratio and size on germination and sex ratio

Results

Germination of seeds collected from natural populations

Inbreeding effects on fecundity, germination, and sex ratio

Experimental population sex ratio and size affected offspring germination and sex ratio

Discussion

Germination higher in seeds from small and highly female populations

Maternal sex affects fecundity in crosses with varying degrees of inbreeding

Understanding link between population sex ratio and size in L. siphilitica

CHAPTER 4: CONCLUSIONS

High visitation levels did not lead to high fecundity

Germination was lower in large natural populations than small

Germination was affected differently in outcrosses and supplementally pollinated females

Germination levels were similar in inbred and outcrossed experimental populations

Paternal fitness was affected by array sex ratio

Inbreeding does not appear to be a link between population sex ratio and size

Complex interactions between population sex ratio and size exist

Future studies

LITERATURE CITED
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE &amp; TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1 Population sex ratio &amp; size trends in <em>Lobelia siphilitica</em></td>
<td>7</td>
</tr>
<tr>
<td>Figure 1.2 Image of pollinator with <em>L. siphilitica</em></td>
<td>8</td>
</tr>
<tr>
<td>Figure 2.1 Herrick Aquatic Ecology Research Facility map</td>
<td>37</td>
</tr>
<tr>
<td>Figure 2.2 Pollinator visits/flower/hour in 2008</td>
<td>38</td>
</tr>
<tr>
<td>Figure 2.3 Female fecundity</td>
<td>39</td>
</tr>
<tr>
<td>Figure 2.4 Paternity analysis</td>
<td>40</td>
</tr>
<tr>
<td>Figure 2.5 Pollen flow between arrays</td>
<td>41</td>
</tr>
<tr>
<td>Figure 2.6 Relative fertility of the sexes</td>
<td>42</td>
</tr>
<tr>
<td>Figure 3.1 Proportion germination of seeds from natural populations</td>
<td>61</td>
</tr>
<tr>
<td>Figure 3.2 Proportion germination of seeds from experimental crosses of varying degrees of inbreeding</td>
<td>62</td>
</tr>
<tr>
<td>Figure 3.3 Germination and sex ratio of seeds produced from experimental arrays</td>
<td>63</td>
</tr>
<tr>
<td>Figure 3.4 Natural population size variation across years</td>
<td>65</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE &amp; TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1 Microsatellite primers</td>
<td>34</td>
</tr>
<tr>
<td>Table 2.2 ANOVA of visitation results</td>
<td>35</td>
</tr>
<tr>
<td>Table 2.3 Microsatellite descriptive statistics</td>
<td>36</td>
</tr>
<tr>
<td>Table 2.4 Natural populations of <em>Lobelia siphilitica</em>: size &amp; sex ratio</td>
<td>56</td>
</tr>
<tr>
<td>Table 3.1 Experimental arrays: seed weight and germination</td>
<td>59</td>
</tr>
<tr>
<td>Table 3.2 Comparative analysis: seed weight and germination</td>
<td>60</td>
</tr>
</tbody>
</table>
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I would like to dedicate this thesis to the memory of my mother. It was at her insistence to play outside that I discovered the natural world and gained a life-long curiosity about what was in it and how it worked.
CHAPTER 1
INTRODUCTION

The reproductive biology of gynodioecious plants has been a topic of importance since Darwin (1877). Gynodioecy, where both female and hermaphrodite plants occur within a population, is hypothesized to be an intermediate stage in the evolution from hermaphroditism to completely separate sexes (Charlesworth and Charlesworth 1978; Lloyd 1982; Delph 2003; Dorken and Mitchard 2008). The maintenance of unisexuals in populations with hermaphrodites is difficult to understand given that they have one, rather than two avenues for fitness gain (Darwin 1877). Therefore, understanding what processes maintain gynodioecy is important for understanding why this transitions has occurred almost 100 times in flowering plants (Barrett 2002).

Populations of gynodioecious species vary in the proportion of females they contain. Understanding what causes population sex ratio variation can help explain how gynodioecy is maintained, and why gynodioecy sometimes evolves to dioecy and sometimes does not. Population sex ratios should reflect the relative fitness of the sexes, measured by comparing the maternal fitness of female plants to the maternal fitness of hermaphrodite plants. Females should only be maintained in the population if their fitness is, on average, twice that of hermaphrodites, because hermaphrodites potentially enjoy dual reproductive fitness through both female and male reproduction (Dorken and Mitchard 2008). However, hermaphrodite fitness can be reduced by resource costs of
pollen production and by the fitness costs of self-fertilization and inbreeding, neither of which affect females (Eckhart and Chapin 1997; Bailey 2002; Glaettli and Goudet 2006). This lower hermaphrodite fitness can allow females to be maintained within gynodioecious populations at a lower threshold of relative fitness.

The two main hypotheses for the maintenance and spread of females in gynodioecious populations are based on the advantages of (1) reproductive compensation and (2) obligate outcrossing (reviewed in Charlesworth 1999). Under the reproductive compensation hypothesis, females of a species are better than hermaphrodites at acquiring and directing resources and thereby increase the number and/or quality of the offspring they produce, specifically because they can use resources that would otherwise be spent on pollen production. For instance, females of many species have a higher relative seed production than hermaphrodites (Shykoff 1988; Sakai and Weller 1991; Agren and Willson 1991; Ashman 1992; Asikainen and Mutikainen 2003; Case and Ashman 2007; Lopez-Villavicencio et al. 2007). However, females of *Daphne laureola* (Thymelaceae) do not consistently produce more seeds than hermaphrodites (Alonso et al. 2007), suggesting other mechanisms may contribute to gynodioecious breeding systems.

According to the outcrossing advantage hypothesis, the relative fitness of females is theoretically increased if hermaphrodites self-fertilize and suffer from inbreeding depression (Lewis 1941; Lloyd 1974; Charlesworth and Charlesworth 1978). Support for this hypothesis exists with several gynodioecious species (Kesseli and Jain 1984;
Charlesworth and Laporte 1998; Charlesworth 1999; Dudle et al. 2001; Emery and McCauley 2002; and Keller and Schwaegerle 2006). While the relative fitness of the sexes is affected differently by resource acquisition and inbreeding disparity, it can also be influenced by external forces such as stochastic events and ecological factors.

Studying population sex ratio variation across environmental gradients is particularly informative because ecological correlates of sex ratio variation point to the agents of natural selection affecting the breeding system. The sex ratio of many gynodioecious species has been shown to vary with environmental contexts such as low resources (Bailey 2002; Case and Barrett 2001; Barr 2004), high altitude (Alonso et al 2007), herbivory (Ashman and Penet 2007), and population size (Caruso and Case 2007). Understanding how ecological factors alter sex ratio involves studying how they may affect females and hermaphrodites differently, because sex-specific responses to context would result in changes in the relative fitness of the sexes.

I chose to examine two factors that may contribute to the link between population size and sex ratio in natural populations: pollination biology (Chapter 2) and seed germination (Chapter 3). First, both factors should be sensitive to ecological context. For example, pollinators tend to be more attracted to larger populations containing more flowers (Eckhart 1992); harsh habitats cause lower density and lower visitation rates, and altitudes change the composition of pollinating fauna (Alonso et al. 2007). Second, both pollination biology and seed germination can contribute to variation in plant population size because pollination sufficiency directly contributes to seed production, while germination influences recruitment of new individuals. Finally, both have the potential to
affect sex ratio through relative fitness of the sexes. Females and hermaphrodites are expected to differ in their response to changes in pollination because hermaphrodites can self-fertilize but females cannot, resulting in sex differences in both seed production and seed quality, the latter of which can have direct consequences for seed germination (e.g., Glaettli and Goudet 2006; Keller and Schwaegerle 2006).

This thesis is divided into two main data chapters. Chapter 2 explores the effects of variation in population sex ratio and size on pollination biology, and how pollinator behavior affects female and male fitness in populations of varying sex ratio and size. I predicted that pollinators would prefer to visit plants in large experimental populations, and hermaphrodite flowers over females. I expected to find higher seed production in plants from large populations because of the higher potential for pollen deposition. I also expected that females would produce more fruits and more seeds per fruit than hermaphrodites because females allocate all gametic resources to producing ovules while hermaphrodites must split gametic resources between pollen and ovule production (Eckhart and Chapin 1997). I expected to find lower levels of inbreeding in females than hermaphrodites since females are obligate outcrossers while hermaphrodites are capable of self-fertilization.

Chapter 3 examines the effects of variation in population sex ratio and population size on seed germination. Because seed germination, as an estimate of recruitment, can feed into population size and is potentially affected by population sex ratio, I expected to find that large populations have higher recruitment, which would be supported if seeds from large populations had higher germination than from small. I also expected to see
similar sex ratios in the greenhouse generation to the parental population in nature, and that seeds from females and those produced from low levels of inbreeding would have higher proportion of germination than those from hermaphrodites or from high levels of inbreeding.

The work presented in this thesis investigates the causes and consequences of population sex ratio variation in *Lobelia siphilitica* (Lobeliaceae; see Figure 1.2). This species presents an interesting test case for studying the evolution of gynodioecy, because multiple ecological and breeding system factors covary simultaneously (Caruso and Case 2007). The proportion of females within *L. siphilitica* populations varies negatively with population size, where small populations contain more females on average, while larger populations typically have between 0-15% females, and most populations contain fewer than 100 individuals. Additionally, small populations tend to show much higher variability in sex ratio than the larger populations (Figure 1; Caruso and Case, 2007).

I chose *Lobelia siphilitica* as a study organism because it is a gynodioecious species that exhibits variation in population size and sex ratio across its range. Additionally, *L. siphilitica* is also a good study organism because, as a perennial herb that grows in moist soils along wet meadows and ditches in eastern North America from southern Canada through the Appalachians to North Carolina (Johnston 1992), populations are readily found in Ohio and West Virginia. Because of its vigorous growth and reproduction, where each plant can produce up to 100 flowers and each fruit can contain up to 1000 seeds (Johnston 1992), I expected to be able to measure variation in fecundity and pollination. However, both population sex ratio and population size vary
with latitude, where populations in the southern part of the species range tend to be small and female-biased. Therefore, it is not clear whether population size contributes to sex ratio variation, vice versa, or whether some other environmental factor contributes to latitudinal variation in both size and sex ratio.

The work examined here provides a unique contribution to the study of how plant sexual system transitions may occur, as well as how population sex ratio and size affect germination and pollination biology and how germination and pollination biology affect population size and sex ratio.
Figure 1.1. Relationship between population sex ratio and number of individuals in natural populations of *Lobelia siphilitica*. Data are from Caruso & Case 2007, plus additional populations sampled during this thesis work. The proportion of female plants tends to decrease as population size increases, and smaller populations tend to have a wider range of female frequencies.
Figure 1.2. Image of *Bombus spp.* pollinating female *Lobelia siphilitica* plant (foreground) in experimental population at HAERF, 2008.
CHAPTER 2

Effects of variation in array sex ratio and size on pollinator visitation and parental fecundity in gynodioecious Lobelia siphilitica

Abstract

Variation in population size and sex ratio in gynodioecious L. siphilitica populations may affect pollinator behavior if pollinator preferences are context-dependent. In this study, I used experimental arrays to assess the effects of population size (30 and 90 plants) and sex ratio (10 and 80% female) on pollinator behavior and on female and male fitness. While large arrays received higher total visitation, individuals in small arrays showed higher levels of multiple paternity. Array sex ratio influenced pollinator behavior, where individuals in the hermaphrodite-biased arrays received more total pollinator visits and produced a higher number of seeds per fruit. Hermaphrodite individuals in the high female arrays sired the highest number of offspring. Finally, plant sex influenced total seed production, where female plants overall produced more seeds than hermaphrodites. The relative fecundity of females and hermaphrodites was similar in most arrays, though hermaphrodites had higher fitness than females in the large, high female array, suggesting evidence for the lack of large, high female populations in nature. My results show a complex relationship between population characteristics and pollinator behavior, as well as an explanation for the maintenance of females in relatively small populations.
Introduction

Pollinator behavior is influenced by population characteristics such as the number of individuals or the number of flowers per plant (Totland et al. 1998; Vaughton and Ramsey 1998; Hiraga and Sakai 2007), sex ratio (Ashman et al. 2000; Aizen 2001, Hansen and Totland 2006; Cuevas et al. 2008), and patch size (Hansen and Totland 2006). Populations with a higher number of individuals tend to attract more pollinator visits per flower because they provide an opportunity for greater number of foraging bouts in a smaller distance (Fritz and Nilsson 1994). Therefore, if large populations are receiving higher visitation than small populations, it is likely that individuals in those populations would produce more offspring through increased pollen deposition (Brown and Kephart 1999).

Pollinator behavior can influence the relative fitness of the sexes one sex is preferred over the other, thus allowing pollinator behavior to affect population sex ratio. Populations with a higher proportion of hermaphrodites tend to attract more pollinator visits per flower because hermaphrodites provide both pollen and nectar rewards (Ashman et al. 2000). In this way, a population high in hermaphrodites could benefit from higher visitation than a population with many females (Ashman and Diefenderfer 2001). Therefore, I predict that patterns of pollen movement, i.e. pollen load and outcrossing, would differ between populations of varying sex ratios.

Interactions between the effect of population sex ratio and population size on pollinator activity, and the effect of pollinators on population sex ratio and size, have been studied in some gynodioecious species (Widen and Widen 1990; Klinkhamer et al.
1994). For example, specific pollinator preferences for one sex may contribute to variation in population sex ratio and size through higher fecundity of the favored sex (Shykoff 1992, Eckhart 1992). Pollinator behavior may influence the levels of inbreeding if pollinators visit multiple flowers on the same plant (Molano-Flores 2002; Orellana et al. 2005), which would likely increase with decreasing number of plants. The selective pressures mediated by pollinators may maintain females within gynodioecious populations to avoid inbreeding of hermaphrodites as explained by natural selection (reviewed in Charlesworth 1999). Therefore, pollinator behavior as influenced by population sex ratio and size may act as a mechanism for the maintenance of females.

To measure the effects of population sex ratio and size on pollinator behavior and vice versa, both female and male fitness must be measured. Previous work has found that greater female fitness, which can be measured simply through analyzing fruit and seed production, is often correlated with higher pollinator visitation (Klinkhamer et al. 1990). Male fitness of hermaphrodites can be analyzed through measuring gene flow, paternity, and levels of self-fertilization using molecular markers. These estimates of male fitness of hermaphrodites have been measured in other gynodioecious species with enzyme loci (Maki and Masuda 1993; Maki 1993), allozyme loci (Kohn and Biardi 1995; Sakai et al. 1997; Taylor et al. 1999), and microsatellites (Wolff et al. 1988; Koorevaar et al. 2002; Garcia et al. 2005; Glaettli et al. 2006).

Here, I measured the effects of variation in population sex ratio and size on insect visitation and fitness of female and hermaphrodite Lobelia siphilitica plants using experimental arrays as artificial populations. In L. siphilitica, the two sexes likely have
different pollinator attractiveness, visitation, and resource allocation (Johnston 1991), and therefore estimation of only female fitness would show biased results of relative fitness of the two sexes. Therefore, I measured fruit production, seed production, male siring success, multiple paternity, and correlated paternity.

I was interested in how population sex ratio and size affected individual plant attractiveness to pollinators and the resulting relative fecundity. I predicted higher visitation per flower to plants in large arrays and those with high proportion of hermaphrodites because those populations would offer more rewards to visitors (Mustajarvi et al. 2001). Next, I was interested in how population sex ratio, size, and pollinator visitation affected the maternal fitness of females and hermaphrodites. I predicted that population sex ratio, size, and level of visitation would affect fecundity by finding lower female fitness when the proportion of pollen donors, i.e. hermaphrodites, is low, consistent with pollen limitation (Brown and Kephart 1999; Andriu et al. 2007), and female fitness would increase with population size. Because there is typically a trade-off between seed size and seed number, I accounted for both of these factors. I was finally interested in how levels of paternity were affected by the varying population sex ratios and sizes. I predicted that hermaphrodite individuals that received more visits per flower per hour would have a higher number of offspring than individuals with fewer visits through higher pollen removal and subsequent deposition. I also predicted that populations with fewer hermaphrodites would yield fewer effective fathers per offspring, as in Kohn and Biardi 1995, and that offspring produced would more likely have fathers nearer to the female plant. My work attempts to explain the effects of variation in array
sex ratio and size on pollinator visitation and parental fertility in gynodioecious *Lobelia siphilitica*.

Methods

Study species

*Lobelia siphilitica* (Lobeliaceae) is a gynodioecious herbaceous perennial, where females and hermaphrodites co-occur in natural populations. Populations can be found in ditches, wet meadows, and openings in wet woods throughout the Appalachian mountains of North America from the Carolinas to southeastern Canada and stretching west to Illinois (Johnston 1991). Each *L. siphilitica* plant produces numerous blue zygomorphic flowers with unfused corolla tubes along 1 to 6 spikes (Figure 1.2), which open acropetally; fruits contain ten to several hundred seeds (Johnston 1991a, b). Flowers are not strongly sexually dimorphic except for the anther cylinder, which is dark and pollen-filled in hermaphrodites but is empty and white in females; corollas of perfect flowers are only slightly wider than pistillate flowers (Caruso 2004; A.L. Case and A. Hill, unpub. data). Perfect flowers are strongly protandrous, and the duration of the male phase depends primarily on the rate of pollen removal (Dudle 1999), typically occurring within several days of anthesis (Johnston 1991a). Pistillate flowers are receptive to pollen receipt on the first day of anthesis, and nectaries are located at the base of each flower (Johnston 1991b). Adult plants typically perennate as rosettes and flower August-October. The seeds they produce germinate in early summer, and may or may not flower in their first year (pers. obs.). Numerous insects visit the species, including bumblebees
(Bombus; Apidae) honey bees (Apis; Apidae), carpenter bees (Xylocopa; Apidae), syrphid flies (Syrphidae), and solitary bees (Halictidae; personal obs).

**Study Site**

I placed experimental population arrays of *L. siphlitica* in the Herrick Aquatic Ecology Research Facility at Kent State University (HAERF; 41°08’N, 81°20’W). The fenced-in facility is approximately 1 acre in size and roughly rectangular, therefore I placed the plot locations in the corners, approximately 100 meters apart (Figure 2.1). The HAERF provided seemingly appropriate habitat for *L. siphlitica*, with moist flat areas that slope into wetlands and ponds, though no native *L. siphlitica* plants have been observed there. Other flowering species at the HAERF included *Aster lateriflorus*, *A. pilosus*, *Bidens cernua*, *Cirsium vulgare*, *C. arvense*, *Daucus carota*, *Dipsacus fullonum* (sylvestris), *D. laciniatus*, *Eupatorium perfoliatum*, *Euthamia graminifolia*, *Lythrum salicaria*, *Polygonum lapathifolium*, *P. pensylvanicum*, *Sagittaria latifolia*, *Solidago canadensis*, *S. nemoralis*, *S. patula*, *S. rugosa*, *Symphyotrichum pilosum*, *Vernonia gigantea*, (http://bioweb.biology.kent.edu/Research/Aquatic_Ecology_AERF.html and personal obs.).

**Plant Culture**

For the 2007 pollination experiment, I used plants that were grown from seeds of 15 randomly selected plants of unknown sex in Bend View Metropark (BV) near Toledo, OH. This native population had the highest proportion of females (56%) of 14 closely
studied populations throughout Ohio and West Virginia in the previous flowering season (see Chapter 3), thus increasing the potential number of female offspring I would have available for experiment. Plants used in the 2008 pollination experiment were grown from seeds produced by the open-pollinated experimental plants in the 2007 experiment. Seeds for both years were cold-stratified at 4°C for 8 weeks in winter and germinated at 24°C in a 12:12 h light:dark cycle in spring. Seedlings were transplanted to individual pots in the greenhouse at Kent State University in summer of each year, fertilized weekly, and kept saturated with water through flowering. I recorded the sex of each plant based on the sex of the first two flowers produced.

Timing of experiment

The arrays were monitored during five consecutive days in late August 2007 (August 21st to August 26th, 2007) and eight days in late August-early September 2008 (August 30 to September 8, 2008). Ohio native populations of L. siphilitica were flowering at this time, so I assumed that pollinators would behave similarly in my experimental arrays to native populations.

Experimental design

I created four unique combinations of two array sizes (30 vs. 90 plants) and two sex ratios (10% vs. 80% female) to assess the effects of population size and sex ratio on pollination. These experimental population size classes were the best approximation of natural populations that was logistically possible with the number of individuals of both
sexes in the grow-out for both 2007 and 2008. Assuming a positive linear relationship between pollinator visitation and population size, I predicted that the number of plants in small versus large arrays would allow me to detect a meaningful effect of population size on pollinator visitation. The population sex ratios, 10% and 80% female, have been observed in natural populations of *L. siphilitica*. Although 80% female is atypically high, this dramatic difference in array sex ratios increases the likelihood of detecting differences between sex ratio treatments.

Plants were randomly assigned to arrays, except that I minimized the number of siblings included within each array (particularly pairs involving hermaphrodites) to minimize and equalize the potential for biparental inbreeding among treatments. Plants were arranged at a constant density, approx. 8 cm apart, and were randomized by genotype but stratified by sex so that the sex ratio was consistent throughout the array. To account for edge effects and potential spatial effects within arrays, I rotated each tray of plants within each array on each consecutive observation day. I observed that the four corners of the HAERF differed in the amount of vegetation cover and incident light. To minimize variation caused by location, the four experimental gardens were randomly assigned to a different corner of the HAERF on each observation day, such that each corner of the site hosted each of the four arrays for at least one day by the end of the experiment in 2007 (five-day observation period) and two days in 2008 (eight-day observation period).

A single array designated for hand-supplementation (10 females and 10 hermaphrodites) was located at the center of HAERF, approximately equidistant from all
experimental arrays (Fig. 1). During each observation day, I collected the pollen in one flower from each hermaphrodite in the hand-pollinated array and from additional plants growing in the greenhouse. I mixed the pollen, and pollinated all receptive stigmas on all plants in the supplementally pollinated array each day using a fine-tipped paint brush.

Prior to setting plants in arrays in the HAERF, I measured the height of each plant and marked all open flowers with latex paint on the back of the calyx such that the paint was not visible from the front of the flower. During the experiment, I marked all newly opened flowers with a new color of paint daily. I also marked flowers on the day of senescence with the corresponding day’s paint color so that floral longevity and daily display size could be determined. For the hand-pollinated array, I also marked a paint dot on each flower for each day that the flower was hand-pollinated. Flowers were hand-pollinated 2-3 consecutive days during the experiment until the stigma shriveled to ensure stigmas were saturated. During the 2008 experiment, I also painted a dot on hermaphrodite flowers on the day of sex phase shift.

**Pollinator observations**

I performed preliminary pollinator observations from 7 am to 7pm for two days (August 18 and 19, 2007) prior to the experiment to determine peak visitation times. During the five observation days in 2007 and eight days in 2008, I monitored the four experimental arrays between 9:30 am and 12:30 pm daily, which corresponded with peak visitation (data not shown). I randomized the time and order of observation by array and counted the total number of open flowers on each plant prior to observing visitation. I
monitored visitation to each small array once per day and each large array a third at a
time for three periods each day so that each plant was observed for 15 minutes each day.

I tallied the number of legitimate visits to each flower during each 15 minute
observation period. I considered a visit to be “legitimate” if the pollinator entered the
flower from the front to reach the nectaries at the base of the flower, where they are likely
to contact the sex organs. This contrasts with nectar thieving, where pollinators steal
nectar through the side of the flower, between upper and lower lobes of the corolla tube,
without contacting the reproductive organs. In 2008, I recorded legitimate visits and
thieving separately, and found that the relative frequency of thieving was high in all
arrays, but did not differ between arrays (data not shown), and therefore I excluded
thieving data from calculations of overall visitation rates. I determined the visitation rate
as the number of legitimate visits per flower per hour for every plant for each day during
the experiment.

The distribution of the visitation data was highly skewed because there were
many plants that did not get visited at all, and a wide range of visitation rates for the rest
of the plants. Because no statistical transformations improved normality, I analyzed this
dataset in two steps. I first analyzed visitation data by categorizing plants as “1” if visited
and “0” if not visited on any given observation day using a split-plot 3-way ANOVA
with array size, sex ratio, and individual plant sex in a full factorial design, with
population size and sex ratio tested over the array. Visitation status was treated as a
categorical variable in the analysis. I also accounted for effects of variation between
years, locations, and days as random effects. To analyze differences between plants that
were visited, I subset the plants that had been visited at least once (i.e., with a value of “1” in the first analysis); this dataset was normally distributed. I analyzed the total number of visits per flower per hour as a continuous variable using a mixed-model split-plot 3-way ANOVA of array size, sex ratio, and individual plant sex in a full factorial design, testing population size and sex ratio over the array. I also accounted for effects of variation between years, locations, and days as random effects.

*Analysis of female reproductive success*

After the pollination experiment was completed, I returned all plants to the Kent State University greenhouse. Plants were quarantined and treated with insecticides and fungicides as needed, and plant height was measured to determine the average height of each plant during the experiment.

At fruiting, approximately two weeks later, I collected each mature fruit into an individual paper envelope. I determined fruit set by dividing the number fruits per plant by its total number of flowers open during the observation period. To account for differences between seed weight and seed number, I weighed exactly 100 seeds from each of two randomly selected fruits and weighed all the seeds from each fruit separately for all fruits produced per plant in 2007. In 2008, I determined weight per seed for each female plant by weighing exactly 100 seeds from each of three randomly selected fruits and then weighing all seeds from each of those fruits separately to estimate total number of seeds produced. For both years, I estimated total female fecundity by multiplying the total fruits produced by the estimated number of seeds per fruit. Fruit set, seeds per fruit,
and total fecundity were all used as estimates of female fitness. However, I was unable to assess differences among hermaphrodites for female fitness in 2007, because many of the hermaphrodites did not enter pistillate phase during the 5-day experiment. To rectify this, in 2008 I marked hermaphrodite flowers with three colors of paint: one for the day of anthesis, one for the day the flower became pistillate, and one for the last for the day of senescence, and plants were observed for eight days.

I analyzed female fitness for each measurement factor (fruit set, weight per seed, estimated number of seeds per fruit, weight of all seeds per fruit, and total number of seeds produced per female plant) using a series of 3-way ANOVAs with array size, sex ratio, and individual plant sex, as well as the interactions of these factors, as fixed effects. Population size and sex ratio were nested within pollination treatment (either open-pollinated or supplementally-pollinated). The model also included the interaction between plant sex and pollination treatment. The model accounted for random variation between years and the random assignment of individual plants to arrays nested in size and sex ratio.

_Estimating male fitness with microsatellites_

Fruit and seed production are estimates of female fitness, which are straightforward factors to measure. I assessed male fitness of hermaphrodites by estimating paternity of the offspring produced in the 2007 experimental arrays. Microsatellites are useful markers for assessing levels of paternity because of their hypervariability and large number of alleles per locus (Teixeira and Bernasconi 2007).
I genotyped a total 300 offspring from each experimental array, by sampling offspring proportionately based on relative contribution of each mother to the total seed crop in the 2007 experimental arrays, where more offspring were genotyped from mothers with higher fecundity. In this way, I was able to assess differences in male fitness in relation to the total number of offspring produced per array. I genotyped all of the parents used in the 2007 experimental arrays, so the entire pool of female and hermaphrodite genotypes were known.

I collected tissue from seedlings from the 2007 arrays into 96-well plates, performed a modified CTAB DNA extraction (http://openwetware.org/wiki/Mimulus:DNA_Extraction_Protocol), and amplified microsatellites with multiplexed labeled primers using polymerase chain reaction (PCR) to amplify a specific fragment of DNA using four primer pairs (Table 2.1). Locus B2aC7, first developed for *L. boykinii* by R.R. Moreno et al. (unpub. ms) at the University of North Carolina at Greensboro, was previously found to amplify well and show allelic polymorphism across populations. Other loci (Lob6, Lob9, and LobTri1) were developed by C. Newton of ATG Genetics (Vancouver, BC, Canada). Each 12 uL PCR reaction contained 2 uL 5x Taq polymerase buffer, 1 uL 2.5 uM dNTPs, 0.8 uL 25uM MgCl2, 0.15 uL of 10uM labeled forward primers, 0.15 uL of 10uM unlabeled reverse primers, and 0.1 uL Taq polymerase. I amplified DNA fragments according to ATG genetics specification on BioRad iCycler thermal cyclers with denaturation at 95° C for 3 minutes, followed by 40 cycles with a denaturation at 95° C for 30 seconds, annealing at 63°C for 30 seconds, and elongation at 72° C for 30 seconds, followed by a
7 minute elongation at 72 °C (Table 2.1). PCR products were checked for accuracy with 1% agarose gel electrophoresis and stored at 4°C until I determined each genotype using a Beckman Coulter CEQ 800 (Fullerton, CA, USA), using fluorescent-labeled primers. Fragment analysis of the DNA consisted of scoring and sizing the large peaks (signal strength of at least 3,000 units), determining individual genotypes, and determining which alleles were shared and which differed among individuals between and within populations.

Genetic data were analyzed using PopGen32 (Raymond and Rousset 1995) to determine allele frequencies, or the proportion of incidence of each allele within the population, and effective number of alleles, or the number of equally frequent alleles that would produce the same level of heterozygosity in the population. I also analyzed genetic data using MLTR (Ritland 2002) to determine the outcrossing rate (t estimate), the partition of the heterozygosity of the individual to the total population (Ft), and the correlation of paternity, as the extent to which siblings share a father (parental F estimate) within and among arrays. Paternity was assigned using Cervus software (www.fieldgenetics.com/pages/aboutCervus_Overview.jsp) to determine most likely male parent with known female parent (Kalinowski et al. 2007). Cervus first determined allele frequencies based on the parental population, then simulated the number of offspring that could be assigned male parents at 95%, 80%, and most likely confidence levels, accounting for various aspects of the dataset including number of female and hermaphrodite plants, level of inbreeding, and the number of loci typed and mistyped. In cases where more than one father was determined to be the most likely parent, each was
given a fraction of that offspring to estimate the total number of offspring likely sired by each hermaphrodite plant. I compared the array of the maternal plant with the array of the most likely hermaphrodite parent plant, the possibility of a hermaphrodite selfing to produce offspring, and the level of visitation to hermaphrodites to determine if hermaphrodites with higher visitation sired more offspring.

*Estimating hermaphrodite total fecundity: seed production plus paternity*

To determine the total fecundity of hermaphrodites in the arrays, I added the total number of seeds produced plus the total number of offspring that each hermaphrodite most likely sired. In this way, I was able to estimate the total fitness of each individual in this experiment. I analyzed the effect of visitation on the total fitness of the hermaphrodites using a one-way ANOVA. I also determined the effect of array sex ratio and size on seed production plus number of offspring sired in a two-way ANOVA split by array, accounting for the pollination treatments as well. Additionally, I performed a Tukey’s HSD to determine which mean values were significantly different from each other.

**Results**

*Pollinator visitation is affected by array size, sex ratio, and individual plant sex*

Pollinators preferred hermaphrodites, but the strength of this preference differed by array size. Using the categorical visitation data, a higher proportion of hermaphrodite
plants were visited relative to females \((df: 1, \chi^2: 14.551, P: 0.0001)\), and visited a higher proportion of plants of both sexes where hermaphrodites were abundant compared to the high-female arrays \((df: 1, \chi^2: 11.598; P: 0.0007)\). Fewer females were visited than hermaphrodites in the small arrays, yet I found no difference between the sexes in the large arrays \((df: 1, \chi^2: 4.7994, P: 0.0285)\).

Because the visitation data collected in 2007 were not duplicated with each array at each location, and there were strong effects of array location on visitation \((df: 3; F: 62.90; P: <0.0001)\), I analyzed visitation rate data separately by year. In 2007, pollinator visitation was significantly affected by plant sex, where females received fewer visits per flower compared to hermaphrodites (Table 2.2). There were no other significant treatment effects on visitation rate.

Pollinators preferred large and low female arrays. In 2008, I found that large arrays received higher visitation than small (Table 2.2). Low female (10%) arrays also received significantly higher visitation than high female arrays (Table 2.2). I also found that hermaphrodites had higher visitation in the low female arrays (Table 2.2), while there was no difference between sex ratio treatments in visitation rates to females. Finally, I found that a complicated relationship exists between all three factors’ effects on visitation (Table 2.2, Figure 2.2). All other factors and interactions in the models were non-significant.

*Female fecundity affected by array sex ratio*

Total flower number throughout the experiment appeared to influence the
proportion of flowers that produced fruits. The mean number of flowers per plant that became pistillate during the experiment was significantly higher in female than hermaphrodite plants \( (df: 1; F: 11.25; P: 0.0009) \), and in large arrays than small arrays \( (df: 1; F: 5.53; P: 0.0191; \) Figure 2.3a). This trend in flower number could affect fruit set and total seed production differently in the supplementally pollinated and open-pollinated arrays. The mean proportion of flowers that produced fruits was significantly higher for female than hermaphrodite plants \( (df: 1; F: 13.12; P: 0.0003; \) Figure 2.3b); no other factors in my model were significant with respect to fruit set.

There appeared to be a trade-off between fruit set and the number of seeds per fruit because the significant effects on fruit set and the number of seeds per fruit are diminished when all seeds from all fruits per plant are bulked (Figure 2.3d). I found little variation in the mean weight per seed, where individuals in the low female arrays produced marginally significantly heavier fruits overall \( (df: 1, F: 3.72, P: 0.0543) \). The number of seeds per fruit was significantly higher for individuals in the low female sex ratio than high female arrays \( (df: 1; F: 4.64; P: 0.0319; \) Figure 2.3c). Finally, I found support for pollen limitation in the open-pollinated arrays because the total number of seeds produced was marginally significantly higher in individuals in the supplemental pollen treatment than those in the open pollen treatment \( (df: 1; F: 3.50; P: 0.0622) \). I also found that hermaphrodites produced marginally significantly more seeds in the supplementally pollinated treatment than in the open pollinated treatment, while females showed no difference \( (df: 1, F: 2.91; P: 0.0885; \) Figure 2.3d). All other factors were non-significant.
Assessment of male fitness

The different estimates of male fitness of hermaphrodites appeared to be influenced by array size and sex ratio. Using PopGen32 to analyze the microsatellite data, I found the allele frequencies for each locus (Table 2.3). I also found that observed levels of heterozygosity were lower than the expected levels for Lob9, and higher than the expected for the other loci (Table 2.3). I found that there were high levels of outcrossing within and among the arrays based on the microsatellite analysis using Ritland’s MLTR, where multilocus $t$ estimates ranged from 0.868 to 1.2, but were not significantly different from 1 in any of the arrays. The overall $F$ estimate, or the single locus inbreeding coefficient of female parents was -0.074, which was not significantly different from 0. The $F_{IT}$ value, or the partition of the heterozygosity of individuals compared to the total population was not different from 0, suggesting that individuals were highly heterozygous and likely outcrossed (Table 2.3).

After assigning paternity to all offspring produced by the 2007 arrays with most likely assignment using CERVUS, I found that hermaphrodites in the supplementally pollinated array sired marginally significantly fewer offspring from experimental arrays than did hermaphrodites in the open-pollinated arrays ($df$: 1; $F$: 3.67; $P$: 0.0577; Figure 2.4a). I found no significant effect of array size or sex ratio, nor the interaction of these two factors, or of visitation on the variation in number of offspring each father most likely sired. There does appear to be a biologically significant effect of sex ratio on the number of offspring per father, where hermaphrodites produced more offspring when they were scarce ($df$: 1; $F$: 2.38; $P$: 0.1253; Figure 2.4a).
In assessing the total number of possible fathers to each female plant, I found that female plants in the small arrays had significantly higher number of sires to their offspring than plants in the large arrays (\(df:1; F: 4.35; P: 0.0406; \) Figure 2.4b). I found no significant effect of array sex ratio, nor an interaction between population size and sex ratio.

The proportion of female plants that produced at least some full siblings was significantly higher in the small arrays (\(df: 1; F: 10.79; P: 0.0011\)). I also found a significant interaction between array size and sex ratio, where mothers in the low female arrays produced fewer full sibling pairs than mothers in the high female arrays, with respect to size of the arrays (\(df: 1; F: 11.05; P: 0.0009; \) Figure 2.4c). Finally, I found that the mean distance of pollen flow in this experiment, based on the array from which the most likely father was located, ranged from approximately 58 meters (large, low female array) to 136 meters (small, high female array; Figure 2.5).

**Relative fitness of the sexes: total fitness of hermaphrodites vs. females**

Because I only obtained paternity data for offspring produced in 2007, I was only able to compare relative fitness of females and hermaphrodites for that year. Hermaphrodites each sired a similar number of offspring (Fig 2.6a), though hermaphrodites in the large arrays and the supplementally pollinated array parented more offspring as mothers vs. fathers, while hermaphrodites in the small arrays sired more seeds than they produced (\(df: 4; F: 2.73; P: 0.0319\); Tukey’s HSD test: \(P: 0.05\); Figure 2.6a). This suggests that hermaphrodites in the large arrays were female in terms of
Within each array, the relative fertility of the sexes, as measured by the proportion of the total number of offspring produced, was not significantly different between the sexes in most of the arrays. However, in the large, highly female array, hermaphrodites produced a higher number of the offspring than females ($df: 1; F: 17.61; P: <0.0001$; Figure 2.6b).

Discussion

Increased pollination success in large populations does not appear to be correlated with individual fertility. In terms of experimental population size, larger arrays received higher visitation than small. This result supports my hypothesis that large populations are more attractive to pollinators than small due to the larger number of plants. Even so, higher visitation did not lead to an increase in the number of offspring produced either maternally or paternally by plants in the large populations.

Population sex ratio appears to influence pollinator behavior, but those effects do not explain patterns of reproductive success. Low female arrays received higher visitation than high female arrays, supporting my prediction that pollinators would prefer populations high in hermaphrodites because of their dual rewards. This is similar to a study that showed pollinator selection for highly hermaphroditic patches within a population of gynodioecious Fragaria virginiana (Ashman et al. 2000). While sex ratio of the populations influenced pollinator visitation, it did not appear to affect maternal or
paternal fertility, suggesting pollinator sex ratio preferences may not alter reproductive success.

Individual plant sex within the experimental arrays appears to influence pollinator behavior but not plant fertility. My results indicate that pollinators preferred hermaphrodites over females, regardless of array size or sex ratio, which supported the hypothesis that pollinators prefer hermaphrodites due to their dual rewards (nectar and pollen). This is similar to previous studies that showed pollinator preference for pollen-presenting rather than stigma-presenting flowers of gynodioecious *Knautia arvensis* (Larsson 2005), and higher visitation to hermaphrodites than females in arrays of varying sex ratios of gynodioecious *Kallstroemia grandiflora* (Zygophyllace; Cuevas et al 2008). While pollinators preferred hermaphrodites, there appeared to be no effect of individual plant sex on reproductive output. My results indicate no evidence of a trade-off between seed size and seed number, and no evidence of higher total number of seeds produced by female plants, as predicted. Therefore, my results are not consistent with the reproductive compensation hypothesis for female maintenance in gynodioecious populations of *L. siphilitica*. Many studies have shown higher seed production by females than hermaphrodites (Shykoff 1988; Sakai and Weller 1991; Agren and Willson 1991; Ashman 1992; Asikainen and Mutikainen 2003; Case and Ashman 2007; Lopez-Villavicencio et al. 2007). The results from this study may be different from others since hermaphrodites in this study received higher levels of pollinator visitation than females, which may have contributed to increased maternal reproduction by those hermaphrodites, diminishing any effects of female reproductive compensation that may normally occur.
To address the interactions between array size, array sex ratio, and individual plant sex, I found that these did interact to influence pollinator behavior and fertility. First, the interaction between array sex ratio by individual plant sex, where hermaphrodites received higher visitation when they were common, further supports the pollinator preference prediction discussed earlier. Next, the three-way interaction among array size, sex ratio, and individual plant sex suggests that a complicated association with populations and pollinators exists. These effects on pollinator behavior, with preference for hermaphrodites as well as large and low female populations, may help to explain the trend I see between population sex ratio and size in *L. siphilitica*. If pollinators cause hermaphrodite individuals in the large and low female populations to produce and sire more offspring, then the population mean fitness may be higher, and therefore may cause the population to increase in size. This may explain why large, high female populations of *L. siphilitica* have not been found.

The overall maternal fitness of hermaphrodites in the 2007 arrays was low compared to hermaphrodites in 2008 (data not shown). One possible explanation is that fewer hermaphrodite flowers entered the pistillate phase during the five-day observation period of the 2007 experiment. Therefore, fewer fruits were produced by hermaphrodites in 2007. Because I observed pollinator visitation as well as phase transition of flowers on all plants in 2008, I was able to more accurately assess the effects of population size, sex ratio, and maternal sex on hermaphrodite maternal reproductive success.

Hermaphrodite male fertility could have been measured differently in this experiment. However, the offspring sampling technique I used maximized the ability to
determine levels of multiple paternity. Sampling even numbers of offspring by maternal plant would allow better approximation of siring success. The results I found, suggesting that individuals in the large arrays showed lower multiple paternity and less correlated paternity than individuals in the small arrays, is likely due to the sampling design for this study. Because I genotyped approximately 300 offspring per array, a higher proportion of offspring from each mother in the small arrays were sampled than from mothers in the large arrays, i.e. few mothers with many genotyped offspring will produce the same total number as many mothers with few offspring genotyped for each. Nevertheless, genetic analysis suggests that there is a high level of outcrossing occurring within and among the arrays. The populations may not have appeared to be distinct to the pollinators, since pollen flow between the four corners of the HAERF appeared to be high. Definitive paternity could be assigned to each offspring at a 95% confidence level if an LOD score was at or below the simulated critical LOD, and these genetic data suggest that I did not have enough power to determine the single best father for each offspring produced. However, I was able to determine the most likely male parent for all offspring genotyped for at least two of the four microsatellite loci at much lower confidence levels, and found high levels of multiple paternity within mothers as well as high outcrossing among the populations. I also found no significant effect of array sex ratio or size on the number of offspring sired per hermaphrodite, regardless of its level of visitation.

The relative fitness of the sexes for the 2007 arrays showed that hermaphrodites in the large, high female array were more fit than females in that array, while no significant differences existed between the sexes in the other arrays. However, I did find
a non-significant, yet possibly biologically important trend of higher offspring production by the more numerous sex in the experimental arrays. This would suggest that the sex ratio of natural populations would remain consistent across years. Additionally, I found that there was no trade-off between female and male fitness in hermaphrodites in the arrays. Most hermaphrodites in the large arrays and the supplementally-pollinated array produced the majority of their offspring through female fitness, while hermaphrodites in the small arrays produced most of their offspring through siring. This suggests that hermaphrodites in large populations are functionally female in their reproduction. Therefore, any females in a large population would likely suffer from pollen limitation and produce few offspring. Furthermore, if hermaphrodites produce mainly hermaphrodite offspring due to the sex determination system, the sex ratio of the next generation would become more hermaphrodite biased. Therefore, it is unlikely that large, highly female populations would be stable, and this may help to explain why I do not find large, highly female populations of *L. siphilitica* in nature.

These results suggest a complicated relationship between population demographics and pollinator behavior. Pollinators may be the mechanism behind variation in population size and sex ratio through different effects on the sexes, leading to differences in the relative fitness of the sexes. Pollinator preference may contribute to population size through increased selection for plants in large populations, though these effects may be negated through effects of seed production (Ashman and Diefenderfer 2001; Cuevas et al. 2008). My results show that pollinator selection affects and is affected by differences in population sex ratio and size and that these effects could help
explain the sexual system patterns observed in gynodioecious species such as *L. siphilitica*. 
Table 2.1. *Lobelia siphilitica* microsatellite primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Repeat</th>
<th>Primer Sequences (5’-3’)</th>
<th>Number of alleles</th>
<th>Allele size range (bp)</th>
</tr>
</thead>
</table>
| Lob6   | (GTAT)$_{13}$ | F: TTGGTTGTACACTCACTGAGCA  
R: AAATGTGTCATGCTTTGA | 9 | 126-158 |
| LobTri1| (CAA)$_{11}$  | F: TATGCAATTTAGCCCCTGTT  
R: CCCCAAGTGATTGGGACTAA | 18 | 160-211 |
| Lob9   | (CATA)$_{10}$ | F: AGGGGCAAGTTGCTGTTTT   
R: CCCATTGTCAACCTCTTCGT | 8 | 174-202 |
| B2aC7  | (TC)$_{10}$  | F: CTCACCTCCGGAGAAAAGT  
R: CCATTCCTTTTGAACCTTCA | 11 | 198-218 |
Table 2.2. ANOVA Results for main effects on visitation in 2007 and 2008. The number beside main effect represents degrees of freedom, the top number under the visits/flower/hour represents the $F$ value, and below that is the $P$ value.

<table>
<thead>
<tr>
<th>ANOVA model</th>
<th>Visits/flower/hour 2007</th>
<th>Visits/flower/hour 2008</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Fixed effects)</td>
<td>(df denom= 1110)</td>
<td>(df denom = 1884)</td>
</tr>
<tr>
<td>Array size (df = 1)</td>
<td>1.11</td>
<td>5.67</td>
</tr>
<tr>
<td></td>
<td>0.2930</td>
<td>0.0174</td>
</tr>
<tr>
<td>Array sex ratio (df = 1)</td>
<td>1.43</td>
<td>20.43</td>
</tr>
<tr>
<td></td>
<td>0.2315</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Individual plant sex (df = 1)</td>
<td>3.85</td>
<td>7.92</td>
</tr>
<tr>
<td></td>
<td>0.0500</td>
<td>0.0050</td>
</tr>
<tr>
<td>Size x Sex ratio x sex (df = 1)</td>
<td>2.01</td>
<td>5.37</td>
</tr>
<tr>
<td></td>
<td>0.1562</td>
<td>0.0241</td>
</tr>
</tbody>
</table>
Table 2.3. Descriptive statistics for the four microsatellite loci used in this experiment.

Sample size (number of individuals genotyped), actual number of alleles, effective number of alleles, allele frequency range, observed and expected heterozygosity, and Fit, the level of inbreeding of an individual compared to within the total population, among experimental arrays.

<table>
<thead>
<tr>
<th>Locus</th>
<th>N</th>
<th>Actual number of alleles</th>
<th>Effective number of alleles</th>
<th>Allele Freq. Range</th>
<th>Obs het</th>
<th>Exp het</th>
<th>Fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lob6</td>
<td>1390</td>
<td>9</td>
<td>3.5629</td>
<td>0.0022 - 0.2957</td>
<td>0.5856</td>
<td>0.7198</td>
<td>0.1809</td>
</tr>
<tr>
<td>LobTri1</td>
<td>1330</td>
<td>18</td>
<td>5.3752</td>
<td>0.0015 - 0.2805</td>
<td>0.7233</td>
<td>0.8146</td>
<td>0.1043</td>
</tr>
<tr>
<td>Lob9</td>
<td>1096</td>
<td>8</td>
<td>3.865</td>
<td>0.0036 - 0.3385</td>
<td>0.8449</td>
<td>0.7419</td>
<td>-0.1379</td>
</tr>
<tr>
<td>B2aC7</td>
<td>1210</td>
<td>11</td>
<td>5.0157</td>
<td>0.0008 - 0.3298</td>
<td>0.7008</td>
<td>0.8013</td>
<td>0.1281</td>
</tr>
<tr>
<td>Mean</td>
<td>1256</td>
<td>11.5</td>
<td>4.4547</td>
<td></td>
<td>0.7137</td>
<td>0.7694</td>
<td>0.07</td>
</tr>
<tr>
<td>St. Dev</td>
<td></td>
<td>4.717</td>
<td>0.8766</td>
<td></td>
<td>0.1063</td>
<td>0.0457</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1 Map of Herrick Aquatic Ecology Research Facility (HAERF) at Kent State University, Kent, OH containing experimental populations of *L. siphilitica*. White circles represent locations of open-pollinated populations, and the orange circle represents the location of the supplementally pollinated population.
Figure 2.2. Mean visits per flower per hour to female and hermaphrodite plants in 2008 experimental pollination arrays (least squares means ± 1 SE). Visitation in 2008 differed significantly by array size, sex ratio, the interaction between array sex ratio and plant sex, and the 3-way interaction. Numbers in each bar indicate the number of plants sampled, and bars sharing the same letter are not significantly different.
Figure 2.3. Average female fecundity from 2007 and 2008 combined data: (a) number of pistillate flowers per plant (total number of flowers per female and number of flowers that entered pistillate phase for hermaphrodites), (b) proportion of fruits per pistillate flower, (c) estimated number of seeds per fruit, and (d) estimated total number of seeds produced per mom. Bars indicate means ±1 SE. Numbers in each bar indicate the number of plants sampled, and bars sharing the same letter are not significantly different.
Figure 2.4. Paternity analysis. (a) *Male siring success*: the number of offspring for which each hermaphrodite was chosen as the most likely father; (b) *Multiple paternity*: the total number of most likely fathers assigned to the offspring of each female plant; and (c) *Correlated paternity*: the mean proportion of mothers with at least one set of full sibling offspring. Numbers in each bar indicate the number of plants sampled.
Figure 2.5. Pollen flow between arrays. The y-axis indicates the number of offspring genotyped for each of the five experimental arrays (grouped along the x-axis); the color of the bars indicates the location of the sire for offspring produced. Asterisks denote offspring produced by hermaphrodites within the same array as the female plant.
Figure 2.6. Relative fertility of the sexes in 2007 experimental arrays. (a) *Functional gender*: Proportion of fitness gained through male vs. female function for hermaphrodite individuals in experimental populations of *L. siphilitica*. Male fitness was measured as the number of offspring sired, while female fitness was measured as total seed production. (b) *Relative fertility within each array*: Total offspring produced in each array by individual plant. Individuals in the supplementally pollinated array produced a higher number of offspring than individuals in the open pollinated arrays, and hermaphrodites in the large, high female array produced more offspring than females in that array. Numbers in each bar indicate the number of plants sampled, and bars sharing the same letter are not significantly different.
a. 

Supplementally pollinated plants

<table>
<thead>
<tr>
<th>Experimental Population Size</th>
<th>Proportion Offspring Produced Through Male Fitness</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% female 80% female Small (30 plants)</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>10% female 80% female Large (90 plants)</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Supplementally pollinated plants</td>
<td>0.8 ± 0.3</td>
</tr>
</tbody>
</table>

b. 

Female plants  
Hermaphrodite plants

<table>
<thead>
<tr>
<th>Experimental Population Size</th>
<th>Total Number of Offspring Produced (Seeds + Sired)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% female 80% female Small (30 plants)</td>
<td>5000 ± 1000</td>
</tr>
<tr>
<td>10% female 80% female Large (90 plants)</td>
<td>15000 ± 2000</td>
</tr>
<tr>
<td>Supplementally pollinated plants</td>
<td>30000 ± 5000</td>
</tr>
</tbody>
</table>
Chapter 3

Population size, but not inbreeding or population sex ratio, affect germination in natural and experimental populations of gynodioecious Lobelia siphilitica

Abstract

Population sex ratio, population size, and inbreeding may affect the size of a population because of their effects on fecundity and recruitment. This work describes experiments with Lobelia siphilitica on how individual plant fitness is influenced by the proportion of female plants (sex ratio) as well as the number of plants (size) within a population, and how varying degrees of inbreeding influences the relative fitness of the sexes. I measured germination in seeds collected in natural and experimental populations, and in crosses of varying degrees of inbreeding. I expected to find a positive relationship between proportion germinated seeds and population sex ratio and size, and a negative relationship between proportion germinated seeds and inbreeding. I found that seeds collected from small natural populations had higher germination than those in large, and no variation in germination was explained by population sex ratio. In seeds collected in experimental populations of low (10%) and high (80%) female sex ratio and small (30) and large (90) population size, I found no effect of population sex ratio, size, or plant sex on proportion germinated seeds. In the manipulated crosses, I found no effect of inbreeding on germination, though females consistently produced heavier seeds.
with higher germination rates compared to hermaphrodites. This work suggests that germination may be more influenced by population size and maternal plant sex than by inbreeding or population sex ratio.

Introduction

Quantifying relative fitness of the sexes is an important factor in understanding variation in population sex ratios (Sakai et al. 1997; Byers et al. 2005; Case and Ashman 2007). Females may be maintained in gynodioecious populations if they achieve higher fitness relative to hermaphrodites (McCauley and Brock 1998; Nilsson and Ågren 2006). It is my goal to understand how the relative fitness of the sexes is influenced by variation in population sex ratio and size.

Population sex ratio varies in many gynodioecious species, ranging from 0% to 100% female (Godley 1955; Connor 1963; Ganders 1978; Dommée et al. 1978; Webb 1979; Krophne et al. 1980; Delph 1990; Barrett 1992; McCauley et al. 2000; Caruso and Case 2007). However, the relationship between population sex ratio and size is not well understood. This relationship has been shown to be negative for some gynodioecious species (Byers et al. 2005; Nilsson and Ågren 2006; Caruso and Case 2007), where larger populations have a lower proportion of female plants. Furthermore, the relationship between population sex ratio and size could affect the relative fitness of the sexes and their parental contributions to the recruitment of new individuals into the population (Byers et al. 2005; Nilsson and Ågren 2006; Alonzo et al. 2007; Caruso and Case 2007). All of these studies show correlation between population sex ratio and size, but not
causation.

It is unclear whether population sex ratio causes variation in population size, or vice versa. To determine the direction of this relationship, one can measure the relative fitness of the sexes in populations of varying sizes and sex ratios. One measurement of fitness is the number of offspring recruited into the population, and while germination does not inevitably lead to recruitment, it is necessary for new individuals to join a population, and can be used to estimate maximum rates of recruitment. Therefore this study focuses on this estimate of fitness, since population size is affected by recruitment of new individuals to the population, and population sex ratio is affected by the number of individuals of each sex that survive and reproduce (Delph and Mutikainen 2003).

One important factor to examine is how fecundity and recruitment affect population sex ratio. It is understood that females are maintained in gynodioecious populations through reproductive compensation and/or outcrossing advantage (reviewed in Charlesworth 1999). According to the reproductive compensation hypothesis, females have a higher relative fitness than hermaphrodites through better acquisition and direction of resources (Dawson and Geber 1999). The second hypothesis states that females produce better quality offspring because the offspring are the result of obligate outcrossing, while the offspring of hermaphrodites could be self-fertilized (reviewed by Sakai and Weller 1999). Differences between outcrossing levels and resource allocation in females and hermaphrodites may explain why populations vary, since females can only outcross while hermaphrodites can either outcross or self-fertilize. Self-fertilization can lead to the accumulation of deleterious alleles within the population and reduce overall
viability and fitness. Self-fertilization can reduce fecundity as well; seeds of out-crossed, sib-crossed, and self-fertilized hermaphrodites had lower germination rate and percent survival than out-crossed and sib-crossed *Silene acaulis* females (Keller and Schwaegerle 2006).

Germination success may be affected by female sex or levels of inbreeding, and consequently affect sex ratios in gynodioecious populations. Female fitness is usually measured in terms of seed production, through this does not account for differences in germination between seeds produced by female vs. hermaphrodite mothers. If females produce a higher proportion of viable seeds than hermaphrodites, females would have higher relative female fitness, and would be maintained within a gynodioecious population, thus compensating for their lack of male fitness. Studies have found that self-fertilized *Silene vulgaris* hermaphrodites not only produce offspring with lower germination and survivorship (Emery and McCauley 2002), but also produce a higher number of female offspring than outcrossed hermaphrodites or females (Bailey and McCauley 2005). The levels of self-fertilization of hermaphrodites within a population have also been shown to increase as the proportion of female plants increase (Sun and Ganders 1986), which could further affect population sex ratio through the decreased fitness of hermaphrodites as mothers. Future population sex ratios would be influenced by the current sex ratio of each population, since females in gynodioecious species are more likely to produce female offspring, based on the cyto-nuclear interaction that causes male-sterility (Couvet et al. 1998). Also, the progeny sex ratio of hermaphrodite mothers has been shown to be significantly affected by population size, while progeny sex ratio
from female mothers was significantly affected by the population sex ratio in *Lobelia spicata* (Byers et al. 2005).

The goal of this study was to understand how maternal fitness of individuals is influenced by the size and sex ratio of populations, and how the relative fertility of the sexes is influenced by varying degrees of inbreeding. In terms of population size, large populations should produce better seeds because they maintain higher visitation rates than small populations (Chapter 2). In terms of population sex ratio, highly female populations should produce better seeds because females are obligate outcrossers. Females must produce more or better quality seeds than hermaphrodites to be maintained within a population because hermaphrodites can reproduce through ovules and pollen while females can only reproduce through ovules (Dorken and Mitchard 2008).

Inbreeding may affect germination through inbreeding depression, which could influence the relationship between population sex ratio and size. The goal for work presented here was to investigate whether population size, sex ratio, and levels of inbreeding affect germination success in natural and experimental populations of *Lobelia siphilitica*.

Previous work with *L. siphilitica* showed a trend of increased effects of inbreeding depression in hermaphrodite lineages when measuring germination and later growth stages (Mutikainen and Delph 1998). Therefore, I focused on the effects of varying degrees of inbreeding on germination levels by performing self, sib, and cross pollinations among individuals from two natural *L. siphilitica* populations. I hypothesized that hermaphrodites would produce seeds with lower germination than females, regardless of cross type, and that seeds of self-fertilized hermaphrodites would
show lower rates of germination compared to hermaphrodites for the other two crosses. Under cyto-nuclear sex determination in *L. siphilitica* (Dudle et al. 2000), the sex of the maternal plant influences the sex of the offspring produced. Therefore, I assessed the sex ratio of the offspring from three datasets (from natural populations, from greenhouse hand-pollinated crosses, and from the experimental populations varying in sex ratio and size) to determine if population sex ratio, size, or inbreeding affect the sex ratio of the next generation. If recruitment, measured by experimental germination, was higher for offspring of one sex over the other, the sex ratio of the population would change over time. The underlying dynamics of the relative fitness of the sexes could explain why populations of *L. siphilitica* vary in size from a few plants to several hundred plants and in sex ratio from 0 to nearly 100% female (Caruso and Case 2007). The levels of seed production, recruitment, and survivorship could provide insight into how relative fitness of the sexes directly affects population sex ratio and size, which will help to further understand *L. siphilitica* as a gynodioecious plant.

Methods

*Study species: Lobelia siphilitica*

*Lobelia siphilitica* is a gynodioecious perennial herb that grows in moist soils along wet meadows and ditches in eastern North America from southern Canada through the Appalachians to North Carolina (Johnston 1992), and populations are readily found in Ohio and West Virginia during flowering season. Most populations are small, with less
than 100 individuals, and contain more females on average than large populations, which typically have between 0-15% females. Small populations tend to show much higher variability in sex ratio than large populations as well (Caruso and Case, 2007; Figure 1). Plants can produce up to 100 flowers during the flowering season from late July through September, and each fruit produced during the fruiting season in October and November can contain up to 1000 seeds (Johnston 1992).

**Germination of seeds collected from natural populations**

In fall 2006, I located 14 populations of *Lobelia siphilitica* containing at least ten flowering individuals in Ohio and West Virginia. I counted and determined the sex of every flowering individual in all populations to calculate the sex ratio and size of each population. Population size (number of individuals) and sex ratio (proportion female) were recorded in August and September 2006 ($N=14$ populations per year; Table 3.1). I returned to those populations in fall of 2007 and 2008 to assess population traits. During the fall 2006 fruiting season, I collected three fruits from each of 15 randomly selected maternal plants of unknown sex from each population into paper envelopes, allowed them to air dry, and stored the seeds at 4°C.

To break seed dormancy and stimulate germination, I cold-stratified 300 seeds per female plant by placing them in Petri dishes between two layers of filter paper moistened with distilled water at 4°C for 12 weeks. Seeds were then removed from cold stratification and placed in an environmental growth chamber at 24°C with 12 hour light/dark cycles in March and April 2007. I put seeds on two layers of wet filter paper in Petri
dishes and maintained wetness with distilled water daily. I scored the number of new germinants in each batch every day for at least 30 days to assess proportion of germinated seeds. Dishes with new germinants at the 30-day cut-off were allowed to continue germinating until there were no new germinants for seven consecutive days, and I assumed that any seeds remaining ungerminated after at least 30 days were inviable. I analyzed the proportion of seeds germinated per maternal plant using a 1-way ANOVA with population, and then determined correlations between proportion germinated seeds with population size and sex ratio in JMP 7 (SAS Institute 2007). At least 60 seedlings from each maternal plant were transplanted into growth trays with germination mix, and grown to the rosette stage in a growth chamber for one month. Randomly selected individuals from two populations were grown to flowering in Cone-tainer Rocket pots (Stuewe and Sons, Inc, Tangent, OR, USA) in a greenhouse at Kent State University, Kent, OH, USA with standing water for the following experiment, and the sex of each plant was determined by assessing the sex of at least two flowers per plant. Sex ratio was calculated as the proportion of female plants.

Effects of Inbreeding on Fecundity, Germination, and Sex Ratio

I grew offspring from two female-biased populations, Bend View Metropark (BV; 56% female, Lucas County, OH) and Kaylor Road (KR; 37% female, Tuscarawas County, OH), in a greenhouse at Kent State University. I randomly selected three hermaphrodites and three females from each of two maternal families from each population to serve as parents in a series of controlled crosses ($N=12$ plants x two
populations = 24 female parents). Perfect flowers of hermaphrodites were emasculated and allowed become receptive for approximately 24 hours before pollen was applied. I generated three sets of offspring per female parent varying in the degree of inbreeding: sib-cross, within-population outcross, and self-fertilization. I hand-pollinated at total of six flowers per plant (total of 144 crosses). Each female was pollinated by three hermaphrodite maternal sibs (“sib-cross”), and with the three hermaphrodites from the other maternal family from the same population (“out-cross”). Hermaphrodites were self-pollinated with pollen from another flower on the same plant (“self-cross”), sib-crossed with the two hermaphrodite maternal sibs, and outcrossed with pollen from the three hermaphrodites from the other maternal family. I could not perform between-population crosses to measure effects of biparental inbreeding because there was no overlap in flowering time between the BV and KR populations. Each pollination was repeated with new flowers until at least one fruit was successfully produced, or until the cross had failed three times.

After approximately one month, I collected all mature fruits into paper envelopes. I counted and weighed 100 seeds from each fruit to determine average weight per seed, then estimated the number of seeds per fruit based on the bulk weight of all seeds from each fruit. In winter of 2007, I cold-stratified 100 seeds per cross and scored germination and sex ratio as described in the previous experiment. I analyzed fecundity measurements using a nested mixed-model ANOVA with maternal sex and cross type nested within maternal sex as the main effects. I also accounted for effects of the two populations, female and hermaphrodite families, and individual female and
hermaphrodite plants as random effects.

*Effects of experimental population sex ratio and size on germination and offspring sex ratio*

To analyze the effects of maternal sex and experimental population size and sex ratio on germination, I measured the proportion of germinated seeds from each maternal plant from the experimental populations described in Chapter 2. These included 4 open-pollinated experimental arrays and one supplementally pollinated array of plants.

I sampled 200 seeds from each maternal plant (both sexes) after estimating total seed production and number of seeds per fruit. Seeds were sampled proportionately among fruits, such that more seeds were selected from fruits containing more seeds. Seeds were cold treated, placed on filter paper moistened with distilled water and enclosed in plastic Petri dishes placed in a growth chamber set at 24°C with 12 hour light/dark cycles and watered daily. Dishes were scored for new germination each day for a minimum of 30 days after initial germination, as described above.

I analyzed both the rate and the proportion of germination of seeds collected from mothers of experimental populations with a 3-way ANOVA, where a full factorial cross of experimental population size category, sex ratio category, and individual plant sex were the main effects. The model also accounted for variation in the number of visits each mother received, the maternal family of the plant, and the pollination treatment of the maternal plant (open-pollinated or supplementally-pollinated).

Germinated seeds were transplanted to plug trays to establish root systems, were
transplanted into Cone-tainer Rocket pots after approximately 1 month, and were moved to a greenhouse where they began flowering after about three months of growth. The sex ratio of the offspring produced in the experimental populations was recorded based on the sex of at least two flowers per offspring. I analyzed the sex ratio of the offspring produced with a split plot 3-way ANOVA with a full factorial cross of experimental population size, sex ratio, and female plant sex as the main effects, accounting for the pollination treatment in all variables dealing with array size or sex ratio, and performing a split plot analysis assigning each individual to its array, nested within array sex ratio and size, as a random variable.

Results

Germination of seeds collected from natural populations

Germination timing in this experiment appeared to be consistent across populations, with most seeds germinating within the first three days and a steep decline in number of new germinants per day after that. The 14 natural populations were significantly different from each other in terms of proportion germinated seeds (df=13, 561; $F=30.3$, $P<0.0001$). This significant population effect was marginally explained by the size of the population where seeds were collected, categorized as either small (<100 plants) or large (≥100 plants). Contrary to expectation, seeds collected from smaller populations had a higher likelihood of germinating than seeds from larger populations, and the two smallest populations produced seeds with significantly higher germination
than any of the other population (Figure 3.1). The proportion of females within each population did not significantly affect germination (Table 3.2a).

**Inbreeding effects on fecundity, germination, and sex ratio**

Of the fecundity variables, i.e. weight per seed, number of seeds per fruit, and estimated total number of seeds produced, only the weight per seed was significantly different among the treatments, where females produced significantly heavier seeds than hermaphrodites, regardless of cross type (Table 3.2b). All other effects were non-significant. The proportion of germinated seeds was also higher for female mothers than hermaphrodite mothers, regardless of cross type (Table 3.2b; Figure 3.2). The sex ratio of the offspring produced by the crosses was not significantly affected by cross type ($df$: 3; $F$: 0.36; $P$: 0.7804) or maternal sex ($df$: 1; $F$: 0.29; $P$: 0.6056).

**Experimental population sex ratio and size affected offspring germination and sex ratio**

I found that germination of seeds produced by plants in the 2007 experimental pollination arrays was marginally significantly higher when they were supplementally pollinated (Table 5c; Figure 3.3a). All other factors in the model were non-significant.

The proportion of female offspring produced in the experimental populations was significantly higher for female than for hermaphrodite mothers ($df$: 1; $F$: 16.61; $P$: <0.0001). All other components of the model were non-significant. It is interesting to note that hermaphrodite mothers in the large, highly female experimental population and the supplementally-pollinated population produced only hermaphrodite offspring (Figure
Discussion

These experiments demonstrated that individual plant sex, population size, and not levels of inbreeding, affect germination in natural and experimental populations of *Lobelia siphilitica*. Additionally, plant sex, population sex ratio, and population size affected offspring sex ratio.

*Germination varies with natural population size but not sex ratio*

For the seeds collected in natural populations, I expected to find that larger populations would have higher proportion of germinated seeds, potentially explaining a large number of individuals through high recruitment. However, I found a negative relationship between proportion of germinated seeds and population size. An explanation for this could be that large populations are constricted by pressures such as competition for resources in the environment or soil microclimate (Hovatter 2008). One other explanation for higher germination in small populations than large could be that small populations tend to occur in the southern portion of the range (Caruso and Case 2007), raising the possibility that latitudinal variation in an external factor, such as temperature, may affect natural population size. My results are consistent with another experiment showing the same relation between germination and population size on soil from these
same 14 natural populations, which also found that germination was higher in seeds collected from small populations, regardless of the soil on which it was grown (Hovatter 2008). Increase in size of natural populations over time could support this hypothesis. Over the course of monitoring populations for three years (2006 to 2008), I have seen no significant trends in population size change over time (Figure 3.4).

I expected that highly female populations would produce seeds with higher proportion germinated seeds than populations with many hermaphrodites through the additive effects of individual female's reproductive compensation and outcrossing advantage. However, my results indicate that population sex ratio did not significantly affect the proportion of germinated seeds. This finding is supported by previous work which found more significance of plant sex than the sex ratio of the population the seeds came from (Delph and Mutikainen 2003; McCauley and Olsen 2003). This means that maternal investment by females is higher than that of hermaphrodites, regardless of the demographics of the population in which they occurred.

**Maternal sex affects fecundity in crosses with varying degrees of inbreeding**

In the manipulated crosses with varying degrees of inbreeding, I found that females produced seeds with higher average weight than hermaphrodites, regardless of cross type. There is usually a trade-off between seed size and number of seeds produced (Webb 1999; Shykoff et al 2003), though I found no significant differences between female and hermaphrodite mothers in terms of seed production. This is consistent with
previous studies showing that females produced heavier seeds than hermaphrodites in other gynodioecious species (Lafuma and Maurice 2006), which is consistent with the reproductive compensation hypothesis for female maintenance in gynodioecious populations.

My results also show that the seeds of females germinated in a marginally significantly higher proportion than seeds of hermaphrodite mothers, regardless of cross type which suggests that female mothers are somewhat more fit than hermaphrodite mothers. This could affect natural population sex ratio and size through higher recruitment by female mothers. My results are consistent with previous studies showing that seeds produced by females have higher germination than those by hermaphrodites in *Sidalcea oregana* spp. *spicata* (Ashman 1992a); *Ochradenus baccatus* (Wolfe and Shmida 1997); *Silene acaulis* (Delph and Mutakainen 2003); and *Thymus loscosii* (Lamiaceae; Orellana et al. 2005).

Surprisingly, I did not find lower germination in seeds produced by inbreeding. This is different from previous studies using other gynodioecious species, in which seeds produced by inbred hermaphrodites had lower germination rates than seeds produced by sib-crossed or out-crossed hermaphrodites (Emery and McCauley 2002; Glaettli and Goudet 2006; Chang 2007). My results may be skewed due to the unknown level of relatedness of the plants used as parents, since the plant families were selected from wild-collected fruits in populations with no prior measurements taken. If the plants from different female families were to some extent related, then their offspring would be biparentally inbred rather than fully outcrossed.
I recommend that future studies focus on measuring fitness at later life stages, such as height at flowering, days to flowering, number of leaves, total flower number, and flower size, to assess delayed effects of inbreeding, like those that were found in other gynodioecious species (Koelewijn and Van Damme 2005; Glaettli and Goudet 2006a, b; Keller and Schwaegerle 2006; Chang 2007). Next, I may not have detected an effect of inbreeding on germination if there is no inbreeding depression. Biparental inbreeding, which could contribute to non-significant effects of the crosses, could not be ruled out because I was unable to perform between-population outcrosses. Future studies could use clones of individuals in which to perform these crosses to assure known genetic heritage.

Understanding link between population sex ratio and size in *L. siphilitica*

The data presented here help to understand the link between population size and sex ratio in *L. siphilitica*. Small populations produced seeds with higher germination than large populations, while female mothers from controlled crosses produced a higher proportion of germinated seeds than hermaphrodite mothers. Taken together, these data could suggest that small populations with many females may increase in population size more quickly than large populations, and the sex ratio of these populations will increase in females. However, this would suggest that large populations should contain many females, which is not consistent with the sex ratio of natural populations I have sampled.

Because I found no significant effect of inbreeding and a negative relationship with population size and proportion germinated seeds, and because fecundity at the level
of germination success level does not reflect fitness at the adult stage, my results suggest that the relationship between population sex ratio and size may be influenced by factors other than germination success and inbreeding.

This work also may assist in the understanding of the mechanisms maintaining gynodioecy. My data show support for the resource allocation hypothesis of female maintenance, where females produced seeds with a higher mean weight than hermaphrodites and higher proportion germinated seeds than hermaphrodites. This suggests that more research should be done to understand fitness at later life stages in gynodioecious plants, especially survival across years in natural populations.
Table 3.1. Natural population sizes and sex ratios of 14 populations in Ohio and West Virginia, 2006.

<table>
<thead>
<tr>
<th>Population Code</th>
<th>Population</th>
<th>Location</th>
<th>County, State</th>
<th>Total</th>
<th>Proportion Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO</td>
<td>Bonnivale</td>
<td>N 39 10'21.7</td>
<td>Wood Co., WV</td>
<td>30</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W 81 29'38.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL</td>
<td>Blue Bend</td>
<td>N 37 55'25.6</td>
<td>Greenbrier Co., WV</td>
<td>38</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W 80 15'95.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV</td>
<td>Bend View</td>
<td>N41 26'55.65</td>
<td>Henry Co., OH</td>
<td>39</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W 83 47'36.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>Storm Drain</td>
<td>N 38 08'68.1</td>
<td>Fayette Co., WV</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W 81 09'27.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OG</td>
<td>Olive Green</td>
<td>N 39 44'62.6</td>
<td>Guernsey Co., OH</td>
<td>53</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
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<td>W 81 36'91.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>Pearson</td>
<td>N41 38'25.95</td>
<td>Lucas Co., OH</td>
<td>61</td>
<td>0.41</td>
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<tr>
<td></td>
<td></td>
<td>W 83 26'5.06</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MH</td>
<td>Mystery Hole</td>
<td>N 38 07'64.4</td>
<td>Fayette Co., WV</td>
<td>84</td>
<td>0.15</td>
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<td></td>
<td></td>
<td>W 81 08'53.2</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>JW</td>
<td>Jennings Woods</td>
<td>N 41 05' 32.5</td>
<td>Portage Co., OH</td>
<td>119</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W 81 11' 19.2</td>
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<td>AC</td>
<td>Apple Creek</td>
<td>N 40 40'7.79</td>
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<td>153</td>
<td>0.01</td>
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<td></td>
<td></td>
<td>W 81 48'40.12</td>
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<td></td>
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<tr>
<td>CP</td>
<td>Campbell Prairie</td>
<td>N41 32'8.58</td>
<td>Lucas Co., OH</td>
<td>200</td>
<td>0</td>
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<td></td>
<td></td>
<td>W 83 50'26.11</td>
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<td>RT</td>
<td>Rt. 62/60</td>
<td>N 40 28'12.5</td>
<td>Stark Co., OH</td>
<td>204</td>
<td>0.005</td>
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<td></td>
<td></td>
<td>W 81 58'51.5</td>
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<tr>
<td>KR</td>
<td>Kaylor Road</td>
<td>N40 29'47.63</td>
<td>Tuscarawas Co., OH</td>
<td>240</td>
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<td></td>
<td></td>
<td>W 81 38'52.02</td>
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<td></td>
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<tr>
<td>WC</td>
<td>Wabash-Cannonball</td>
<td>N 41 33'23.6</td>
<td>Lucas Co., OH</td>
<td>300</td>
<td>0</td>
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<td></td>
<td>W 83 52'9.32</td>
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<tr>
<td>BW</td>
<td>Buckwheat</td>
<td>N 39 31'51.36</td>
<td>Preston Co., WV</td>
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<td>0.04</td>
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<td></td>
<td>W 79 38'11.24</td>
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</table>
Table 3.2. Comparative analysis of seed weight and percent germination.  a.) Regression of the proportion germination of seeds collected from natural populations. (N.B. I did not weigh seeds from natural populations prior to germination.)  b.) ANOVA of seed weights and germination from crosses of varying degrees of inbreeding.  c.) ANOVA of seed weights and germination from experimental populations.

<table>
<thead>
<tr>
<th></th>
<th>Main effect</th>
<th>Weight per seed</th>
<th>Proportion germination</th>
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<tbody>
<tr>
<td><strong>a. Natural populations</strong></td>
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<td></td>
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<tr>
<td>Population size (N=14)</td>
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<td></td>
<td>(r: 0.4602)</td>
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<tr>
<td></td>
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<td></td>
<td>(P: 0.0978)</td>
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<tr>
<td>Population sex ratio (N=14)</td>
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<td></td>
<td>(r: 0.1660)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(P: 0.5706)</td>
</tr>
<tr>
<td><strong>b. Crosses of varying degrees of inbreeding</strong></td>
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<td></td>
</tr>
<tr>
<td>Cross type ((df: 3))</td>
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<tr>
<td></td>
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<tr>
<td><strong>c. Experimental populations</strong></td>
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<tr>
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<tr>
<td></td>
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Figure 3.1. Proportion germinated seeds in seeds collected from 14 natural populations of *L. siphilitica* in Ohio and West Virginia in 2006. Population size ranged from 38 to 333 individual plants. Data points represent mean proportion germination ± 1 standard error among 24 - 45 female sibships collected from each population. Proportion germinated seeds decreased as population size increased. Asterisks indicate that the two smallest populations had significantly higher germination (Student’s t-test: *t*: 1.9640; *a*: 0.05) than the other populations. The line of best fit shows a linear regression (*r*: 0.4602; *P*: 0.0978) using population means (*n*=14).
Figure 3.2. Mean proportion germinated seeds from manipulated crosses of female and hermaphrodite mothers. Varying degrees of inbreeding did not affect proportion of germinated seeds produced by manipulated crosses of *L. siphilitica*. Female mothers produced seeds with higher proportion of germination than hermaphrodite mothers, regardless of cross type. Numbers in each bar indicate the number of female families sampled.
Figure 3.3. Offspring germination and sex ratio from seeds produced in experimental populations (see Ch. 2 for details).  a) Proportion germinated seeds of mothers in 2007 experimental arrays. b) Offspring sex ratio of female and hermaphrodite mothers. Numbers in each bar indicate the number of maternal families sampled.
a. Proportion germinated seeds in offspring from experimental populations

b. Proportion female offspring from experimental populations
Figure 3.4. Natural population size variation from 2006 to 2008. (N: 11 populations; see Table 4). Change in population size: a) 2006 vs. 2007, and b) 2007 vs. 2008. The trend line shows a 1:1 ratio representing stable population size.

a.

![Graph showing population size in 2006 vs. 2007.](image)

b.

![Graph showing population size in 2007 vs. 2008.](image)
Chapter 4

Conclusions

The goal of this thesis was to understand how the demographic factors of *Lobelia siphilitica* population size and sex ratio affect pollinator behavior, female and male fitness, and germination. In the second chapter, I explored the effects of varying sex ratio and size on pollinator behavior in experimental populations. The chapter also focused on estimates of female fitness in terms of fruit set and seed set of both sexes, as well as male fitness, or siring success, of hermaphrodites. The study compared the relative fitness of the sexes in populations of varying sex ratio and size to help aid in the understanding of the correlation between population sex ratio and size. In the third chapter, I investigated how germination of seeds collected in natural populations was affected by varying population sizes and sex ratios, as well as the germination of seeds produced in the experimental populations. It also focused on the fitness differences between females and hermaphrodites in controlled crosses in terms of maternal production and effects on germination. When the results of the two studies are combined, they create a broader depiction of the natural pollination and breeding system for *L. siphilitica*.

*High visitation levels did not lead to high fecundity*

In the second chapter, I expected to find higher pollinator visitation to the hermaphrodites due to their dual rewards (nectar and pollen), higher visitation to large populations because of the higher number of flowers (Eckhart 1992), and higher
visitation to low female populations because of the higher proportion of hermaphrodite plants. I found higher visitation to hermaphrodites when they were common and higher visitation to large arrays than small arrays. However, this higher visitation did not necessarily translate into higher fecundity of the individuals in those arrays, since I found higher total seed production by females, regardless of the experimental population they were in. This is in contrast to a recent study on gynodioecious *Kallstroemia grandiflora* where they found higher visitation to hermaphrodites, but no effect of female sex or sex ratio on visitation, fruit set, or seed set (Cuevas et. al. 2008). Hermaphrodites produced more seeds in the supplementally pollinated treatment than in the open pollinated treatment, which may have resulted from decreased visits to pistillate phase flowers of hermaphrodite open-pollinated plants. While monitoring pollinator visitation, I did not indicate the sexual phase of the hermaphrodite flowers. In this way, hermaphrodites in the open pollinated arrays may have suffered from pollen limitation while females did not. Future studies should indicate whether hermaphrodite flowers are in the male or female phase.

Female resource allocation to producing better, i.e. heavier, seeds than hermaphrodites has been discussed as one aspect of female maintenance in gynodioecious populations (Lafuma and Maurice 2006). Females in the manipulated cross experiment produced heavier seeds than hermaphrodites, regardless of cross type, though the weight per seed of individuals in the experimental populations did not differ between sexes, population sex ratio or size treatments, or pollen treatments. However, females did produce a higher ratio of fruits to flowers, leading to a higher level of
resource allocation to maternal reproduction. Additionally, hermaphrodites in the supplementally pollinated experimental population produced more seeds per plant than hermaphrodites in the experimental arrays, while females showed no difference. This may suggest that inbreeding or resource allocation stress may play a role in hermaphrodite seed production. Furthermore, my results indicate that hermaphrodites from natural populations may not produce as great a proportion of viable seeds as females, which may explain why some populations are small with many females.

Germination was lower in large natural populations than small

I found a negative relationship between natural population size and the proportion of germinated seeds, which contradicted my expectations. Large populations would need high levels of recruitment in order to become and remain large. Alternatively, small populations with high levels of recruitment may increase in size rapidly over time. This result could also be explained through cumulative effects, where a large population in which all individuals could produce seeds with low germination would make a higher total number of new germinants than a small population in which all individuals produced seeds with complete germination. Next, a link between population size and inbreeding level may affect germination, since small populations may have purged prior genetic load and therefore produce better seeds than large populations still maintaining genetic load. Higher germination in smaller populations may also be the result of decreased inter- and intraspecific competition for resources and pollen, since small populations may be in recently disturbed sites. One other explanation for this
phenomenon could be that the artificial population size category shows affects on germination and visitation differently, where all populations that I deemed different categories in fact behave similarly. Future studies should assess germination from more populations from a broader range of population sizes in different regions to account for variation in germination across the species’ range, and to determine what the relationship between population size and germination truly is.

Germination was affected differently in outcrosses and supplementally pollinated females

Sex ratio did not significantly affect germination in natural populations, but significantly affected germination success of individuals in the experimental arrays. I found a great reduction in comparing the germination success of offspring from females in the out-crossed experiment with the germination success of that of the supplementally-pollinated females. Females in the manipulated crosses in the greenhouse produced offspring with approximately 75% germination success (Figure 3.2), while females in the supplementally pollinated experimental population in the field produced offspring with approximately 48% germination success (Figure 3.3a). Females from these experiments were from the same source population, and some of them were at least half siblings.

This dramatic reduction in proportion germinated seeds could be from sub-optimal growth conditions for plants in the experimental populations, which were outside for two to three weeks, while the manipulated cross plants remained indoors during the entire experiment and were provided with resources for optimal growth and reproduction. Another explanation could be that females in the supplementally-pollinated experiment
were under stress of nectar production for pollinator visitors, and put fewer resources into producing quality seeds. This explanation, however, would not be consistent with the theory that females evolved in unfavorable conditions (Campbell 2000; Dorken and Mitchard 2008). One way to test this is to use clones of identical ages, heights, flower numbers, etc. to replicate the experiments and determine if the germination of offspring from one set of females is reduced.

The same general trends of the effects of plant sex and pollen treatment affected visitation rate similarly to proportion germinated seeds in the experimental populations, though the factors affecting visitation did not significantly affect germination (see Figure 2.2 and Figure 3.3a). Germination was highest in the seeds collected from hermaphrodites in the supplementally pollinated array, contrary to the trend evident in manipulated crossing study and in the open-pollinated arrays. This could be the result of increased allocation of hermaphrodites in the supplementally pollinated array to maternal fecundity, as in Atlan et. al. (1991). Some evidence for this exists because the hermaphrodites in the supplementally pollinated array sired fewer offspring than hermaphrodites in the other arrays. This result could also be due to my sampling method, since I did not genotype the maternal offspring of plants in the supplementally pollinated array. My findings do not support results from work done on *Geranium maculatum* which showed higher seed production, size per seed, and germination in seeds produced by female plants (Chang 2006). Future studies should assess germination from plants of known sexes from a broader range of sizes and sex ratios.
Germination levels were similar in inbred and outcrossed experimental populations

The levels of germination between the experimental populations and the manipulated crosses of varying degrees of inbreeding were remarkably similar. Sampled offspring produced by the experimental populations were predominantly outcrossed (0.006% were definitely the result of self-fertilization), and had high germination. Interestingly, offspring produced by self-fertilized hermaphrodites also had high proportion of germinated seeds. These results suggest that, even while *L. siphilitica* hermaphrodites are capable of self-fertilization with no negative effects on germination, much outcrossing is occurring in natural populations. Another explanation is that individuals in the manipulated cross experiment remained in the greenhouse under optimal conditions, with no stressors, while individuals in the experimental populations were outdoors and may have been affected by stressors like extreme heat, sunlight, and reduced water. Perhaps the stressors indicated the need for selective seed production, while optimal conditions allowed any and all fertilized ovules to set seed. These results may also mean that life stages other than germination are negatively affected by inbreeding (Keller and Schwaegerle 2006), which should be measured in future studies.

Male fitness was affected by array sex ratio

I also found no correlation between pollinator visitation rates and siring success, in which I expected hermaphrodites with higher visitation to sire more offspring. These findings may require further investigation into pollinator visitation at different times of the day when pollinator behavior may be altered. Next, hermaphrodites in the large,
high-female array produced significantly more offspring than the females in that array. This means that when hermaphrodites are scarce, they are capable of becoming predominantly female (Atlan et. al. 1991), which may help to explain why large highly female populations are not found in nature (Caruso and Case 2007). These results are the first to assess both female and male fitness in *L. siphilitica* using seed production and microsatellite techniques, though future studies should use a greater number of microsatellite loci to better determine paternity and selfing.

*Inbreeding does not appear to be a link between population sex ratio and size*

Maternal sex, regardless of inbreeding level, significantly affected seed production and germination in the manipulated crosses of varying levels of inbreeding. I predicted that seeds produced from high levels of inbreeding would have lower proportion of germination because inbreeding increases deleterious alleles. I also expected to find higher proportion germinated seeds produced by females due to their reproductive compensation and/or outcrossing advantage as in Sakai et. al. (1997). I found that out-crossed females in the greenhouse produced seeds with much lower proportion germination than females in the supplementally-pollinated experimental array, while hermaphrodites showed the reverse trend. Again, this could be due to resource allocation shifts in the hermaphrodites of the supplementally-pollinated array from male to higher female fecundity. While I did not find an effect of inbreeding on proportion germinated seeds in my experiment, the range of germination success was similar to that of my experimental arrays, in which I measured a high level of outcrossing. This may
mean that inbreeding does not have an effect on germination success in *L. siphilitica*.

*Complex interactions between population sex ratio and size*

These results contributed to the overall knowledge of *L. siphilitica*, a model gynodioecious species, by demonstrating that females can be maintained in gynodioecious populations through higher germination rates and higher female fitness. This was the first manipulated study to observe pollinators in experimental populations of *L. siphilitica*, and I found significant effects of size, sex ratio, sex ratio by sex, and size by sex ratio by sex on pollinator behavior.

My results indicate that pollinators are influenced by demographic factors of gynodioecious populations, showing higher levels of visitation in large low female arrays. This pattern of visitation led to lower seed production by females in the large, highly-female population, which may help to explain why large, highly-female populations do not exist in nature. This work is also the first to measure inbreeding and paternity in experimental populations of *L. siphilitica* using microsatellites. I determined that, while *L. siphilitica* is a self-compatible species, inbreeding did not affect female fecundity or seed germination. My work found high levels of outcrossing using the microsatellites, as well as high level of multiple paternity and high levels of pollen movement within and among our experimental populations.

These two sets of experiments, when taken together, show that, even though pollinators prefer large populations, seed production and germination of seeds from large populations is relatively low. My data also suggest that plant sex, and not inbreeding,
significantly affects population demography, since inbreeding does not appear to be prevalent in nature. Finally, my data show that hermaphrodites in large populations act more like females than hermaphrodites in small populations, so that when hermaphrodites are rare in large populations, females are likely not producing many offspring. This would suggest a further explanation for why large, highly-female populations do not exist in nature (Caruso and Case 2007).

**Future studies**

Future work should include pollinator behavior studies of natural populations and experimental populations that more closely resemble natural population ranges in size and sex ratio. This would help to determine the influence of population size and sex ratio on pollinator behavior in the natural setting. Along with this, future studies should continue to observe natural populations of *L. siphilitica* to determine variation across years, and monitoring of plots containing marked individuals could help to determine individual plant longevity and population recruitment. This would help to determine if small populations are staying small or are in fact increasing in number, as would be predicted based on the results of this study. Because I did not find a significant effect of inbreeding on germination, I would recommend future studies to measure the effects of inbreeding at later life stages, such as age at flowering, flower number, leaf number, max height, which have been documented in other gynodioecious species (Sakai et al. 1997; Kephart et al. 2001).
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Willis Lab DNA extraction protocol:

http://openwetware.org/wiki/Mimulus:DNA_Extraction_Protocol
