THE EVOLUTION AND MAINTENANCE OF GYNODIOECY IN *GERANIUM MACULATUM*

by

MEGAN L. VAN ETTEN

(Under the Direction of Shu-Mei Chang)

ABSTRACT

Gynodioecy is thought to be the most common transition from hermaphroditism to dioecy, which is a major evolutionary transition in plants. There are three major stages in this transition: the initial invasion of females, the maintenance of females, and the masculinization of hermaphrodites. The research presented here addresses each stage to examine this process in *Geranium maculatum*, an herbaceous perennial. I found that the initial invasion requires a large seed fitness increase by females. This is partly due to the nuclear control of sex, which requires that females have at least twice the seed fitness as hermaphrodites to successfully invade, and to increased pollinator discrimination against females when they are rare. The maintenance of females is influenced by seed fitness differences between the sexes. Due primarily to differences in seed production and flowering frequency, I found that females are expected to be maintained in all populations examined, but only when including sex differences throughout the entire lifecycle of a plant. Seed production differences in part may be due to the lower selfing and biparental inbreeding found in females or due to the sexes living in different environments within populations. On the other hand, I found that pollinator discrimination may decrease seed production, lowering females' relative fitness. Thus,

the maintenance of females is influenced by several different factors. The last step to dioecy, the masculinization of hermaphrodites, does not appear to occur in this species due to selection favoring more flowers, which increases both pollen and seed fitness. Without a tradeoff between pollen and seed fitness, it seems unlikely that dioecy will evolve in *G. maculatum*. Thus, in this species, the initial invasion of females may be difficult, but once established gynodioecy appears to be stable, despite sex being under nuclear control.

INDEX WORDS: gynodioecy, reproductive allocation, Geranium, selection analysis, kriging, frequency dependent selection, mating system, demography, functional gender.

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Dedication

To my husband.

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TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Breeding systems are one of the most evolutionarily important features of plants (Barrett 2003). Ranging from hermaphroditic, with both male and female function within the same flower, to dioecy, with separate male and female plants, breeding systems influence a wide variety of processes. For example, separating sexual functions often leads to higher reproductive costs for females. As a consequence, females may be smaller, grow slower and have lower survival rates (Obeso 2002). The breeding system also has consequences for genetic variation. Separating male and female function may decrease the amount of selfing, thereby increasing the heterozygosity of individuals (Charlesworth and Charlesworth 1987). This may influence the phenotypic variation within species, and even their ability to colonize new habitats (Carlquist 1966). Thus, understanding the evolutionary forces behind the origin and maintenance of breeding systems deepens our understanding of past pressures and future evolutionary potential of a species as well as insight into evolution in general.

The transition from one extreme, hermaphroditism, to the other, dioecy is most likely mediated through gynodioecy, a breeding system in which female and hermaphroditic plants coexist (Charlesworth and Charlesworth 1978). The first step in this process is that females invade hermaphroditic populations. For this to occur, females must have higher seed production than hermaphrodites because they lose all of their

potential pollen fitness (Charlesworth and Charlesworth 1978; Lloyd 1974). If this requirement is met, females may be maintained in populations, with their frequency dependant in part on the relative fitness of their seeds. As the frequency of females increases, hermaphrodites should be gaining more of their fitness through pollen donation, which may select for traits increasing pollen fitness. This gradual increase in male function may lead to the evolution of pure males if there is a trade-off between male and female fitness, thereby becoming a dioecious population (Charlesworth and Charlesworth 1978).

The broad goal of my dissertation was to examine each of these steps, invasion, female maintenance and masculinization in *Geranium maculatum*, an herbaceous perennial. These steps can be broken down into several broad questions. First, in chapter 2 I investigated the genetic control of sex, which determines the fitness needed for female invasion and influences the dynamics of the further evolution of gynodioecy. Second, in chapter 3 I measured the seed fitness of the sexes in natural populations to determine if seed production differences explain the high frequency of females we find in natural populations. Third, in chapters 4-6 I investigated other factors that may contribute to female maintenance, including mating system differences, environmental differences and pollinator preferences. Fourth, in chapter 7 I investigated the last step towards dioecy, the masculinization of hermaphrodites. With this extensive set of results, our understanding of how gynodioecy is maintained in a species is greatly enhanced.

Genetic Control

Determining the genetic control of sex is an important step because it determines the level of fitness females need to invade and be maintained in populations (Charlesworth and Charlesworth 1978; Charlesworth 1981; Lloyd 1974). Additionally, it influences population dynamics, particularly the sex ratio. There are two types of genetic control that have been found in gynodioecious species. Less common is pure nuclear control where one or more nuclear loci control sex. Under this type of control, females must have twice the seed fitness of hermaphrodites to compensate for the lack of pollen fitness (Charlesworth and Charlesworth 1978). With nuclear control, the frequency of females is primarily based on their relative seed production. More common is cyto-nuclear control. In this case, male sterility (i.e. femaleness) is determined by a mitochondrial mutation (called a cytoplasmic male sterility factor, or CMS) while a nuclear gene(s) can restore male function (called restorers, Budar and Pelletier 2001). Due to the maternal inheritance of the mitochondria, and therefore male-sterility, in most angiosperms (but see McCauley et al. 2007; Pearl et al. 2009), the lack of pollen production does not affect the fitness of the CMS haplotype. Thus, under this type of control, females must only have a slight increase in seed production to increase in frequency. Because of the interaction between the male-sterility gene and the restorer, the frequency of females depends on both their relative seed production and the frequency and fitness of the restorer allele(s) (Delph and Wolf 2005). Most theoretical models predict frequent fluctuations in the frequency of females under this system (Bailey and Delph 2007a; Delph et al. 2007; Frank 1989; Gouyon et al. 1991).

Within these two broad types of genetic control there is much variation among species in the number and dominance of loci involved. Nuclear controlled species seems to have fewer numbers of loci. For example, *Fragaria virginiana* only has one locus controlling sex (Ahmadi and Bringhurst 1991) as does *Phacelia linearis* (Eckhart 1992b), although they differ in the dominance of femaleness. Alternatively, cyto-nuclear systems can be quite complex. For example, in *Plantago lanceolata* it has been suggested that for one CMS haplotype there are five restorer loci, three dominant and two recessive (Van Damme and Van Delden 1982). Similarly, in *Silene vulgaris* several restorers have been suggested (Ehlers et al. 2005). These results indicate that the genetic control of sex can be complex and species specific. None of the several gynodioecious *Geranium* species have been thoroughly examined for the type of genetic control, although several researchers have suggested that sex is under cyto-nuclear control (Asikainen and Mutikainen 2003, C.F. Williams pers.com.). Through reciprocal crosses and crosses between females and hermaphrodites, in chapter 2 I investigated the type of genetic control as well as the number and dominance of loci involved.

Relative Fitness

The relative seed fitness of females is important in the initial invasion of females as well as their maintenance. Depending on the type of genetic control, females must have either a slight seed fitness advantage to a twofold advantage to invade and be maintained in populations. This can be achieved through producing more seeds and/or better seeds. Most studies have found a female seed fitness advantage (Shykoff et al. 2003), which ranges from just above 1x to 20x the seed production of hermaphrodites.

Several studies have also found an increase in seed size (Shykoff et al. 2003), and a few have found that progeny from females germinate and survive better (e.g. Ashman 1992; Chang 2006). This information can then be incorporated into models to examine the population dynamics, such as the sex ratio. One approach that is not used often is demographic modeling, which can easily incorporate information on all life stages to get a more comprehensive view of fitness differences and their influence on the sex ratio (Caswell 2001). In chapter 3, I used data collected from field populations to examine seed fitness differences between females and hermaphrodites.

Factors Affecting Relative Fitness

While knowledge of the amount of seed fitness compensation that females achieve is important, the mechanisms leading to this increase is equally, if not more, important. Historically there are two hypotheses for how females achieve higher seed fitness. First, because females cannot self pollinate, they may have higher seed quality due to lower inbreeding depression (Chang 2007; Darwin 1877; Glaettli and Goudet 2006; Schultz and Ganders 1996). The importance of this factor depends upon the amount of selfing in hermaphrodites, the strength of inbreeding depression as well as the amount of biparental inbreeding. Second, because females do not have to produce male structures, the resources saved can be used for producing more or better seeds (Ashman 1994; Darwin 1877; Eckhart 1992a). The importance of this factor is dependent upon the availability of resources and the amount of the resources saved from not producing male structures. While these two mechanisms may explain the higher seed fitness in some species, several more ecologically based hypotheses have been proposed. For example,

in *F. virginiana* females have higher seed production because they are more tolerant to spittlebug damage than hermaphrodites, increasing their relative seed fitness (Cole and Ashman 2005).

In chapters 4-6 I examined several factors that may influence the relative fitness of females. First, I examined selfing rates and the spatial genetic structure to determine the amount of selfing and the potential for biparental inbreeding (mating with relatives). Second, I examined the light and moisture environments in natural populations to determine if sexes are located in different environments and how this may affect their reproduction. Third, I examined how pollinator discrimination against females may decrease their relative seed production. Using the information gathered in these chapters, we can get a better idea of what is influencing female fitness and to better understand when/where gynodioecious populations may occur.

MALENESS

The last step in the evolution of dioecy through gynodioecy is the masculinaztion of hermaphrodites. From theory, it is expected that hermaphrodites in populations with females will be male-biased in their fitness gained and that as the frequency of females increases, their "maleness" should increase (Lloyd 1976). Empirical results support the theoretical predictions in several species. For example, in *Geranium sylvaticum*, hermaphrodites had lower seed production in populations with a higher frequency of females (Asikainen and Mutikainen 2003). However, despite theory, there are many species in which it appears that gynodioecy does not result in dioecy. Gynodioecy in *Geranium* is an example of this; while there are several gynodioecious species, there are

no dioecious ones (Fiz et al. 2008). To investigate the last step in the transition to dioecy, in chapter 7 I examined the maleness of hermaphrodites and how the sex ratio affects maleness.

The research presented here is one of the more extensive sets of data on a gynodioecious species. Through investigation of the entire process from hermaphroditism to dioecy, we better understand what factors are influencing each step. This research is unique because it focuses on how the biology of the species influences the evolution and maintenance of gynodioecy. Results from extensive research, such as presented here, are revolutionizing how we think about gynodioecy and are providing important insights into how and when dioecy may evolve.

CHAPTER 2

THE GENETIC CONTROL OF SEX IN A GYNODIOECIOUS SPECIES, *GERANIUM MACULATUM*[1]

[1] Van Etten, M.L. and S-M. Chang. To be submitted to *Heredity*.

Abstract

Gynodioecy, the co-occurrence of females and hermaphrodites within a population, has been of interest to biologists because despite losing half of their potential fitness, females can remain in populations. Theory has shown that to compensate for their lost pollen fitness, females must have higher seed fitness than hermaphrodites. The increase in fitness required for female establishment depends on the genetic control. There are two types of genetic control: nuclear, which requires females to have twice the seed fitness of hermaphrodites, or cyto-nuclear, which requires females to have only slightly more seed fitness than hermaphrodites because the male sterility gene is located in the mitochondria, which is generally maternally inherited in angiosperms. We investigated the type of genetic control responsible for sex in *Geranium maculatum*. Through reciprocal crosses between hermaphrodites we concluded that sex is most likely not under cyto-nuclear control. Instead, sex appears to be controlled by at least two nuclear loci.

Introduction

Gynodioecy, the co-occurrence of females and hermaphrodites within populations, has been of interest to biologists because it is considered to be the most likely intermediate step from hermaphroditism to separate sexes (Charlesworth and Charlesworth 1978). Theoretical models have suggested that females can be maintained in populations if they have higher seed fitness than hermaphrodites (Charlesworth and Charlesworth 1978; Lloyd 1974). This requisite seed fitness increase can be from producing more and/or better quality seeds. Most studies have found that females

produce more seeds than hermaphrodites (Shykoff et al. 2003), in some species reaching 20 times as many (Wolfe and Shmida 1997). The type of genetic control determines how much of a seed fitness increase is needed for females to be maintained. Thus, knowledge of the genetic control of sex is important in predicting the evolution of gynodioecy within a species.

There are two broad types of genetic control that have been found in gynodioecious species. First, nuclear control is where sex is determined entirely by loci on the chromosomes. Because alleles at nuclear loci are passed on through both pollen donation and seed production, for the female causing allele to spread, females must have more than twice the seed fitness as hermaphrodites to compensate for their loss of pollen fitness. The second type of genetic control, generally called cyto-nuclear, involves a gene in the mitochondria that causes male-sterility (a cytoplasmic male sterility factor, or CMS) and a nuclear gene that restores male function (called a restorer). Because in angiosperms the mitochondria is usually inherited from the ovule donor, for the CMS haplotype to spread, females only need slightly higher seed fitness than hermaphrodites to be maintained. Thus, there is a large difference between the types of genetic control in the increase in female fitness needed for the maintenance of females.

In addition to the maintenance of females, the type of genetic control also potentially influences the sex ratio and stability of gynodioecy. Under nuclear control, theory has shown that an increase in the fitness compensation of females increases the proportion of females in the population (Charlesworth and Charlesworth 1978; Lloyd 1974). On the other hand, under cyto-nuclear control, the sex ratio is determined by many additional factors. Similar to nuclear control, the higher the female fitness

compensation is, the more females there should be in a population. However, the sex ratio also depends upon the frequency of the restorers and the fitness of individuals with the restorers (Delph and Wolf 2005). Generally, species under cyto-nuclear control can have higher percentages of females than species under nuclear control because of the interactions between the restorer and the cytoplasmic loci (Bailey and Delph 2007b). Finally, the type of genetic control also potentially influences the stability of gynodioecy. Under nuclear control, it is predicted that selection will favor the gradual masculinization of hermaphrodites, eventually leading to pure males (Charlesworth and Charlesworth 1978). However, under cyto-nuclear control, it is thought that this transition to dioecy is more difficult because sex specific genes cannot be linked with the sex determining genes (but see Maurice et al. 1994; Maurice et al. 1993). Knowledge of the genetic control is therefore an important first step in understanding the evolutionary dynamics of gynodioecious species.

Determining the type of genetic control is generally best achieved by comparing the sex ratio of progeny from reciprocal crosses between individuals from distant populations (e.g. Bailey and Delph 2007b). For a given cross between two individuals, the nuclear composition of the resulting progeny will be the same, on average, regardless of which parent served as the ovule donor. In contrast, the cytoplasmic composition may be different because the progeny generally inherit the cytoplasmic genomes from only the ovule donor (but see McCauley et al. 2005). Thus, if the sex ratios of progeny arrays differ depending on the directionality of the crosses, the involvement of a cytoplasmic element in sex determination is concluded. The most powerful type of reciprocal crosses are ones made between distant populations because in order to detect a difference in the

sex ratios, substantial differences in the frequency of females produced are needed. Large differences in the progeny sex ratio may result from a mismatch between a CMS haplotype and the restorers. To maximize the chance that this mismatch occurs, crosses are usually done between distant populations, making it more likely that the populations have different CMS haplotypes and thus do not carry the same restorer.

Using this technique, most species have been found to be under cyto-nuclear control with several different CMS types and restorers. One of the best studied species with regard to the genetic control of sex is *Plantago lanceolata*. Through reciprocal crosses and crosses between the sexes, it has been demonstrated that there are at least 2 different CMS haplotypes in a single population (Van Damme and Van Delden 1982), each with its own set of restorers (Van Damme 1983). Additionally, crosses between hermaphrodites and females have demonstrated that several restorers are involved with restoring a single CMS (Van Damme 1983). Although the majority of species studied being under cyto-nuclear control, there are a few under nuclear control (Ahmadi and Bringhurst 1991; Eckhart 1992b; Godley 1955; Kohn 1989). For example, *Fragaria virginiana* has been shown to have purely nuclear control and is relatively simple, with a single locus controlling sex and femaleness being dominant (Ahmadi and Bringhurst 1991). Sex in *Phacelia linearis* has also been shown to be primarily controlled by a single nuclear gene (Eckhart 1992b). Contrary to *F. virginiana*, femaleness was recessive rather than dominant (Eckhart 1992b). Combined, these results suggest that genetic control for sex expression can be complicated and that it is often species specific.

In this study, we explored the genetic control of sex in *Geranium maculatum*. No studies have yet determined the genetic control of any gynodioecious *Geranium* species,

although several authors have suggested a cyto-nuclear control (Asikainen and Mutikainen 2003, C.F. Williams pers.com.). Through reciprocal crosses we tested whether there is a cytoplasmic component to sex determination. Additionally, through crosses between hermaphrodites and females, we further examined the number of loci involved and the dominance of alleles.

METHODS

Geranium maculatum L. is a gynodioecious, rhizomatous perennial ranging from the South Eastern US to Canada and west to the Great Plains (Radford et al. 1968). Flowering begins in early spring, with flowers being visited by generalist pollinators including bees, flies and butterflies. Hermaphrodites are self-compatible, but natural selfing rates are low, ranging from 0-17% (Van Etten *et al.*, Chapter 4). Inbreeding depression is high and variable between populations (cumulative postdispersal inbreeding depression ranges from 0.38 to 0.84, Chang 2007). Seeds are dispersed by the elastic dehiscence of the schizocarp, to an average of 3 m from the maternal plant (Stamp and Lucas 1983). Females have small aborted anthers and smaller petals when compared to hermaphrodites (Ågren and Willson 1991; Chang 2006). In natural populations, yearly seed production is higher for females than hermaphrodites (20 – 50% increase, Ågren and Willson 1991; Chang 2006) and seeds from females have a higher germination rate in the greenhouse (Chang 2006). Local populations around Athens, GA range in female frequency from 0-50% (Chang 2006).

Crossing design

To determine if there is a cytoplasmic component of sex determination, reciprocal crosses between hermaphrodites were done. On average, if sex is controlled solely by nuclear elements, reciprocal crosses between hermaphrodites should produce the same sex ratios, regardless of which parent was used as the pollen donor and which was the ovule donor. On the other hand, if there is a cytoplasmic element, the direction of the cross, i.e. which individual was used as the ovule donor, will affect the sex ratio in the progeny arrays. Rhizomes from populations throughout the south eastern US were collected and grown in the greenhouse (Table 2.1). Twenty hermaphroditic plants were chosen and paired with a hermaphrodite from a different population. Distances between populations of the paired individuals ranged from 0.32 km to 206.2 km. Interpopulation crosses were used because there may be population differences in the CMS and restorer loci/alleles, which increases the chance of detecting a cytoplasmic element. Crosses were done by taking 1-2 anthers and rubbing them onto a receptive stigma until the stigma was completely covered with pollen. Hermaphrodites were emasculated as needed prior to stigma receptivity. Seeds were collected once they were fully developed.

To better determine the number of loci involved and to understand differences among populations, plants collected from a subset of 5 populations were chosen for a more in-depth crossing design. From each population, two hermaphrodites and several (1-3) females were chosen, depending upon availability. Crosses were made between individuals from the same and from different populations as was possible given the number of available flowers. We were not able to make crosses for all possible combinations because of non-overlapping flowering between some pairs of plants due to

low flower number within a given flowering bout and inconsistent flowering. There were 24 sets of within population crosses and 4 sets of among population crosses between hermaphrodites in addition to the reciprocal crosses, and 51 and 9 for FxH.

Progeny scoring

A subset of the seeds from crosses were planted to determine the sex ratio in the progeny arrays from each cross. Most of the seeds from the reciprocal crosses were planted in order to obtain large sample sizes for each cross (from 14-129, average=65). For the within/among population crosses, up to 50 seeds per cross were planted. A total of 3961 seeds were planted. Preparation and planting of seeds followed the methods in Chang (2006). After 3-4 months of growing, plants were transplanted into 10.2 cm pots and their locations randomized. After another month of growth, leaves were removed and the plants placed into 4°C cold room to simulate winter. After 1-3 months of cold stratification, plants were placed back into the greenhouse, where they would grow for 3-5 months before placing back into the cold room. This cycle was repeated between 3-5 times until the majority of the surviving plants had flowered twice.

To determine the sex of individuals, flowers were monitored for at least two flowering bouts. Each time, at least two flowers per plant were scored for sex, not including the first flower. If the first several flowers were female, the remaining flowers produced were also monitored. There were several cases of intermediate flowers or plants. Flowers with both sterile and fertile anthers within a single flower were scored as intermediate flowers. This type of intermediate generally transitioned quickly into normal hermaphroditic flowers, in which case the individual was scored as hermaphrodite. However, some plants consistently produced intermediate flowers and

were scored as intermediate individuals. There were also several cases of individuals producing a large proportion of female flowers with a few intermediate flowers. These individuals were also scored as intermediate. The data analyzed here exclude plants that have only flowered once (~48% of the germinated seeds) because of individuals switching from female to hermaphrodite between flowering bouts.

Data analysis

To determine if there was a cytoplamic component to sex determination, the reciprocal crosses were tested to see if the direction of the cross affected the progeny sex ratios. As is common in genetic studies of gynodioecious plants (Eckhart 1992b; Koelewijn and Van Damme 1995a; but see Van Damme 1983), intermediates were grouped with the hermaphrodite category because they produce pollen. The sex ratios from each pair of reciprocal crosses were compared using a chi-squared test in SAS (SAS Inc. 2000). Significant differences between reciprocal crosses in the progeny sex ratio would indicate a cytoplasmic component to sex determination.

To determine the number of loci involved, sex ratios from all of the crosses were compared to expected sex ratios under different models of genetic control (see Table 2.2 for predicted sex ratios). The possible models of genetic control are derived from those hypothesized for other species (primarily from Molina-Freaner and Jain 1992). These models range from a single locus (either cytoplasmic or nuclear) to multiple loci and from cytoplasmic to nuclear. Table 2.2 lists the genotypes possible for both sexes for each model of genetic control. Using these genotypes, the possible sex ratios obtained from crosses with females (F) and crosses between hermaphrodites (H) are listed. For example, if sex is determined entirely under cytoplasmic control (model 1), females will

pass on their male-sterile cytoplasm to all of their progeny, leading to all progeny from a female ovule donor being female (0:1 H:F progeny sex ratio). On the other hand, progeny from hermaphrodite ovule donors will all have the fertile cytoplasm and all be hermaphrodites (1:0 progeny sex ratio). Using a chi-squared test, the observed sex ratio can be compared to those expected for a given model. If the observed sex ratios do not match those predicted, then the model does not adequately describe the data. The model used in the previous example can be ruled out because all crosses with female ovule donors produce sex ratios that are significantly different from a 0:1. Each additional model was tested in a similar manner. If a model adequately described the sex ratios observed from the crosses, a more in-depth analysis was done. Since many individuals are crossed with a variety of other individuals, there are some cases in which a genotype can be predicted based on a particular genetic model. The predicted genotype can then be compared to the predicted genotype obtained for that individual from other crosses to better determine the fit between that model and the data. Only crosses with more than 10 scored individuals were used in testing the models.

RESULTS

Testing cyto-nuclear control

Results from reciprocal crosses provide no support for a cytoplasmic component to sex determination. The simplest model of sex determination is a pure cytoplasmic control (model 1 in Table 2.2), in which case females would produce all females and hermaphrodites would never produce females. Our data do not support either of these expectations because female ovule donors produced between 4-80% females and

hermaphrodite ovule donors produced between 1-17% females (Fig. 2.1, Table 2.2). A slightly more complicated model of one CMS with a dominant restorer (model 2 in Table 2.2) is also not supported. In this model, crosses with females will produce a 1:1, 1:0 or 0:1 ratio of hermaphrodites to females and crosses between hermaphrodites will produce a 1:0, 3:1, 1:1 or 0:1 ratio and the sex ratio in the progeny from reciprocal crosses may differ depending upon the direction of the cross. Our data did not support this model either because crosses with females often produced a 3:1 ratio (Table 2.2), crosses between hermaphrodites produced ratios not predicted by this model (e.g. 15:1, 7:1, Table 2.2), and there were no significant differences between reciprocal crosses (Table 2.3).

While a model with one CMS and two restorer loci (model 4 in Table 2.2) better explained the data, there were many inconsistencies. In this model, females have a CMS and are homozygous recessive for both restorer loci. Crosses with females can produce ratios of 3:1, 1:0, 1:1, or 0:1 depending on the genotype of the hermaphrodite. Crosses between hermaphrodites can produce ratios of 1:0, 15:1, 7:1, 3:1, 1:1 or 0:1 and the sex ratio of the progeny from reciprocal crosses may differ depending on the direction of the cross. Although the range of sex ratios found in our results is better encompassed by this model (i.e. 15:1 and 7:1), there were some inconsistencies. First, in our crosses with females, a 1:0 or 0:1 ratio was never produced, which this model predicts (Table 2.2). Second, this model predicts that some reciprocal crosses between hermaphrodites should be different depending upon the direction, but no significant differences were found in reciprocal crosses (Table 2.3). Thus, while this model better predicts the range of sex ratios found, there is no evidence that there is a cytoplasmic component involved.

Testing nuclear genetic control

In light of the weak support of a cytoplasmic contribution to sex, nuclear models of genetic control were tested. The data did not support a single locus, female dominant model of control (model 6 in Table 2.2) because hermaphrodites occasionally produced females and the crosses with female ovule donors consistently produced too few females (model predicts 1:1, observed 3:1). A single locus, hermaphrodite dominant model of control (model 5 in Table 2.2) was also not supported because progeny from crosses with hermaphrodite ovule donors were not always 1:1 or 3:1 and crosses with female ovule donors often produced a 3:1 ratio (model predicts 1:0 or 1:1).

Models with two nuclear loci better explain the data. A two locus, duplicate recessive model (model 7 in Table 2.2) would explain the frequent 3:1 progeny arrays with females as ovule donors as well as the 15:1 and 7:1 ratios with hermaphrodites' as pollen donors. However, this model predicts many crosses between females and hermaphrodites should produce 1:0 ratio, which was never found in our data. Additionally, when it was possible to predict the exact genotypes based on this model, there are many cases where an individual's predicted genotype based on one cross does not match that predicted from another cross, suggesting that this model does not fit the data well.

Similar problems were encountered when testing a model with two complimentary loci (model 8 in Table 2.2), in which an individual is female if it has two recessive alleles at either of the two loci. While this model predicts most of the progeny sex ratios observed in our results (e.g. 15:1 or 7:1), predicted genotypes for an individual often do not match depending on which cross is used. For example, crosses involving

5C3 as the maternal individual suggest that it is $M_1m_1m_2m_2$ because it is the only female genotype that could produce a 3:1 ratio. Depending on the hermaphrodite it is crossed with, progeny should be 1:0, 3:1 or 3:5. However, when crossed with IS3 the progeny sex ratio is either a 1:1 or 5:3 ratio, which is not expected given the predicted maternal genotype. Thus, our data also do not completely support this model.

A two locus epistatic model (model 9 in Table 2.2) proposed by Lewis and Crowe (1956) also fits the sex ratios well. In this model, females have at least one dominant allele at the first loci and are homozygous recessive at the other loci, thus there are two possible female genotypes, Mmhh and MMhh. Crosses with Mmhh will produce sex ratios of 1:0, 1:1, 5:3 or 3:1 depending on the genotype of the hermaphrodite while crosses with MMhh will be either 1:0 or 1:1. Because of the variety of sex ratios predicted in crosses with Mmhh, this model better describes the range of sex ratios found in our results than other models (e.g. crosses with 5C3 or 52.2.6). However, as with the other models, predicted genotypes do not always match depending on which cross is used to make the predictions. Thus, while this model better fits the sex ratios found in our crosses, it does not completely predict the data.

Intermediates

Many of the crosses produced some proportion of intermediate plants. Intermediate individuals had several different phenotypes, which can be grouped into two categories. The first category, hereafter I1, is those individuals that were intermediate early but stabilized to a single sex. This category included plants that produced female or intermediate flowers early and hermaphrodite flowers later in the flowering season. Additionally, some plants in this category produced only female flowers the first

flowering season and hermaphrodites flowers the second and following seasons. The second category, hereafter I2, is intermediates that did not stabilize to either a female or hermaphrodite sex. It included plants that consistently produced a mixture of sterile and fertile anthers within a single flower. Additionally, it included plants that first produced hermaphrodite flowers and later produced female or intermediate flowers.

Crosses with females and between hermaphrodites produced substantial amounts of intermediates (HxH=12.2%, FxH=22.3%, Fig. 2.2). Most intermediates fell into the I2 category, primarily from plants producing a large number of intermediate flowers in one or both flowering seasons. Crosses with females as the ovule donor produced significantly more intermediates than crosses between hermaphrodites (Fig. 2.2, $F_{1,59}$=8.18, P=0.006). For crosses between hermaphrodites, the percentage of intermediates produced increased with the distance between parents used in the cross (slope=0.002, t=2.35, d.f.=1, P=0.02, Fig. 2.3), while the percentage of hermaphrodites decreased (slope=-0.002, t=-2.25, d.f.=1, P=0.03, Fig. 2.3). There was no relationship with distance from crosses with females as the ovule donor (%H: P=0.90, %I: P=0.63, %F: P=0.93).

DISCUSSION

In this study, we explored the type of genetic control responsible for sex determination in *Geranium maculatum*. We found that sex is not under a simple genetic control, involving at least two and possibly more loci. Additionally, we found a significant proportion of the progeny from crosses had an intermediate sex phenotype.

Testing the control of sex

Our results indicate that sex is not determined by a single locus, either in the cytoplasm or the nucleus, paralleling results from most other species (Belhassen et al. 1991; Eckhart 1992b; Koelewijn and Van Damme 1995a; Koelewijn and Van Damme 1995b; Van Damme 1983; Van Damme and Van Delden 1982). For example, the most well studied species with regard to the genetic control is *Plantago lanceolata*. Through an extensive number of crosses, two CMS types have been found within a single population (Van Damme 1983). For one CMS type, it was proposed that there are at least two dominant and three recessive restorer loci. For the other CMS type, three dominant restorer loci were proposed. Despite this complex model proposed for sex determination in this species, there remained inconsistencies in the progeny arrays. This example illustrates both the complexity of sex determination and the difficulty in determining the precise number of loci.

One of the difficulties in determining the number of loci is that once a model with more than one locus is considered, the ability to distinguish among different models becomes more difficult. This is because the difference in the predicted sex ratios becomes more subtle and therefore, a larger sample size must be used to statistically distinguish between them. For example, model 8, a nuclear 2-locus complementary model, can be distinguished from model 7, a nuclear 2-locus duplicate recessive model, because under model 8 there may be a 15:1 ratio (6.25%F), whereas under model 7 the closest ratio is 7:1 (12.5%F), a difference of only ~6% females. This small difference between the two models limits the ability to statistically tell the differences between these predicted sex ratios without very large numbers of progeny. The situation is similar

when trying to determine whether progeny sex ratios differ in reciprocal crosses, which would indicate cyto-nuclear control. For example, in our results, a cross in one direction produced a 5.9% female progeny array while the other direction produced a 0% female progeny array. Because the difference is so small, the sample size must be high to distinguish statistically whether they are different (from a power analysis of a 6% difference: more than 100 progeny per cross must be scored for a 60% chance of statistically finding a difference). In an attempt to find a cross between individuals that would produce a bigger difference in the progeny sex ratios if sex is under cyto-nuclear control, we have crossed hermaphrodites from populations in Georgia to ones from New York. Few of these progeny have flowered, but most crosses have produced no females (10 out of 12 crosses) or few females (33%), similar to crosses between near populations. These results suggest that sex is unlikely to be under cyto-nuclear control.

Consistent with the results from reciprocal crosses, there are several other lines of evidence that suggest that sex is not under cyto-nuclear control. First, a model of cyto-nuclear control with 2 restorers predicts that some crosses with females should produce all female progeny ($r_1r_1r_2r_2$+(C) x $r_1r_1r_2r_2$+(F)) and some should produce all hermaphrodites ($r_1r_1r_2r_2$+(C) x $R_1R_1R_2R_2$, $R_1R_1r_2r_2$ or $r_1r_1R_2R_2$), neither of which we found in any of our crosses. While it is possible that none of the hermaphrodites we used had these genotypes, it seems unlikely that four out of seven possible hermaphrodite genotypes we not included in our crosses. Second, under cyto-nuclear control it is predicted that some populations will have a population sex ratio above 50% female, but no populations of *G. maculatum* found are above 50% (Van Etten, personal observation). In fact, most populations have low frequencies of females (10-20%). Third, under cyto-

nuclear control, the distance between populations used in the cross should increase the percentage of females produced because of an increased chance of finding a mismatch between the CMS and restorer. However, there was no relationship between the distance and the proportion of females produced, even in crosses with female ovule donors. Together, these lines of evidence suggest that the genetic control is not cyto-nuclear.

Intermediates

We found a high frequency of intermediates in our crosses, especially in crosses with female ovule donors. Additionally, there was an increase in the proportion of intermediates produced as the distance between populations increased. Intermediates have been found in almost every gynodioecious species examined (references in Andersson 1999). Often these plants are ignored in analyses, but some research has explored their genetic control, primarily in species under cyto-nuclear control. There have been two hypotheses proposed for intermediates. First, it has been suggested that a mixture of female and hermaphrodite flowers or inflorescences can be explained by lineage sorting of different mitochondria haplotypes if sex is under cyto-nuclear control (Andersson 1999). Second, intermediate sex phenotypes may be caused by the partial restoration of the CMS type (references in Andersson 1999). This second hypothesis can be extended to systems with nuclear control of sex because the efficacy of each sex determining locus may not be the same or there may be epistatic interactions between loci. This hypothesis could explain the observation that females produce more intermediates. Presumably, intermediates are caused by some mixture of female and hermaphrodite specifying alleles. In crosses with females, there is guaranteed to be some female specifying ones, leading to more intermediates than crosses between

hermaphrodites. It may also explain the increase in intermediates with the distance between populations because there may be a greater chance of combining alleles/loci that differ in their ability to produce one sex or the other in crosses between distant populations. Further research is needed to determine the genetic basis of intermediates in order to understand their role in the maintenance of gynodioecy.

Regardless of the mechanism behind the production of intermediates, the high frequency of intermediates observed in this study does not match the frequency we observe in local natural populations. Surveys of natural populations from which the plants were collected have found little to no intermediates (Van Etten, pers. obs.: 2-6%). Additionally, in a 3-year study using 3 natural populations located around Athens, GA, we never observed a single case of sex switching of over 250 plants and no intermediate flowers were ever observed (Van Etten and Chang, in prep). In contrast, a study of natural populations in Illinois found much higher frequencies of intermediates (Ågren and Willson 1991: 20-27%I), which are similar to the results found in our crosses. These contradicting results could occur if there is stronger selection against intermediates in natural populations in Georgia, which is at the edge of the species range, so that fewer intermediates reach flowering than in Illinois. Studies comparing survivability among hermaphrodites, females and intermediates should be done to determine if selection is occurring and whether it is stronger at the range boundaries. It is possible that the high frequency of intermediates found in our crosses compared to the populations in which they were collected is due to the nature of the crosses. While we primarily did interpopulation crosses, most individuals in local populations will be from intrapopulation crosses, which may lead to fewer intermediates. Similar crosses between

individuals from the same population should be done to determine if they also produce a large proportion of intermediates.

Future work

To better understand the genetic control of sex in *G. maculatum*, more crosses are needed. In particular, a larger sample of long distance crosses between hermaphrodites is needed to confirm that sex is not under cyto-nuclear control. To better understand the number of loci involved and how they may interact, selfing and/or backcrossing of the progeny would be useful. The results from our study can be used to determine which crosses would be most informative for these later generational crosses. An additional avenue to be explored is analysis of the progeny sex ratios using quantitative genetics. Our results suggest that this approach may apply to *G. maculatum* because of the complex inheritance of sex and the nearly continuous range of sex expression. There have been two notable attempts at modeling sex determination as a quantitative trait. The first study compared variation in progeny sex ratios due to the maternal and paternal individual from crosses within and among populations (Taylor et al. 2001). They found that in crosses between populations, there was a larger effect of the maternal individual compared to crosses within populations, presumably because the CMS types differed between populations. The second study modeled sex determination as a threshold trait controlled by many additive loci (Ehlers et al. 2005). As more "male" alleles are present in an individual, the "maleness" increases until a threshold is met and the individual is a hermaphrodite. Using maximum likelihood techniques, the "maleness" of individuals was predicted from crosses in *Plantago coronopus*, *Thymus vulgaris* and *Silene vulgaris,* which fit the observed progeny sex ratios well (Ehlers et al. 2005). Additionally, this

model predicted well the frequency of intermediates, which are easily accommodated in the analysis. Both of these analyses require an extensive set of reciprocal crosses (between individuals within and between populations), which were not yet available for our system. In the future, analyses like these may help in determining the details of the genetic control in *G. maculatum*, and may lead to a more prominent incorporation of the intermediate sex.

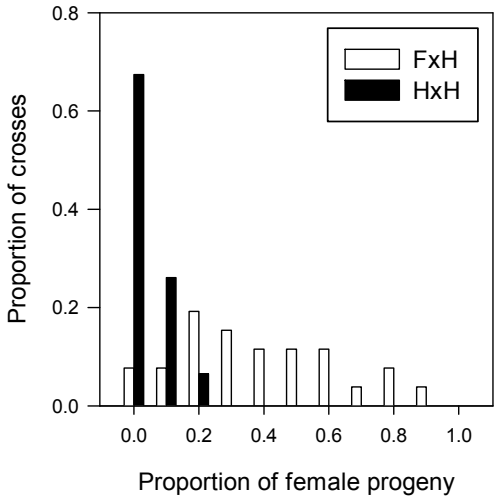


Figure 2.1. Distribution of the sex ratio (proportion female) of progeny in crosses with females (white bars) and between hermaphrodites (black bars).

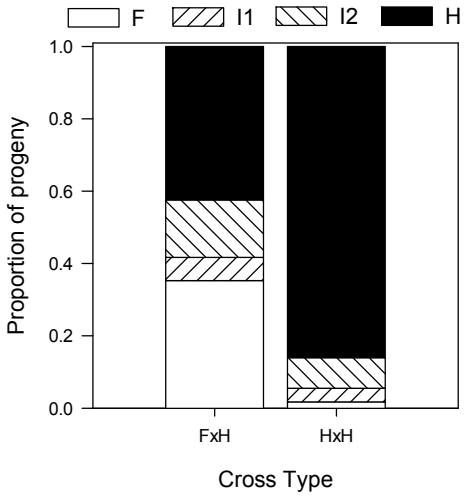


Figure 2.2. Proportion of progeny of each sex type (refer to results for description of intermediates) from crosses between hermaphrodites and females (FxH) and between hermaphrodites (HxH).

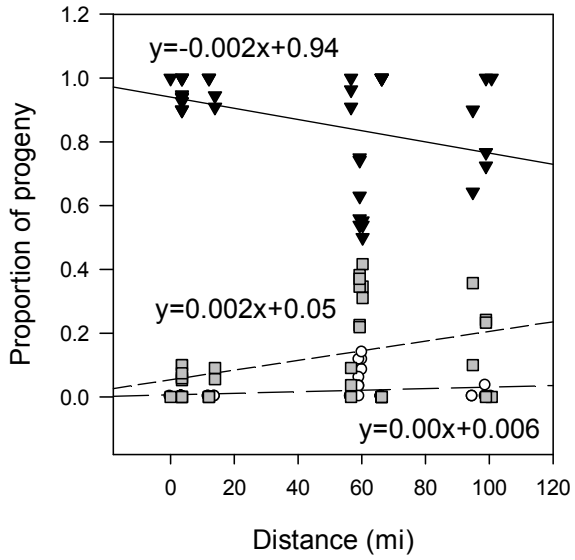


Figure 2.3. The proportion of intermediates (gray squares) increases with the population distance bewteen parents while the proportion of hermaphrodites declines (black triangles) and females remains the same (white circles) in crosses between hermaphrodites.

Table 2.1. Sex ratios and locations of each population from which plants were collected.

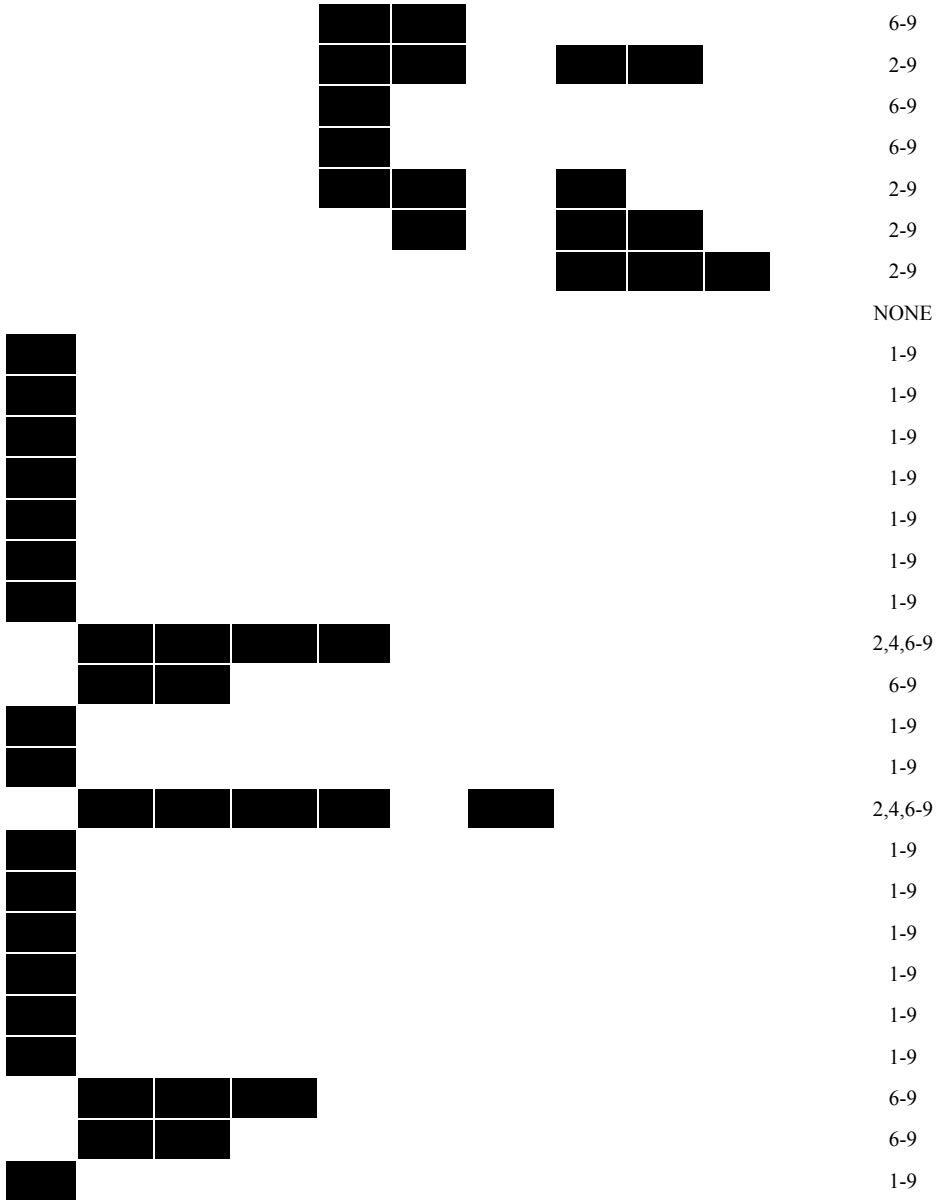
Population	Sex ratio (%F)	Location
52.1	0	GA
MP	35	GA
OT	50	GA
BH	10-20	GA
52.2	30-40	GA
GC	10-20	GA
GG	0	NC
SB	3	NC
IS	7	NC
JK	0	NC

Table 2.2 (next page). Predicted sex ratios (H:F) based on several genetic models compared to the observed sex ratios from all crosses. Models proposed to explain the genetic control are followed by the sex ratios that would result from that model for crosses between a female and a hermaphrodite (F) and between hermaphrodites (H). Below these predicted sex ratios are the observed sex ratios for all crosses made. Black boxes under a sex ratio indicate that the observed sex ratio does not differ from that predicted sex ratio using a chi-squared test ($P>0.05$). For each cross, the models with which the observed sex ratio does not conflict with the predicted sex ratios is listed. Msex is the sex of the ovule donor, Mpop and Ppop are the populations where the ovule and pollen, respecitvely, originated from. N is the number of progeny included in the analysis.

Model		Genotypes	H:F	1:0	15:1	7:1	13:3	3:1	5:3	9:7	1:1	3:5	1:3	0:1
			%F	0	6.25	12.5	18.75	25	37.5	43.75	50	62.5	75	100
1	Cytoplasmic	F=(C), H=(F)		H										F
2	Cyto-nuclear 1 dominant restorer	F=(C)rr, H=others		F H				H			F H			F H
3	Cyto-nuclear 1 recessive restorer	F=(C)Rr, H=others		H							F			
4	Nuclear hermaphrodite dominant	F=mm, H=others		F H				H			F			
5	Nuclear hermaphrodite recessive	F=Mm, H=mm		H							F			
6	Cyto-nuclear 2 dominant restorer	F=(C)$r_1r_1r_2r_2$, H=others		F H		H	H	F H			F H			F H
7	Nuclear 2 locus duplicate recessive	F=$m_1m_1m_2m_2$, H=others		F H		H	H	F H			F			
8	Nuclear 2 locus complimentary	F=$M_1\_\ m_2m_2$, $m_1m_1M_2\_$, $m_1m_1m_2m_2$, H=others		F H	H			F H		H	F	F	F	
9	Nuclear 2 locus epistatic	F=M_hh, H=others		F H		H	H	F H	F		F			

Maternal	Paternal	Msex	Mpop	Ppop	N	F%	H%	I%	Matches model:
17C8	BH2	F	OT	BH	10	80.00	0.00	20.00	8
17C8	IS3	F	OT	IS	17	76.47	5.88	17.65	8
52.2.6	8H10	F	52.2	MP	14	42.86	28.57	28.57	6-9
52.2.6	BH2	F	52.2	BH	17	23.53	58.82	17.65	6-9
52.2.6	IS20	F	52.2	IS	17	47.06	35.29	17.65	2-9
52.2.6	IS3	F	52.2	IS	20	25.00	40.00	35.00	6-9
52.2.6	SB24	F	52.2	SB	17	17.65	52.94	29.41	6-9
52.2.8	8H10	F	52.2	MP	15	53.33	6.67	40.00	2-9
52.2.8	BH2	F	52.2	BH	19	52.63	5.26	42.11	2-9
52.2.X6	52.2.9	F	52.2	52.2	13	15.38	84.62	0.00	6-9
5C3	52.2.18	F	MP	52.2	11	36.36	45.45	18.18	2-9
5C3	8H10	F	MP	MP	11	18.18	54.55	27.27	6-9
5C3	BH2	F	MP	BH	11	72.73	9.09	18.18	2-9
5C3	IS3	F	MP	IS	23	39.13	47.83	13.04	2-9
5C3	JK1.1	F	MP	JK	15	60.00	20.00	20.00	2-9

5C3	SB24	F	MP	SB	24	29.17	41.67	29.17	6-9
BH7	52.2.18	F	BH	52.2	13	38.46	46.15	15.38	2-9
BH7	8H10	F	BH	MP	23	8.70	73.91	17.39	6-9
BH7	IS3	F	BH	IS	20	15.00	50.00	35.00	6-9
BH7	JK1.1	F	BH	JK	10	30.00	30.00	40.00	2-9
GCF1	8H10	F	GC	MP	21	47.62	23.81	28.57	2-9
GCF1	IS3	F	GC	IS	12	66.67	33.33	0.00	2-9
GCF1	SB24	F	GC	SB	24	4.17	75.00	20.83	NONE
17H3	52.2.18	H	OT	52.2	14	0.00	64.29	35.71	1-9
52.1.22	GG26	H	52.1	GG	10	0.00	100.00	0.00	1-9
52.1.25	GG13	H	52.1	GG	10	0.00	100.00	0.00	1-9
52.1.27	GG28	H	52.1	GG	24	0.00	100.00	0.00	1-9
52.1.28	IS8	H	52.1	IS	20	0.00	100.00	0.00	1-9
52.1.30	IS13	H	52.1	IS	27	0.00	96.30	3.70	1-9
52.2.18	17H3	H	52.2	OT	10	0.00	90.00	10.00	1-9
52.2.18	IS20	H	52.2	IS	29	13.79	55.17	31.03	2,4,6-9
52.2.18	SB24	H	52.2	SB	32	3.13	75.00	21.88	6-9
52.2.18	SB26	H	52.2	SB	27	0.00	62.96	37.04	1-9
52.2.18	SB36	H	52.2	SB	9	0.00	88.89	11.11	1-9
52.2.9	GG11	H	52.2	GG	8	12.50	75.00	12.50	2,4,6-9
8H10	IS29	H	MP	IS	10	0.00	100.00	0.00	1-9
GG11	52.2.9	H	GG	52.2	4	0.00	100.00	0.00	1-9
GG13	52.1.25	H	GG	52.1	27	0.00	100.00	0.00	1-9
GG27	SB26	H	GG	SB	13	0.00	100.00	0.00	1-9
GG29	JK1.H	H	GG	JK	18	0.00	94.44	5.56	1-9
IS13	52.1.30	H	IS	52.1	33	0.00	90.91	9.09	1-9
IS20	52.2.18	H	IS	52.2	23	8.70	52.17	39.13	6-9
IS20	8H10	H	IS	MP	29	3.45	72.41	24.14	6-9
IS20	SB24	H	IS	SB	27	0.00	92.59	7.41	1-9



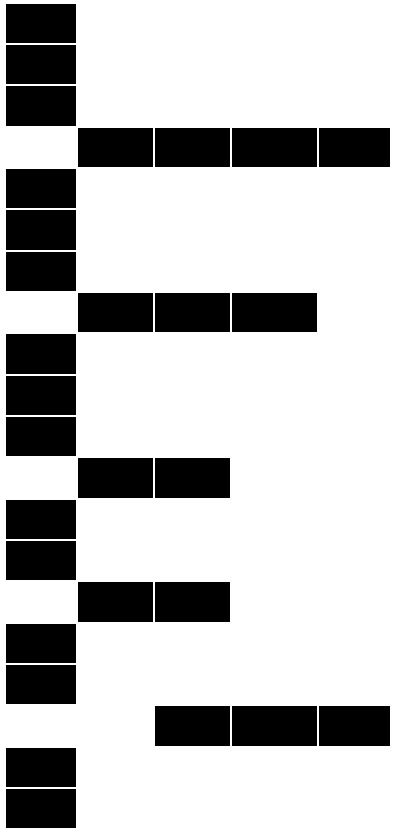
IS22	SB11	H	IS	SB	13	0.00	100.00	0.00		1-9
IS29	8H10	H	IS	MP	30	0.00	76.67	23.33		1-9
IS3	17H3	H	IS	OT	19	0.00	100.00	0.00		1-9
IS3	52.2.18	H	IS	52.2	26	11.54	53.85	34.62		2,4,6-9
IS36	SB40	H	IS	SB	16	0.00	100.00	0.00		1-9
JK1.H	GG29	H	JK	GG	22	0.00	90.91	9.09		1-9
SB11	IS22	H	SB	IS	10	0.00	90.00	10.00		1-9
SB24	52.2.18	H	SB	52.2	52	11.54	53.85	34.62		6-9
SB24	IS20	H	SB	IS	16	0.00	93.75	6.25		1-9
SB24	IS3	H	SB	IS	10	0.00	90.00	10.00		1-9
SB24	SB36	H	SB	SB	12	0.00	100.00	0.00		1-9
SB26	52.2.18	H	SB	52.2	34	5.88	55.88	38.24		6-9
SB26	GG27	H	SB	GG	47	0.00	100.00	0.00		1-9
SB26	IS20	H	SB	IS	24	0.00	100.00	0.00		1-9
SB36	52.2.18	H	SB	52.2	31	3.23	74.19	22.58		6-9
SB36	IS20	H	SB	IS	19	0.00	94.74	5.26		1-9
SB40	IS36	H	SB	IS	17	0.00	94.12	5.88		1-9
BH4	8H10	I	BH	MP	24	16.67	62.50	20.83		2,4,6-9
BH4	IS3	I	BH	IS	20	0.00	85.00	15.00		1-9
BH4	JK1.1	I	BH	JK	23	0.00	86.96	13.04		1-9

Table 2.3. Sex of progeny from reciprocal crosses that produced females. The chi-squared P is the P value that the progeny sex ratio depends upon which parent acted as the ovule donor. Black boxes under the predicted sex ratios (H:F) indicate that the observed sex ratio does not differ from that predicted sex ratio using a chi-squared test. Predicted sex ratios are based on different models of genetic control (see Table2).

						Predicted sex ratios (H:F)				
Maternal	Paternal	H	F	%F	χ^2 P	1:0	15:1	13:3	3:1	9:7
GG11	52.2.9	4	0	0	0.46	■				
52.2.9	GG11	7	1	12.5			■	■	■	■
IS20	52.2.18	21	2	8.7	0.57		■	■		
52.2.18	IS20	25	4	13.8			■	■	■	
SB24	52.2.18	46	6	11.5	0.18		■	■		
52.2.18	SB24	31	1	3.1			■			
SB26	52.2.18	32	2	5.9	0.2		■			
52.2.18	SB26	27	0	0		■				
SB36	52.2.18	30	1	3.2	0.59		■			
52.2.18	SB36	9	0	0		■				

CHAPTER 3

DEMOGRAPHIC DIFFERENCES BETWEEN SEXES AND THEIR EFFECT ON THE SEX RATIO IN A GYNODIOECIOUS SPECIES, *GERANIUM MACULATUM*[1]

[1] Van Etten, M.L. and S-M. Chang. To be submitted to *Ecology*.

ABSTRACT

Gynodioecy, the co-occurrence of females and hermaphrodites, is a breeding system that is the most likely transition from hermaphroditism to separate sexes. To prevent regression to hermaphroditism, the seed fitness of females must be higher than that of hermaphrodites. Though many studies have shown that females often produce more seeds than hermaphrodites, most studies fail to examine the entire life cycle. We compare multi-year seed production and natural germination and survival rates to examine sex differences throughout the life cycle. Additionally, we test how these differences may affect the population sex ratio. We found that while females produce only slightly more seeds than hermaphrodites, they flower more often, increasing their cumulative seed production. On the other hand, we found few differences between females and hermaphrodites in seed germination and survival under natural conditions. Models of the populations predict that females will be maintained at high frequencies and that this frequency depends primarily on adult survival. Our results indicate that only through examining the entire life cycle can we understand the high frequency of females found in this species.

INTRODUCTION

Gynodioecy, the co-occurrence of female and hermaphroditic individuals, is the most likely transition state from hermaphroditism to separate sexes, a major evolutionary transition in angiosperms (Barrett 2003). As such, this breeding system has drawn the attention of evolutionary biologists to explore the question of how and when this evolutionary transition occurs (e.g. Charlesworth and Charlesworth 1978; Darwin 1877;

Lewis and Crowe 1956; Lloyd 1974; Ross 1978). Much of the early work focused on mathematical models involving the seed fitness increase females must have to compensate for the loss of male function. These models have shown that to prevent the loss of females from a population, female seed fitness must be higher than hermaphrodite seed fitness, with the amount dependent upon the type of genetic control (Charlesworth and Charlesworth 1978; Charlesworth 1981; Lloyd 1974). In addition, they also demonstrated that the conditions that maintain a stable gynodioecious system were quite stringent and, therefore, a system is likely to either complete the transition to dioecy through increased male function of the hermaphrodites or to revert back to hermaphroditism through the loss of females. These early theoretical models, thus, suggested that it was very difficult to maintain both females and hermaphrodites within a population and gynodioecy was thought to be transitory (Lewis and Crowe 1956; Ross 1978).

While these early models adequately describe the sex ratio dynamics in some species (e.g. Ashman 2006; Weller and Sakai 2005; Wolfe and Shmida 1997), it has been shown that these models do not predict the observed sex ratios in several other species (e.g. Ashman 1999; Marshall and Ganders 2001). For example, in natural populations of *Sidalcea hendersonii*, there was no detectable female seed production increase, leading to a prediction that females could not be maintained (Marshall and Ganders 2001). Yet, natural populations had as high as 54% females. These deviations from the model predictions are generally attributed to ecological mechanisms (e.g. Ashman 2006; Marshall and Ganders 2001) or the type of genetic control (Bailey and Delph 2007a). Thus, traditional models are lacking in realistic details (either in ecology or in the genetic

control) that may be important in many gynodioecious species. More recent models have been more successful in predicting situations under which gynodioecy is evolutionarily stable. These newer models include frequency dependent effects (Maurice and Fleming 1995), a fitness cost of the restorer allele (Bailey et al. 2003; Delannay et al. 1981) or meta-population dynamics (McCauley and Taylor 1997). For example, seed production should be frequency dependant because as the frequency of females increases, the ratio of available pollen grains to the number of ovules in the population is decreasing (Maurice and Fleming 1995). Thus, at high female frequencies, females may produce fewer seeds than hermaphrodites and limit their increase, which may help to maintain both sexes. While these models are more realistic, they are often species specific and therefore, how widely they can be applied is unclear.

An alternative approach, demographic modeling, is less commonly used in gynodioecious systems, but can provide additional insight for the population dynamics of gynodioecious populations (Caswell 2001; Morris and Doak 1998; Ramula et al. 2007). As opposed to other modeling techniques, demographic modeling does not include as many assumptions, such as non-overlapping generations and equilibrium populations. Thus, this approach is more flexible and should be applicable to many species (Caswell 2001). Another of the advantages of demographic modeling is that it can easily incorporate the entire life cycle and not just the reproductive stage, i.e. seed production. This may be important in gynodioecious species because in addition to producing more seeds, females have also been found to have higher seed quality, a fitness difference expressed in the next generation (Shykoff et al. 2003). Additionally, the sexes may differ in life span, time to reproduction, or frequency of flowering, all of which could easily be

incorporated into demographic models. While traditionally studies have measured seed fitness as a single years' seed production, demographic modeling highlights how fitness is a function of differences in various stages throughout the entire life cycle, and therefore can provide a more comprehensive understanding of the evolution of a system.

Demographic modeling has been successfully used in recent studies to investigate the population dynamics of gynodioecious species. Morris and Doak (1998) used this approach to estimate lifetime reproductive success of a long lived alpine perennial, *Silene acaulis*. Based on fruit production for two years, females tended to produce 2.3-10x more fruit than hermaphrodites. However, after incorporating information about the life span and growth, it was estimated that females produced about 4.4 times as many seeds over their lifetime as hermaphrodites. Thus, through demographic modeling, fitness differences could be extrapolated for a very long lived plant (estimated as >300 years), which would be difficult using other modeling techniques. In another study, Ramula and Mutikainen (2007) used a demographic approach to investigate how inbreeding depression and pollen limitation may affect the maintenance of gynodioecy in the perennial *Geranium sylvaticum*. In their model, pollen limitation affected seed production while inbreeding depression affected several parameters including seed germination and survival. Using this approach, they determined that pollen limitation would have less of an impact on the maintenance of gynodioecy than inbreeding depression because the sex ratio was influenced little by changes in seed production. Only through demographic modeling is this result apparent. In both studies, demographic modeling allowed a more accurate model to be constructed and new insights into the maintenance of gynodioecy were gained.

In this study we use multi-year fitness measurements to create a demographic model to determine the potential long-term fitness differences between females and hermaphrodites of *Geranium maculatum*. Using these estimates in the demographic model, we determined the expected sex ratio and examined how different life stages affect the sex ratio. In particular we asked: 1) using multi-year observations, do females produce more seeds than hermaphrodites, 2) under field conditions, are the seeds produced by females of better quality than those from hermaphrodites, 3) using this data to construct a demographic matrix model, are females expected to be maintained and 4) what parts of the life cycle are most influential on the population sex ratio. We expected that females would produce more seeds and of higher quality, but as in other long-lived perennial species, seed production would have little effect on the equilibrium population dynamics, including the sex ratio.

METHODS

Geranium maculatum L. is a gynodioecious, rhizomatous perennial ranging from the South Eastern US to Canada and west to the Great Plains (Radford et al. 1968). Flowering begins in early spring, with individuals producing on average six flowers per inflorescence (Chang 2006). Flowers are visited by generalist pollinators including bees, flies and butterflies. Hermaphrodites are self-compatible and selfing rates range from 0-17% depending on the population (Van Etten et al., Chapter 4). Inbreeding depression is high and variable between populations (cumulative postdispersal inbreeding depression ranges from 0.38 to 0.84, Chang 2007). Seeds are dispersed by the elastic dehiscence of the schizocarp, to an average of 3 m from the maternal plant (Stamp and Lucas 1983).

Based on field and greenhouse observations, sex appears to be genetically determined. Preliminary data rule out the possibility that sex is controlled entirely by cytoplasmic genes, but the exact genetic control has not yet been determined (Van Etten and Chang, Chapter 2). Females have small aborted anthers and smaller petals when compared to hermaphrodites (Ågren and Willson 1991; Chang 2006). Flower number is approximately the same between sexes in natural populations (Ågren and Willson 1991; Chang 2006) though in one greenhouse study females produce slightly more flowers than hermaphrodites (Van Etten et al. 2008). In natural populations, yearly seed production is higher for females than hermaphrodites (20 – 50% increase, Ågren and Willson 1991; Chang 2006) and seeds from females have a higher germination rate than those from hermaphrodites under greenhouse conditions (Chang 2006). Populations around Athens, GA range in female frequency from 0-50% (Chang 2006).

Three populations were chosen near Athens, GA for demographic measurements. Two populations (OT and OTG) were located in the Georgia State Botanical Gardens (Athens, GA; OT:33˚54'4.92"N, 83˚22'47.34"W; OTG: 33˚54'4.90"N, 83˚22'44.93"W) and the other population was located in the Redlands Wildlife Management Area (Oconee County, GA; RL:33˚45'44.34"N, 83˚15'53.58"W). All three populations were in the understory of piedmont forests with a nearby flowing stream. Previous environmental measurements taken on these populations indicate that RL has higher soil moisture than OT and OTG but similar light availability (Van Etten and Chang 2009).

Seed production for females and hermaphrodites in each population were observed for 3 or 4 years. Because of population differences in density and sex ratio, the method of choosing plants differed for each population. In OT, plants were selected by

first creating a grid of 3 x 3 m squares and at each intercept of the grid lines, the closest female and hermaphrodite were chosen. If there were no plants of a given sex within 3 m from the intercept, that sampling point was not used. This led to 48 females and 48 hermaphrodites chosen in 2006 and monitored through 2009. In OTG and RL, which are less dense than OT, females throughout each population were randomly chosen. Each female was paired with a nearby hermaphrodite. This led to 47 females and 47 hermaphrodites in OTG and 18 females and 32 hermaphrodites in RL that were chosen in 2007 and monitored through 2009. These sampling schemes allowed the majority of the populations' area to be sampled.

Adult plants were monitored for a variety of characters. Survival from one year to the next was monitored. Reproductive measures included flower number, seed number and fruit number. Seeds were collected by covering inflorescences with bridal veil bags to prevent seed dispersal. Leaf number was also counted in 2009 for all populations as an estimate of plant size.

Because of the low number of seedlings observed in natural populations, data on germination and early seedling survival were estimated by creating three experimental plots, which were established near each of the three target populations. Plots were placed in similar environments as the natural populations, but were located at least 50 m from the nearest natural populations to prevent unwanted seed dispersal into the plots. To monitor individual seed germination, seeds collected from female and hermaphrodite plants in natural populations were glued to wooden sticks using Elmer's glue and planted into each of the plots. A pilot study showed that this method does not reduce germination rates of scarified seeds in the greenhouse (80% germination for both glued and the not

glued control). Seeds were planted in OT in the fall of 2007 (N_F=69, N_H=116), and in OTG (N_F=249, N_H=276) and RL (N_F=604, N_H=629) in the fall of 2008. Because we expected seed germination might be too low to obtain sufficient sample sizes for the later life stages, seeds were also germinated in the greenhouse to generate seedlings that were used to estimate survival of this life stage (OT N_F=545, N_H=255; OTG N_F=91, N_H=101; RL N_F=113, N_H=306). Seeds were weighed (except for OT seeds), scarified to facilitate germination and then cold stratified at 4°C in water for 4 weeks. Seeds were then planted in pine bark mix and left in the misting room in the greenhouse for 4 weeks to allow seedling establishment. Germination and leaf number of these young seedlings was measured weekly, but this data was not used in the demographic model. Four weeks after planting, surviving seedlings were planted into experimental plots and their survival and leaf number monitored (called seedlings throughout paper). Seedlings were transplanted in the OT plot in the spring of 2007 and in OTG and RL plots in the spring of 2008.

To determine the survival and reproduction of young plants as well as the sex ratio of progeny arrays from females and hermaphrodites, young plants that spent 2 growing seasons in the greenhouse were also planted in the OT plot (hereafter referred to as juveniles). Seeds that gave rise to these plants were originally collected from OT and their growth conditions were similar to those of seedlings described previously. After ~8 weeks of growth in the greenhouse, plants generally show signs of senescence so their leaves were removed and the plants placed into a 4°C cooler to mimic winter. This process was repeated once more before plants were planted into the OT plot. Survival, clonal growth, leaf number, sex, flower number and seed production were monitored

after planting. Juveniles were only available for OT (N_F=96, N_H=95) and were planted in the spring of 2007.

Data analysis

Reproductive and survival data from adult plants were analyzed to determine if the sexes differed significantly. Sex differences in survival, frequency of flowering and cumulative seed production were tested using an ANOVA with population, sex and population*sex as predictor variables. Differences between sexes in flower number, fruit number, seed number, fruit set (fruits produced/flowers produced) and seed set (seed number/fruit number) were tested using a repeated measures ANOVA with population, sex, population*sex, as the between subjects effects tested against the sums of squares of the plant ID within population*sex effect, and the ID, year, population*year, sex*year, population*sex*year as the within subject effects. If no flowers or seeds were produced, the reproductive traits were zero. Proc glm in SAS was used for these analyses (SAS Inc. 2000).

To examine sex differences in seed quality the following traits were examined: seed germination, seedling survival and growth and juvenile survival, growth and reproduction. The germination rate of seeds planted in the plots was not tested because of the extremely low number of germinants in each plot. Differences between sexes in the germination of seeds from OTG and RL in the greenhouse was tested using an ANOVA with maternal sex, population, tray number and maternal sex*population as predictors and seed weight as a covariate. OT seeds were not included in this analysis because no information on seed weight was collected. Growth of seedlings while in the greenhouse was examined at each measuring time using an ANOVA with maternal sex,

germination tray and population maternal sex*population as predictors and seed weight as a covariate. The effect of seedling plant size on survival and growth in the field was examined using an ANOVA with the survival (binary) of the plant or leaf number as the response variable and maternal sex, population, maternal sex*population as predictors and growth stage as a covariate. Proc glm in SAS was used for these analyses.

To examine the effect of maternal sex on survival and reproduction in juvenile plants, differences in the following traits were tested using a repeated measures ANOVA in Proc glm with maternal sex tested against ID within maternal sex as the between subject effects, and ID, year, maternal sex*year as the within subject effects: survival, flowering, flower number, fruit number, seed number, fruit set, seed set and clonal growth. Differences in leaf number due to maternal sex was tested using a t-test. Too few juvenile individuals flowered to adequately test whether the sex of the juvenile (rather than the maternal sex) affected any of the traits or if there is an interaction between maternal sex and progeny sex.

To combine the effects of all life stages, we created a demographic matrix model, which uses transitions between life stages to determine population growth rates and the composition of the population at equilibrium. These models can be depicted in a life-cycle diagram (Fig. 3.1), with the squares depicting each life stage and the arrows depicting the transitions between life stages. Arrows returning to the same life stage indicate that some individuals remain in that life stage for more than one year. Based on field observations, we created a model with 6 different life stages for each sex for a total of 12 life stages (Fig. 3.1). Because seed germination was observed in both year 1 and year 2 in the OT plot, flowering individuals may contribute both to next years' seedlings

and to a seed bank. Seedlings are modeled as requiring 2 years before becoming juveniles based on field observations, which suggest that seedlings grow very little between years (most seedlings had only 1 small leaf the year after they were planted). A two-year period for the seedling stage is most likely an underestimate of the time individuals spend as very small plants. After the seedling stages, plants may become juveniles. Observations from the experimental plots show that juveniles generally have more and larger leaves than seedlings, resembling adult plants morphologically, but not yet flowering. Plants in the juvenile stage can remain juveniles or transition into flowering. Transition rates between the juvenile stage and either one of the sexes were determined from the sex ratios produced by the juveniles in experimental plots. Flowering plants may either remain flowering the next year, or become non-flowering adults.

Separate life stages for progeny from females and hermaphrodites were used because the quality of the progeny may differ and because transition rates from juvenile to either sex may be very different between progeny from females and hermaphrodites. It has been shown in this species that female progeny have higher germination and survive better than those from hermaphrodites under greenhouse conditions (Chang 2006). To include any potential differences from our data in the model, separate life stages for progeny from females and hermaphrodites must be used. The differences in the transition rates from juvenile to either sex may also be important to the final sex ratio. Progeny from crosses in the greenhouse have shown that hermaphrodites produce almost no females while females produce about equal proportions of females and hermaphrodites

(Van Etten and Chang, Chapter 1). These substantial differences may have a large impact on the final sex ratio and thus require separate life stages.

Transition rates were calculated based on the data collected at both the natural populations and the experimental plots. From the plots, the following transition rates were calculated (with the type of plants used in parentheses): germination rate year 1 (seeds), seed bank germination rate (seeds from OT), seedling survival (seedling year 1), survival of seedlings to a second seedling year (seedlings year 2 from OT), juvenile survival (juveniles year 2 and 3 from OT), juvenile transitions to flowering (juveniles from OT). From the natural populations, the following transition rates were calculated for each population: adult survival, adult flowering frequency and adult seed production. Because germination rates were so low, an average of all the plots was used for each population. Each population was modeled separately with their own seedling survival and adult parameters. The other parameters in the model were obtained from the OT plot, which was the only plot that had juveniles and had more years of data than the other two plots. To obtain a more general model, the populations were pooled into one model using the averages over all populations.

For each of the models, the population growth rate (λ), the stable age structure and the sex ratio were obtained. The population growth rate is equivalent to the net birth rate per individual. It is independent of the composition of the initial population and is obtained directly from the matrix of transitions (Caswell 2001). A value greater than one indicates that the population is growing in number while a value less than one indicates the population is decreasing in number. The stable age structure was also calculated, which is the proportion of the population in each stage class after the proportions

stabilize, so that each year the proportion in each stage is the same. The stable age structure is independent of the composition of the starting population and can be obtained directly from the matrix of transitions. The sex ratio was calculated using the stable age structure as the female flowering plants/(female flowering plants + hermaphrodite flowering plants). The proportion of female flowering plants (rather than the proportion of female adult plants) was used to enable comparisons with the observed sex ratio in natural populations, which is necessarily calculated based only on the flowering plants. The number of times an individual flowers during its lifetime was determined for each sex using a Markov Chain technique, which basically computes the average number of times an individual will pass through a stage before dying (Caswell 2001, performed using PopTools in Excel). This number was then multiplied by the average number of seeds produced by each sex to determine the total reproductive output of an average individual of each sex.

To determine the effect of changes in transition rates on the sex ratio, we conducted a sensitivity and elasticity analysis of the transitions on the sex ratio (Caswell 2001; Veran and Beissinger 2009). A sensitivity analysis is a method used to determine the effect that small changes in transition rates have on population dynamics. For example, we can calculate the sensitivity of the proportion of females and hermaphrodites to perturbation of a parameter in our model. A parameter may change a single transition rate or multiple transition rates. For example, in our model, a change in seed production (the parameter) changes two transition rates: the number of seeds in the seed bank and the number of seedlings. If a parameter changes more than one transition rate, then the sensitivity of each of the affected transitions is added together. Large sensitivities imply

that changes in that parameter would greatly influence the sex ratio while small sensitivities imply there would be less of a change. Sensitivities were calculated using the equations in Veran and Beissinger (2009). Elasticities were also calculated, which essentially are proportional sensitivities. Elasticities can be interpreted as the amount of change in the sex ratio due to a 1% increase in a parameter, such as seed production. For both sensitivities and elasticities, the sex ratio was calculated as female flowering plants/total flowering plants. Thus, a positive sensitivity or elasticity indicates an increase in the proportion of females while a negative one indicates a decrease in the proportion of females. Sensitivities and elasticities were calculated for the matrix model in which the averages across populations were used. Except where noted, all matrix calculations were done in SAS using Proc iml.

RESULTS

Sex differences in reproduction and survival

Females and hermaphrodites produced similar numbers of flowers ($F_{1,168}$=0.57, P=0.45), fruits ($F_{1,166}$=2.24, P=0.13) and seeds ($F_{1,164}$=1.20, P=0.27) when flowering (Table 3.1). However, females had a significantly higher cumulative seed number than hermaphrodites ($F_{1,234}$=12.03, P=0.0006, Fig. 3.2). This result was in part because females flowered more frequently than hermaphrodites ($F_{1,191}$=6.56, P=0.01). Over the 3 or 4 year study period, females produced between 1.6-2.1 times more seeds than hermaphrodites (Table 3.1, Fig. 3.2).

Seed germination

Seeds from both sexes had very low germination rates in the field (0-7.35%, only 33 germinants total from 1,943 planted seeds). Germination the first year after planting from females ranged from 0-4.8% and from hermaphrodites ranged from 1.11-4.31% depending on which plot was used (Appendix 1). In the one plot monitored for additional germination the second year after seed planting, the germination rate was similar between the sexes (F_{mean}=7.35%, H_{mean}=7.21%) and there was a 60% (N=5 seeds) survival rate for the first year following germination.

Seeds planted in the greenhouse had a substantially greater germination rate than those in the field. Although females did not produce significantly heavier seeds ($F_{1,606}$=0.61, P=0.4352, F_{mean}=5.06mg, H_{mean}=4.97), their seeds had a higher germination rate (OTG: F_{mean}=76.92% germination, H_{mean}=71.29%; RL: F_{mean}=93.81%, H_{mean}=91.84%; $F_{1,599}$=4.55, P=0.03). Seedlings from females had more leaves at each measuring time (week 1: $F_{1,599}$=11.87, P=0.0006; week 2 $F_{1,599}$=13.04, P=0.0003; week 3 $F_{1,599}$=8.40, P=0.0039; week 4 $F_{1,599}$=11.67, P=0.0007).

Survival in the field

Although seedlings from females were larger prior to planting in the field, and larger seedlings had a higher survival rate ($F_{1,470}$=15.35, P=0.0001), the maternal sex did not significantly affect leaf number ($F_{1,470}$=0.40, P=0.5257, F_{mean}=1.74, H_{mean}=1.78) or survival to the following year ($F_{1,1084}$=0.17, P=0.6788, F range:21.7-59.21% survival, H range:20-62.6%).

For juveniles, which were grown in the greenhouse for two growing seasons prior to being planted in the field, those from females tended to have lower survival rates

($F_{1,389}$=3.09, P=0.08), with the greatest difference the year after they were planted (F_{mean}=91.67% survival, H_{mean}=94.52%), though this difference was not statistically significant.

Early flowering

During the year in which they were planted (their 3rd growing season), 19-24% of the juveniles flowered. An additional 50-57% flowered the year after they were planted (4th growing season) and 18% the second year after they were planted (5th growing season). Plants from female mothers had a slightly higher flowering rate ($F_{1,195}$=1.94, P=0.17, F_{mean}=36.2% flowering, H_{mean}=29.8%). The plants that flowered produced on average 5.1 flowers (N=192, S.E.=0.22). There was no effect of maternal sex on flower number ($F_{1,120}$=0.07, P=0.79, F_{mean}=5.2, H_{mean}=5.0), fruit number ($F_{1,100}$=0.80, P=0.37, F_{mean}=2.98, H_{mean}=2.59), seed number ($F_{1,98}$=1.28, P=0.26, F_{mean}=9.0, H_{mean}=7.7), fruit set ($F_{1,102}$=0.10, P=0.75, F_{mean}=0.59, H_{mean}=0.58) or seed set ($F_{1,98}$=0.53, P=0.47, F_{mean}=2.9, H_{mean}=2.7) in the juveniles that flowered. The maternal sex also did not affect clonal reproduction ($F_{1,197}$=1.44, P=0.23, F_{mean}=1.34 ramets, H_{mean}=1.50) or the number of leaves ($F_{1,495}$=3.24, P=0.07, F_{mean}=2.8, H_{mean}=2.6).

Matrix models

The populations differed in their population growth rates and the predicted sex ratios (Table 3.2). In both OT and OTG, lambda was less than one, indicating that the population is declining, while RL had a lambda of slightly greater than one, indicating a growing population. The populations also differed in their sex ratio at stable age structure. OT had a much lower expected sex ratio (17% F) than the other two

populations (OTG:33%F, RL:41%). The average matrix model predicts a growing population (λ=1.02) and a stable sex ratio of 24% F.

Using the average model, females had a lower reproductive value than hermaphrodites. Females had a reproductive value of 2.44 while hermaphrodites had a value of 2.58, indicating that hermaphrodites will contribute slightly more to the future generations than females. This is in part due to different expected lifetime production of seeds for females and hermaphrodites. For females, an individual was predicted to flower on average 13.5 times, producing a total of 64.2 seeds. Hermaphrodites on average were predicted to flower 40.7 times, producing a total of 147.9 seeds. This large difference is caused by the lower death rate of adult hermaphrodite non-flowering plants than females (estimated from natural populations as 1.3% vs 10.1%, respectively). When the differences in death rates are removed, females produce on average 100.8 seeds in their lifetime compared to 40.3 from hermaphrodites, which is 2.5 times more seeds than hermaphrodites.

Sex ratio sensitivities and elasticities

Seed production and early survival had little effect on the final sex ratio (Fig. 3.3). Increasing hermaphrodite or female seed production had almost no effect on the sex ratio (a 1% increase in female seed production increased their sex ratio by 1%). The sex ratio was even less sensitive to changes in germination rates, a 1% increase in female seed germination only increased female frequency by 0.2%. There was also little effect of increasing seedling survival on the sex ratio (a 1% increase in seedling survival increased female frequency by 0.9%).

Unlike early survival, changes in the juvenile transitions had a larger effect on the sex ratio. An increase in the survival of female juveniles increased the frequency of females (a 1% increase increases female frequency by 5%). An increase in the survival of hermaphrodite juveniles also increased the frequency of females (a 1% increase increases female frequency by 7%). This unexpected result stems from the frequency of females and the transition rates from juvenile to one of the sexes; when females are at low frequency, the majority of the females in the population are produced from hermaphrodites, and therefore an increase in juveniles from hermaphrodites can increase the frequency of females. When females are more prevalent this result disappears; using the RL model, which predicts 41% females, to calculate sensitivities leads to a more intuitive results (Female juvenile survival Sensitivity=1.23, Hermaphrodite juvenile survival Sensitivity =-0.06).

The sex ratio was most affected by changes in adult traits, with adult survival and the frequency of repeat flowering having the largest sensitivities and elasticities. A 1% increase in female survival increased their frequency by 24%. Similarly, if females flower more often, then their frequency increases (1% increase led to 8.5% increase in female frequency). These strong results could be simply a product of the stage at which the sex ratio is determined, such that any increase in adult survival or the frequency of flowering changes the sex ratio. Additionally, higher survival and frequency of flowering may change the sex ratio through increasing seed production. Some of the results may be explained by this because a 1% increase in the frequency of female flowering increases the proportion of females' progeny in the seed bank, seedlings and juveniles, each by

~9%. If these progeny survive to flowering they could then increase the frequency of females.

DISCUSSION

In this study, we examined demographic differences between females and hermaphrodites and evaluated the effect these differences have on the population sex ratio. We found that while female flowering plants produced only slightly more seeds than hermaphrodites on a yearly basis, females flowered more often leading to higher cumulative seed production. Despite finding only small differences between females and hermaphrodites in germination and survival in early life stages, our models predict that females would be maintained at much higher frequencies than predicted using more traditional models. The population sex ratio was primarily affected by the sex ratio of the progeny and adult survival. These results highlight the necessity of using demographic data, rather than single year seed production, for predicting the evolutionary outcome of gynodioecious systems.

Relative seed fitness

We found that females produced more seeds than hermaphrodites when seed production was measured over multiple years. When examining a single year's data, females would have 1.16-1.75x hermaphrodites' seed production. However, because females reproduce more often, over the 3-4 years observed, females cumulatively had 1.6-2.07x hermaphrodites' seed production. Similar to our results, most studies have found that females produce more seeds than hermaphrodites (Shykoff et al. 2003).

However, few studies have investigated sex differences in the flowering frequency in gynodioecious systems (but see Agren and Willson 1994; Morris and Doak 1998). Sex differences in flowering frequency have been found in dioecious species but with the opposite trend; males tend to reproduce more frequently than females (Lloyd and Webb 1977; Meagher and Antonovics 1982; Thomas and Lafrankie 1993). These differences have often been attributed to females having higher reproductive costs than males, limiting the frequency of reproduction. Following this logic, our results could suggest that hermaphrodites have higher reproductive costs. Indeed, other authors have suggested high reproductive costs in hermaphrodites because they produce seeds and pollen while females only produce seeds (reviewed in Case and Ashman 2005). Alternatively, the higher reproductive frequency of females could be due to females garnering more resources through higher photosynthetic rates or nutrient absorption (e.g. Caruso et al. 2003). However, this does not seem to be the case in *G. maculatum*, at least for photosynthesis (Van Etten et al 2008). Finally, females may flower more frequently at the expense of their life span, which is consistent with our results of females having slightly higher adult mortality. However, even though our study represents one of the longer term studies of gynodioecious species, to adequately estimate life span, we need an even longer study period. Together, evidence from gynodioecious and dioecious species show that estimating seed fitness in perennial species requires more than a single year's seed production.

Surprisingly, we found no differences between sexes in seed mass, which is often used as an indicator of progeny quality (Tremayne and Richards 2000). This is in contrast to most gynodioecious species, in which females have heavier seeds than

hermaphrodites (Shykoff et al. 2003; Zhang et al. 2008). Our results also contradict previous findings in this species that females had slightly heavier seeds than hermaphrodites (Chang 2006). Similar to finding no differences in seed mass, we found few significant differences in progeny quality. Although germination rates have been found to be higher in female progeny in the greenhouse (this study, Chang 2006), there was no consistent difference in the field. In the greenhouse, progeny from females grew more quickly, but once planted into the field there were no differences in their survival or growth. These results suggest that there is the potential for differences in progeny quality under optimal conditions, but these differences may be difficult to detect under harsher or more variable field conditions. A couple of studies have attempted to evaluate progeny quality in the field and have found similar results. Ashman (1992) found that progeny from the sexes differed in the greenhouse in germination and juvenile growth rate but in the field they were only significantly different for juvenile growth. Similarly, Molina-Freaner and Jain (1992) found no difference in seedling survivorship under field conditions. The results from these studies suggest that differences in progeny quality that are often found under greenhouse conditions may not be indicative of differences under natural conditions, which may explain differences between our results and previous studies (Chang 2006; Van Etten et al. 2008).

Sex ratio

Seed production has traditionally been considered as the key factor for predicting sex ratio. For example, everything else being equal a 2x seed fitness will allow females to invade a population. Several theoretical studies extend this basic prediction to allow variation in other factors such as the selfing rate and inbreeding depression (Charlesworth

and Charlesworth 1978). Using the data obtained from this and previous studies, we can calculate the necessary female seed fitness for them to be 1.71 to 1.74x that of hermaphrodites (s=0.17, Van Etten et al, Chapter 4; δ=0.76-0.84, Chang 2007; equation 4 in Charlesworth and Charlesworth 1978). This requirement was met in two of the three populations. However, the sex ratio predicted by this model is much lower than those observed in the natural populations (OT predicted: 1.6-3.0%F, OTG: 14-15%F, RL: 0%F). Even using a selfing rate that corrects for inbreeding depression during seed development (s=0.153 or 0.397, Chang 2007), the sex ratios at most would reach 25%, which is still much lower than the observed sex ratios. Another theoretical model that predicts the sex ratio based solely on the relative seed fitness of the genders (Lloyd 1976) predicts that females would be maintained only in OTG if using seed number as the measure of fitness. These results show that in *G. maculatum*, single year estimates of seed number are not a good measure of seed fitness and cannot be used to make accurate predictions regarding the maintenance of gynodioecy.

The matrix model used in this study predicts a frequency of females substantially higher (ranging from 15 to 50%F) than those from Charlesworths' or Lloyd's models (0-25%F) and are closer to the observed sex ratios (25-50%). The higher sex ratio predicted here is in part due to the matrix model specifically incorporating a more comprehensive measure of fitness, rather than just emphasizing seed production. In addition, the matrix model makes no assumptions of overlapping generations and can incorporate multiple reproductive bouts. Especially in perennial species, violations of the theoretical models' assumptions may influence the predicted sex ratios. Additionally, the matrix model incorporates the specific genetic control through inclusion of the transition rates from

juvenile to either sex. Both Charlesworths' and Lloyd's model used here are based on a simple type of nuclear control, which may not be the case in most gynodioecious species (e.g. Charlesworth and Laporte 1998; Van Damme 1983). The matrix model simplifies this issue by using the progeny sex ratios that actually occur in nature.

Despite the matrix model predicting higher female frequencies than other models, predicted frequencies are still quite different from the natural populations. In particular, the matrix model predicts a much lower sex ratio in OT and a much higher sex ratio in RL than those found in the actual populations. These differences could be caused by several factors. First, the sex ratio predicted by the matrix model is the sex ratio when the proportion of individuals in each stage remains the same between years. In a species, such as *G. maculatum*, with low turnover rates and long maturation times, the time required to achieve this equilibrium may be many generations (Caswell 2001) and hence the natural populations may not yet be at equilibrium. Second, this model is completely deterministic. Both environmental and demographic stochasticity may influence the sex ratio and merit further exploration. Third, the matrix model used here assumes all transitions remain the same. This is clearly not the case for many of the transitions, which may be influenced by the sex ratio. Three factors that may vary with the sex ratio is pollen limitation, the selfing rate (Miyake and Olson 2009) and transitions of juveniles to either sex. In *G. maculatum*, pollen limitation does not appear to be frequency dependant nor does the selfing rate (Van Etten and Chang, Chapter 6, unpublished data). The most important transition rate that may change with the sex ratio is the transition between juveniles to either sex. Under either type of genetic control, the progeny sex ratios will be highly dependent upon the frequency of alleles determining sex, which is

expected to change with different sex ratios. A model that incorporates allele frequency changes in the sex determining loci is needed to determine how a dynamic system may evolve over time.

Sensitivity of the sex ratio

The sex ratio was most affected by the transition from juvenile to either sex and adult survival. These results seem to stem from both the very low germination rates (0-4.82%) and the high survival of adult plants (97.7-99.6% survival). These high survival rates lead to plants having many reproductive bouts once becoming established (~15-40 times). The sensitivities suggest that given the very low germination rates found in this study, increasing the frequency of flowering may increase the total seed fitness more than increasing the seedling survival or germination rates. The transition rates from juvenile to either sex also had a large influence on the sex ratio, but in an unexpected way. An increase in juvenile survival from hermaphrodites increased the female frequency, rather than the hermaphrodite frequency. This result stems from a large portion of the females being produced by adult hermaphrodites when females are at low frequencies. No other studies have directly investigated how changes in demographic traits may influence the sex ratio in plant species. However, though not specifically tested, Ramula and Mutikainen (2007) also found that changes in yearly seed production had little effect on the sex ratio, while changes to germination, juvenile survival and the transition into flowering had greater effects on the sex ratio in *Geranium sylvaticum*. Together, our study and the Ramula and Mutikainen (2007) study show that seed production, which is the focus of many theoretical and empirical studies, had less of an effect on the sex ratio

than expected and that other demographic factors deserve more attention, particularly for perennial gynodioecious species.

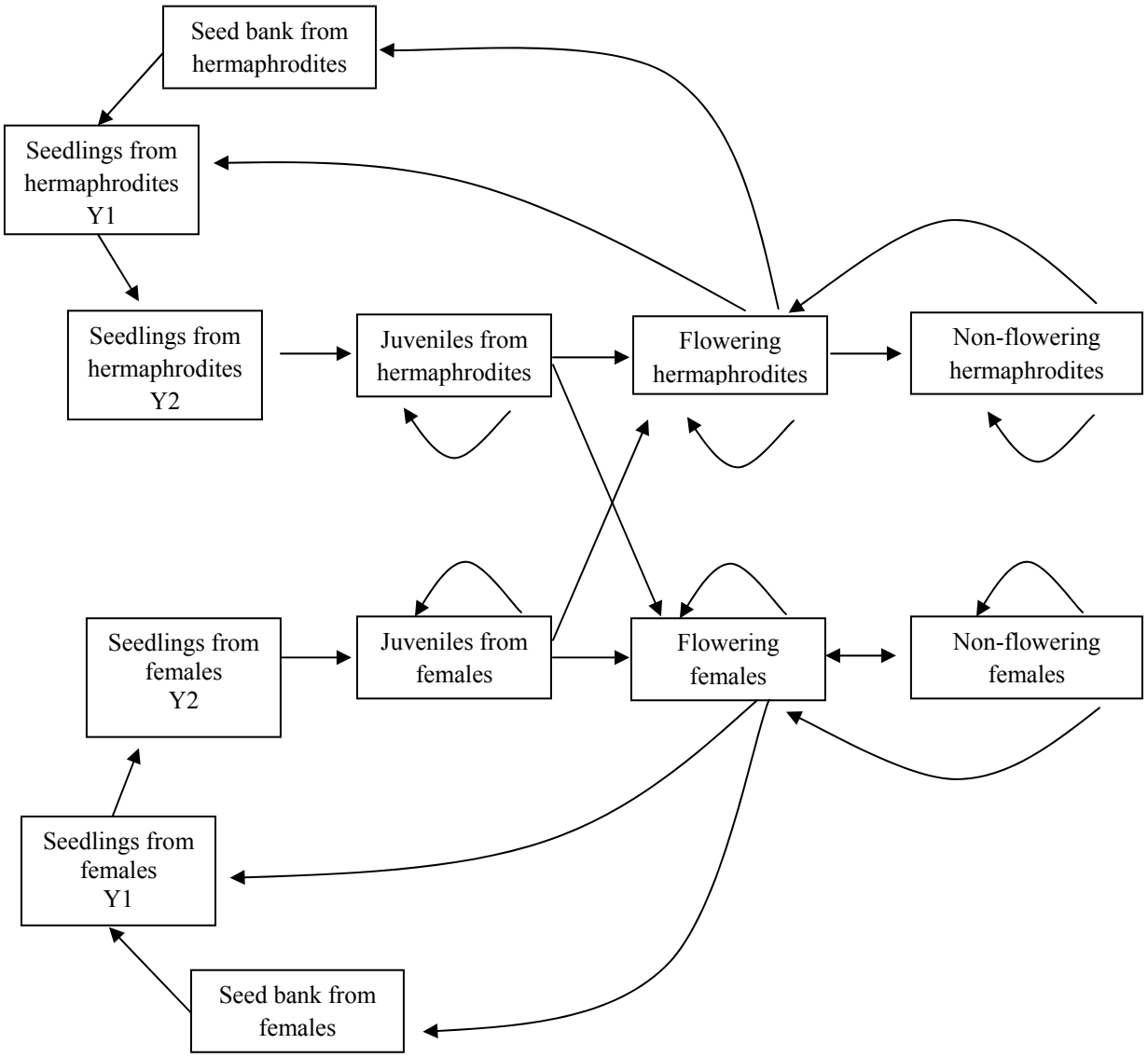


Figure 3.1. Life-cycle diagram of matrix model with contributions from hermaphrodites on the top and from females on the bottom.

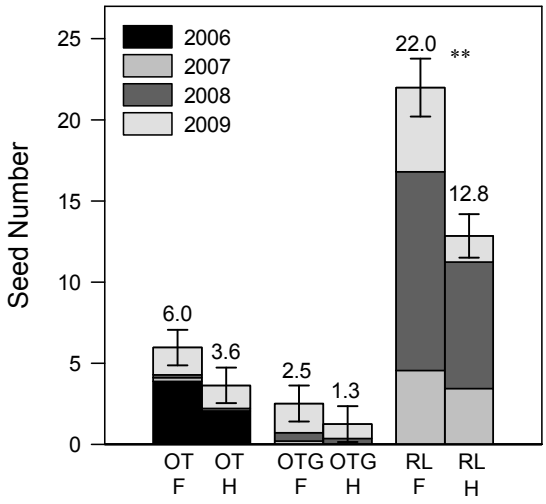


Figure 3.2. Cumulative seed production for females and hermaphrodites in each population. The contribution of each year is shown in a different shade of grey. Values above bars indicate the average cumulative seed number. Error bars indicate 1 SE.

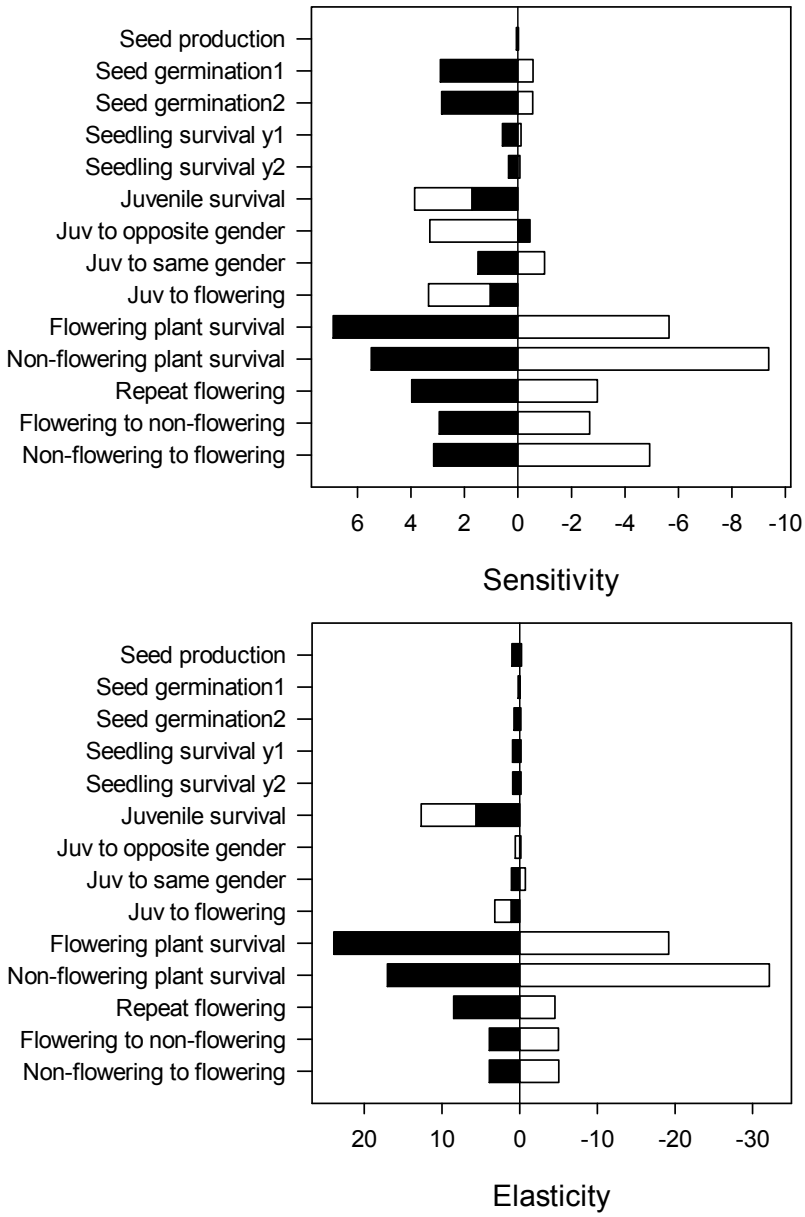


Figure 3.3. Sensitivity (A) and elasticity (B) of the sex ratio to several parameters. Solid bars indicate changes to the female parameter while empty bars indicate changes to hermaphrodite parameters. Positive sensitivities or elasticities indicate an increase in the frequency of females. Stacked bar values represent the sum of female and hermaphrodite values.

Table 3.1. Reproductive measures for females and hermaphrodites in each population. Asterisks under P indicates a significant difference among sexes using a t-test.

Pop	Sex	Flower number	P	Seed number	P	Seed set	P	Fruit number	P	Fruit set	P	Cumulative seed number	P	F/H seed number
OT	F	2.30		0.65		0.20		0.30		0.06		5.19		1.77
	H	2.53		0.48		0.15		0.23		0.05		2.94		
OTG	F	3.01	**	0.78		0.17		0.33		0.04		2.09		2.09
	H	1.70		0.44		0.11		0.15		0.02		1.00		
RL	F	6.52	*	6.87	**	1.18	*	2.33	***	0.27	**	20.06	**	1.60
	H	5.22		4.32		0.84		1.41		0.17		12.50		

Table 3.2. Population growth rates, relative seed number and sex ratio for each of the matrix models examined.

Model	λ	Relative F seed number	Predicted sex ratio (%F)	Actual sex ratio (%F)
OT	0.961	1.37	16.6	50
OTG	0.997	1.75	32.5	50
RL	1.081	1.21	41.3	25
Average of all populations	1.015	1.311	24.4	

Table 3.A1 (next page). Transition probabilities for model averaging over populations, with population specific values in parentheses (OT, OTG, RL).

		Female						Hermaphrodite					
		Seed bank	Seedling 1	Seedling 2	Juvenile	Flowering adult	Non-flowering adult	Seed bank	Seedling 1	Seedling 2	Juvenile	Flowering adult	Non-flowering adult
Female	Seed bank	0	0	0	0	4.76 (2.48, 2.54, 9.27)	0	0	0	0	0	0	0
	Seedling 1	0.07	0	0	0	0.1 (0.05, 0.05, 0.19)	0	0	0	0	0	0	0
	Seedling 2	0	0.45 (0.59, 0.22, 0.55)	0	0	0	0	0	0	0	0	0	0
	Juvenile	0	0	0.73	0.64	0	0	0	0	0	0	0	0
	Flowering adult	0	0	0	0.198	0.61 (0.56, 0.54, 0.75)	0.36 (0.23, 0.25, 0.60)	0	0	0	0.041	0	0
	Non-flowering adult	0	0	0	0	0.38 (0.43, 0.46, 0.25)	0.53 (0.67, 0.73, 0.20)	0	0	0	0	0	0
Hermaphrodite	Seed bank	0	0	0	0	0	0	0	0	0	0	3.63 (1.81, 1.45, 7.64)	0
	Seedling 1	0	0	0	0	0	0	0.07	0	0	0	0.10 (0.05, 0.04, 0.21)	0
	Seedling 2	0	0	0	0	0	0	0	0.45 (0.63, 0.20, 0.53)	0	0	0	0
	Juvenile	0	0	0	0	0	0	0	0	0.70	0.68	0	0
	Flowering adult	0	0	0	0.107	0	0	0	0	0	0.221	0.44 (0.48, 0.45, 0.39)	0.29 (0.26, 0.19, 0.43)
	Non-flowering adult	0	0	0	0	0	0	0	0	0	0	0.54 (0.48, 0.52, 0.61)	0.69 (0.70, 0.81, 0.57)

CHAPTER 4

DIFFERENCES IN THE MATING SYSTEM AND SPATIAL GENETIC STRUCTURE ASSOCIATED WITH THE PRESENCE AND PROPORTION OF FEMALES ON TWO SPATIAL SCALES[1]

[1] Van Etten, M.L., A.C. Deen, J.H. Hamrick and S-M. Chang. Submitted to *Molecular Ecology*, 12/07/2009.

ABSTRACT

Self-pollination is thought to be one of the primary causes of high relatedness between individuals at a local scale, i.e. strong spatial genetic structure (SGS), based on among species comparisons. However, selfing rates may vary within a species and thus may also lead to variation in SGS within a species. Populations of gynodioecious species, which contain both hermaphrodites (who can self) and females, may vary in the population selfing rate due to variation in the sex ratio. Populations or patches with more hermaphrodites should have higher average selfing rates and thus stronger SGS than populations or patches with more females. We compared the selfing rates and SGS for populations with only hermaphrodites to populations with both sexes using six to eleven polymorphic allozyme loci. Surprisingly, we found that hermaphrodites in populations with both sexes have slightly higher selfing rates than hermaphrodites in purely hermaphroditic populations. However, these selfing rate differences did not appear to affect SGS because populations with both sexes had weaker SGS than hermaphroditic populations. Examining populations more closely revealed that patches with fewer hermaphrodites had stronger SGS than patches with more hermaphrodites. This within population variation in SGS may result in the hermaphrodites experiencing biparental inbreeding, which may increase females' relative fitness and thereby help to maintain gynodioecy.

INTRODUCTION

Most plant species show some level of within population genetic structure, with individuals located near each other being more related than ones far apart (Loveless &

Hamrick 1984; Hardy & Vekemans 1999; Vekemans & Hardy 2004). This non-random distribution of genotypes at the local scale (often referred to as spatial genetic structure, SGS) can influence many processes in plant populations. For example, in populations with strong SGS, matings between near-neighbors, which is common in plants, may lead to higher levels of inbreeding and lower seed fitness due to inbreeding depression (Griffin & Eckert 2003). The proportion of matings between related individuals, in turn, can influence the strength of kin selection (Smith 1978; Nakamura 1980), as well as selection on many other traits such as the optimal seed dispersal distance (e.g. Kalisz *et al.* 1999; Jones & Comita 2008). The ubiquitous nature of SGS in plant populations and its potentially far-reaching influence have led to attempts to understand what factors influence the strength and pattern of SGS in natural plant populations.

Two factors especially important for SGS have emerged from comparative studies among species. First, the mating system, the proportion of self vs. outcross mating, is often associated with the strength of SGS. Particularly, the relatedness of near-neighbors tends to be higher in species with higher selfing rates (Hamrick & Nason 1996; Hardy & Vekemans 1999). Second, the amount of gene movement within and among populations influences the strength of SGS, with species that have lower gene movement tending to have higher SGS (Vekemans & Hardy 2004). For example, species with gravity dispersed seeds have twice as much SGS as species with wind dispersed seeds (Vekemans & Hardy 2004). Although these trends have mostly been used to explain differences in SGS among species, these factors may also explain differences in SGS within a species.

One system in which selfing rates and gene movement may differ both within and among populations is gynodioecy, in which female and hermaphroditic individuals coexist within populations. On an individual level, the sexes may differ in the amount of selfing because females cannot self-pollinate while hermaphrodites can, and often do, self-pollinate. The absence of self-pollination in females, and thereby inbreeding depression, is thought to be one of the primary mechanisms increasing female fitness and allowing their maintenance in populations (e.g. Charlesworth 1978). In addition to individual variation in selfing rates, there may be variation between populations depending on the relative frequency of females, which may vary widely in gynodioecious species (e.g. Van Damme & Van Delden 1982; Cuguen *et al.* 1994; Ashman 1999; Delph & Carroll 2001). Several studies have found a relationship between the selfing rate and the sex ratio. However, the relationship differs among species. Results from some studies suggest that that the selfing rate of hermaphrodites and the frequency of females are positively correlated (Sun & Ganders 1988; Wolff *et al.* 1988; Delph 1990; Van Treuen *et al.* 1993), while other studies have suggest a negative correlation (Medrano *et al.* 2005; Cuevas *et al.* 2006). These mixed results have led to two hypotheses. Ashman (2006) explained the positive correlation as being caused by females invading and persisting only in populations with a high selfing rate. Alternatively, Gouyon and Couvet (1987) suggested the negative correlation as being caused by females lowering the population's average selfing rate because they are entirely outcrossing. While these hypotheses are not mutually exclusive, the predicted relationship between the sex ratio and the selfing rate are quite different and it is not yet clear which hypothesis applies more generally. Regardless the sign of the correlation, it is clear that the mating system

can vary among populations depending on the sex ratio, which may lead to variation in SGS.

In addition to the mating system, the SGS can be influenced by other factors, including gene movement and plant density. Both of these factors may vary among populations within a species. For example, it has been shown that the presence of females increases gene movement within (Garcia *et al.* 2005) and among (Moyle 2006) populations. An increase in gene movement, through either increased pollen or seed movement, could lead to weaker SGS in populations with more females due to better mixing of individuals from different families. Although significant SGS has been found in several gynodioecious species (e.g. Gehring & Delph 1999; Laporte *et al.* 2001; Olson *et al.* 2006), no studies to our knowledge have directly investigated whether the sex ratio affects the SGS.

In this study, we took advantage of variation in sex ratio at different spatial scales of a gynodioecious plant, *Geranium maculatum*, to address how sex ratio may influence the mating system and the SGS. Although the SGS can be influenced by several factors, we chose to first investigate the possible effect of mating system differences. We studied mating system differences between two types of populations: monomorphic populations with only hermaphrodites and dimorphic populations with both females and hermaphrodites. In addition, we hierarchically characterized the SGS among populations and between patches within populations. As in other gynodioecious species (Graff 1999; Olson *et al.* 2006), females and hermaphrodites are spatially segregated within populations of *G. maculatum*, forming patches that differ widely in sex ratio (Van Etten & Chang 2009). Similar to differences between populations with different sex ratios, this

sex segregation within populations could lead to within-population variation in the SGS. Specifically, we asked (1) do hermaphrodites self pollinate, (2) is the selfing rate similar between monomorphic and dimorphic populations, (3) does the strength of SGS differ between monomorphic and dimorphic populations and/or (4) between patches within populations with different sex ratios. Based on the lack of morphological differences between hermaphrodites from different population types, we predicted that hermaphrodites would self-pollinate regardless of the presence of females, leading to the average selfing rate being lower in dimorphic populations. Given this, we predicted that SGS would be weaker in dimorphic populations and in female-biased patches within populations because there should be less selfing than in monomorphic populations and hermaphrodite-biased patches.

METHODS

Study species

Geranium maculatum is a gynodioecious, rhizomatous perennial ranging from the southeastern US to Canada and west to the Great Plains (USDA NRSC). Populations are located predominantly in the understory and contain from five to more than a thousand flowering individuals (S-MC pers. obs.). Flowering begins in early spring, with individuals producing on average six flowers per inflorescence (Chang 2006). Flowers are visited by generalist pollinators including bees, flies and butterflies. Each fruit can produce at most 5 seeds, which are dispersed by the elastic dehiscence of the schizocarp, to an average of 3m from the maternal plant (Stamp & Lucas 1983). Based on field and greenhouse observations, sex appears to be genetically determined. Females have small

aborted anthers and smaller petal sizes compared to hermaphrodites (Ågren & Willson 1991; Chang 2006). Local populations near Athens, GA range in female frequency from 0-50% (Chang 2006).

Populations

Seven populations, four monomorphic (M1-M4) and three dimorphic (D1-D3), were included in this study (Table 4.1). For mating system analyses, we sampled from two populations of each type (M3, M4, D1 and D3) and for the genetic structure analyses, we sampled from three populations of each type (M1, M2, M3, D1, D2, and D3). All populations were located in Clarke Co., GA except population M4, which was located in adjacent Oconee County, GA (Table 4.1), with the distance between populations ranging from 100m to 5.44km. Study populations varied in size, density and sex ratio (Table 4.1).

Mating system sampling

Seeds for mating system analyses were collected in the spring of 2004 for D1, D3, and M3 and in the spring of 2008 for M4. 17-19 individuals of each sex were chosen throughout each population and only included individuals that had produced a sufficient number of seeds. Seeds were collected by covering the entire inflorescence with mesh bags until seeds were mature, thus, the seeds used were from multiple fruits. For each individual, 9-20 seeds were randomly selected for extraction (see Table 4.2 for sample sizes). Half of the seeds from D1 were scarified, germinated, and grown in the greenhouse. This procedure, however, was later abandoned because germination rates were low. For the remaining seeds from D1 and for all of the seeds from D3, M3 and M4, ungerminated seeds were used instead. These seeds were prepared for allozyme

extraction by scarifying the seed coat, soaking the seeds in de-ionized water at 5° C for 24 hours to soften the seed coat, and carefully removing the seed coat before placing the seeds in a cold room (5˚ C) for an additional 1 to 2 weeks before extraction. Except the seed coat, mature seeds in this species contain predominantly embryonic tissues, thus, representing the progeny genotype.

Genetic structure sampling

Strategies to obtain ~96 individuals of each sex from each population differed because populations varied in size and density. To sample throughout the populations, 1-3 one meter wide transects were placed across the population (ranging from 10-30m depending on the size of the population, Fig.4.1). For populations with too few plants within the transects (M2, M3 and D2), the transects were widened until they contained the appropriate number of plants. For populations with too many individuals within the transects (D1 and D3), individuals were randomly selected. In populations M1, M2, D1 and D2, three transects were used to sample individuals throughout the population and the patches. In M3, the smallest population, only one transect was used, which encompassed the entire population. In D3, two transects were used to sample from two distinct but connected patches. Plants used in the genetic structure analyses were mapped to the nearest cm by stretching a meter tape down the length of a transect and measuring the distance from the plant to the tape with another meter tape perpendicular to the first. Locations were then converted to x,y co-ordinates to allow analysis of SGS. As mentioned earlier, dimorphic populations often exhibit non-random, patchy distributions of the two sexes. To evaluate whether SGS differs depending on the sex ratio of a patch, we divided the dimorphic populations into two patches (Fig. 4.1): female-biased patches

(60-82% female) and hermaphrodite-biased patches (5-20% female). In D3, there were distinct patches while in the other 2 populations, the patches were less distinct so the exact area of the patches were defined semi-arbitrarily based on field observations. The sex ratio in each patch was obtained using data collected for another study in which all flowering individuals were sexed and mapped.

Genotyping

Seeds and leaves were crushed and enzymes extracted using the extraction buffer of Wendel & Parks (1982). Protein extracts were absorbed onto 4 x 6 mm wicks punched from Whatman 3mm chromatography paper and stored at -70° C for later electrophoretic analysis.

Eleven or twelve polymorphic allozyme loci were resolved for each population for the mating system analyses and eight loci were resolved for the genetic structure analyses. Wicks were placed in 10% starch gels for electrophoresis and enzyme stains were resolved on one of four buffer systems (8-, 4, 11, or MC from Soltis *et al.* 1983) depending on optimal expression: fluorescent esterase (*FE1, FE3*), diaphorase (*DIA1, DIA2*), aspartate aminotransferase *(AAT)*, phosphoglucomutase (*PGM1, PGM2)*, isocritrate dehydrogenase *(IDH1, IDH2),* l-leucine-B-napthyl-amide phosphatase *(LAP),* F-1, 6-diaphosphatase *(F-16),* phosphoglucose isomerase *(PGI),* UTP-glucose-1-phosphate *(UGPP),* and malate dehydrogenase *(MDH).* All stains and buffer systems used were adapted from Soltis *et al.* (1983) except *DIA* and *UGPP*, which were from Cheliak & Pitel (1984) and Manchenko (1994), respectively. Due to differential levels of enzyme activities for some loci and in some individuals, some loci were not scorable for a portion of the individuals. To avoid biasing our analyses due to missing data, we

removed individuals or loci that were missing 30% of the genotypic data from the analysis of that population. In addition, we identified clonal ramets as individuals that had the same multilocus genotype, sex, and were within 1m of each other. For these potential clones, all but one of the individuals were removed from the analysis.

Analysis:

Do hermaphrodites self?

To determine the extent of selfing in hermaphrodites and biparental inbreeding (matings between relatives) in both hermaphrodites and females, several mating system parameters were estimated. These parameters included the multilocus outcrossing rate (t_m) and the mean single locus outcrossing rate (t_s), which were then used to calculate the selfing rate ($s_m=1-t_m$) and the amount of biparental inbreeding (t_m-t_s) for females and hermaphrodites. Selfing rates for females should be zero, however, it is possible to get a non-zero value if variation in the genetic markers is not sufficient to distinguish selfing from biparental inbreeding (Brown 1990; Leclercpotvin & Ritland 1994). Also calculated were the single locus inbreeding coefficient (F), and correlation of paternity (r_p, indicates similarity in the pollen parents of progeny collected from the same seed parent). The effective number of pollen parents, which is the expected number of different pollen parents given the amount of variation observed in seeds produced from a seed parent (Ritland & Jain 1981), was calculated as $N_{ep}=1/r_p$. For each of these measures, 1000 bootstraps were used to estimate the standard deviation, which was then used to test for significant differences between females and hermaphrodites using a t-test, with the number of families being the sample size. All parameters were calculated using the MLTR program, which uses a maximum likelihood technique for parameter estimates

(Ritland 2002). Data from both germinated seeds and ungerminated seeds were included in the analyses. Removing germinated seeds from the analyses increases the outcrossing rate slightly (hermaphrodite t_m increases from 0.902 to 0.967, female t_m increases from 0.956 to 1.010), but does not change the relationship between sexes.

Does the selfing rate differ between population types?

To determine if the selfing rate differed between monomorphic and dimorphic populations, we estimated the previously described mating system parameters to obtain population averages. Because dimorphic populations were sampled to get an equal number of females and hermaphrodites instead of an amount proportional to the frequency of each sex, we calculated the average population parameters using the parameters from each sex weighted by the population sex ratio. The weighted averages were used in a t-test to determine if monomorphic and dimorphic populations differed from each other, with each population as a sample.

Does SGS differ between population types?

We analyzed SGS within populations using correlograms, which characterize the genetic relationship between pairs of individuals separated by various distances (Sokal & Oden 1978a; Sokal & Oden 1978b; Ennos 2000). Genetic relatedness was calculated using the multilocus autocorrelation coefficient, r, described in Smouse and Peakall (1999). This coefficient is bounded by 1 and –1, with positive values indicating that individuals are more related than expected by chance and negative values indicating individuals are less related than expected by chance. The null hypothesis is that any two individuals chosen regardless of their location would be as related to each other as expected by chance, (i.e. $r = 0.0$). If pairs of individuals at a certain distance have

significantly higher or lower relatedness than expected, we conclude that the population is structured. The most commonly observed pattern is that nearby individuals are more related than distant individuals. A relatedness value of 0.5 represents full sibs (share both ovule and pollen parents) and 0.25 represents half sibs (share one parent). For each distance class, the mean genetic relatedness between pairs of individuals at that distance class is calculated, and tested to see if it is significantly different from zero by bootstrapping the genotypes.

To test for differences among populations, we used a heterogeneity test described in Smouse *et al.* (2008). Briefly, we first tested whether the relatedness at each distance class was different between populations in a pairwise manner. To do this, random assortments of individuals are chosen from the pool of both populations and used to calculate relatedness. The difference between the two randomly created populations is compared to that found in the actual populations to determine a p-value for each distance class. These p-values are then combined to determine whether the populations are significantly different over the entire correlogram. Similarly, to determine if monomorphic and dimorphic populations differ in structure, the heterogeneity test was performed on the three dimorphic populations versus the three monomorphic populations. The correlogram analysis and the heterogeneity tests were performed using GenAlEx V6.2b (Peakall & Smouse 2005).

For comparisons with other species, the *Sp* statistic was calculated for each population and averaged (Vekemans & Hardy 2004). It was calculated as $b/(F_1\text{-}1)$ where b is the slope of the decrease in relatedness over the logarithm of the distance between pairs up to 500cm and F_1 is the relatedness at the first distance class calculated according

to Loiselle *et al.* (1995). All analyses involving the *Sp* statistic were performed using SpAGeDi v1.1 (Hardy & Vekemans 2002).

To compare genetic variation between population types, we calculated a set of standard population genetics statistics. For each population, we calculated: average number of alleles per polymorphic locus (AP), observed heterozygosity (H_o), expected heterozygosity (H_e), and Wright's inbreeding coefficient (F_{IS}) and among population structure coefficient (F_{ST}). A difference in heterozygosity between populations was tested using t-tests. H_o, H_e, $F_{IS,}$ and F_{ST} values were tested to determine whether they were significantly different from zero by jackknifing genotypes across individuals. All calculations were performed using GenAlEx V6.2 (Peakall & Smouse 2005).

Does SGS differ between patches of the same population?

To determine if the local sex ratio affected genetic structure, patches within dimorphic populations (female-biased and hermaphrodite-biased patches) were analyzed in the same manner as populations: genetic structure was determined for female-biased and hermaphrodite-biased patches using correlograms, which were compared using the heterogeneity test.

For each patch within dimorphic populations H_o, H_e, F_{IS}, and F_{ST} (patches as the subpopulation and the total population as the total) were calculated and tested to determine whether they were significantly different from zero by jackknifing genotypes across individuals.

RESULTS

Do hermaphrodites self?

Though the average selfing and biparental inbreeding were fairly low (3.9% and 3.5% respectively), populations varied in these parameters (Table 4.2). In M3 and M4, the average selfing rate was effectively zero while in D1 and D3 the selfing rate was 9.8 and 16.5%. Females had a selfing rate of 0.8-4.6%, which can be attributed to the inability to distinguish self-fertilization from biparental inbreeding as discussed in the methods. These values were, however, very close to zero and significantly smaller than selfing rates estimated for hermaphrodites (D1: $t=2.39$, d.f.=48, $P=0.02$; D3: $t=7.61$, d.f.=32, $P<0.001$). Estimates of biparental inbreeding were low in all populations but significantly different from zero in all but population M4 (M3: $t=8.17$, d.f.=18, $P=0.0001$; M4: $t=1.48$, d.f.=15, $P=0.16$; D1: $t=19.98$, d.f.=49, $P=0.0001$; D3: $t=4.56$, d.f.=33, $P=0.0001$), ranging between 2 to 10%. The levels of biparental inbreeding were similar between hermaphrodites and females, with the exception of hermaphrodites in D1 having a higher rate than females and than all other populations. Correlated paternity was similar across populations (ranged from 0.069-0.194) with an average of 0.135, which is equivalent to ~7.42 effective pollen parents per plant. Within a population, hermaphrodites had a higher correlation of paternity than females, resulting in fewer effective pollen parents (N_{ep}) for hermaphrodites than for females (D1: hermaphrodites $N_{ep} = 5.15$, females $N_{ep} =6.85$, $t=2.48$, d.f.=48, $P=0.02$; D3: hermaphrodites $N_{ep} =5.78$, females $N_{ep} =7.35$, $t=3.12$, d.f.=32, $P=0.004$). The inbreeding coefficient was not significantly different from zero in any population, consistent with the low selfing rate estimates.

Does the selfing rate differ between population types?

Although not significantly different between population types, there was a trend that hermaphrodites in dimorphic populations had higher selfing rates than hermaphrodites in monomorphic populations (t=3.25, d.f.=2, P=0.08). In monomorphic populations, hermaphrodites had a selfing rate of effectively zero, while in dimorphic populations hermaphrodites had a selfing rate significantly higher than zero (Table 4.2, D1: t=3.78, d.f.=18, P=0.001; D3: t=8.50, d.f.=16, P=<0.0001). This slightly higher selfing rate of hermaphrodites in dimorphic populations led to dimorphic populations having a slightly higher average selfing rate, 7.2-11.2%, than the monomorphic populations (t=2.90, d.f.=2, P=0.10).

Does SGS differ between population types?

Even though significant SGS was found in all populations, monomorphic populations had higher relatedness between near neighbors than dimorphic populations. Across all populations, the average relatedness between individuals that are 75 cm apart (the smallest distance class) was 0.216, approximately that of half sibs, i.e. they share one parent (Fig. 4.2). In all populations there was significant structure until between 150-300 cm. The average distance at which relatedness between paired individuals became zero, an indication of the patch size of genetic structure, was approximately 350 cm. The *Sp* ranged from 0.026-0.11, with an across population average of 0.050. When examining population types separately, monomorphic and dimorphic populations differed significantly at the smallest distance class, with the average relatedness being 0.235 and 0.159, respectively (Fig. 4.3, r(75) P=0.04, overall P=0.16). Monomorphic and dimorphic populations were similar in the distance at which relatedness between paired

individuals was zero (Fig. 4.3, monomorphic population mean=340cm, dimorphic population mean=368cm).

Overall, individuals in all populations were highly heterozygous, with the expected heterozygosity ranging from 0.329 – 0.429 for the polymorphic loci used in these analyses (Table 4.3). The inbreeding coefficient (F_{IS}) was only significantly different from zero in one population (M1 F_{IS} = -0.166, χ^2=13.56, d.f.=5, P=0.02), and was negative, indicating an excess of heterozygotes. The overall F_{ST} value was moderate (0.159), indicating only moderate genetic differentiation among populations.

Does the SGS differ between patches of the same population?

Analyzing patches within dimorphic populations for differences in SGS showed that in two of the three populations (D2 and D3) female-biased patches had higher relatedness at the smallest distance classes than hermaphrodite-biased patches, leading to stronger structure (Fig. 4.4; D1: overall P=0.1; D2: r(75) P=0.05, r(150) P=0.03, overall P=0.01; D3: r(75) P=0.01, r(150) P=0.01, r(225) P=0.01, overall P=0.01). Observed heterozygosity, expected heterozygosity and the inbreeding coefficient were similar between patches in all populations (Table 4.4). In two populations, (D2 and D3) the F_{ST} between patches was significantly different from zero indicating genetic differentiation between patches.

DISCUSSION

This study investigated the relationship between the population sex ratio, the mating system and the spatial genetic structure at two spatial scales. We found that though the populations are all relatively close to one another geographically, selfing rates

of hermaphrodites were not consistent across populations, but rather, tended to vary with the population type. Additionally, we found that the relationship between sex ratio and SGS differed depending on the spatial scale examined. At the population level, populations with females (dimorphic populations) had lower relatedness between near neighbors than populations without females (monomorphic populations). In contrast, in two of the populations at the within population level, patches with more females had stronger structure than patches with fewer females.

Sex ratio and mating system

We found that some hermaphrodites indeed produced selfed seeds, but at low rates and only in dimorphic populations. Mating system analyses in other gynodioecious species have found from 0-100% selfing in hermaphrodites, with an average between 30-50% selfing (Collin & Shykoff 2003). Thus, our results of between 0-17% selfing are less than most other studies. Interestingly, we found that the selfing rate tended to be higher in populations with females. Similar results have been found in other studies (Vantreuren *et al.* 1993; Medrano *et al.* 2005; Cuevas *et al.* 2006). For example, hermaphrodites of *Limnanthes douglasii* in dimorphic populations had higher selfing rates than ones in monomorphic populations (Kesseli & Jain 1984). Similarly, in two Hawaiian *Bidens* species, hermaphrodite selfing rates were higher in populations with lower hermaphrodite frequencies (Sun & Ganders 1986). These results have been hypothesized to be caused by the selfing rate influencing the female frequency - populations with less selfing would result in females having less of a relative fitness advantage due to the lack of inbreeding depression (Ashman 2006). However, another explanation is that in the dimorphic populations, there is less outcross pollen available to

each individual because the pollen produced by hermaphrodites is shared with female plants, perhaps lowering the ratio of outcross to self-pollen on the stigmas of hermaphroditic plants leading to a higher selfing rate. Indeed, a recent experimental study using *Silene vulgaris* showed that as the frequency of females increased, so did the selfing rate of hermaphrodites (Miyake & Olson 2009). Alternatively, the selfing rate differences may be caused by another factor that is confounded with population type. In the populations used for the mating system analyses, dimorphic populations were denser than monomorphic populations. Higher densities have been shown to decrease the selfing rates because pollinators visit fewer flowers per plant before moving to the next plant, reducing geitonogamous pollen transfer (Kunin 1993; Karron *et al.* 1995). However, our results show the opposite trend, the selfing rate increased with increased density. Further experimental studies would assist in determining if the selfing rate differences seen are caused by the sex ratio or density or if the selfing rate is the cause of the sex ratio.

Sex ratio and genetic structure at the population level

Given that dimorphic populations have higher selfing rates, we would expect that those populations would be more structured, with nearby individuals more closely related. Our results, however, do not support this prediction – dimorphic populations actually had significantly lower average relatedness at small distances compared to monomorphic populations. The lack of effect of the mating system on SGS may have several causes. First, in many gynodioecious species females produce more seeds than hermaphrodites (reviewed in Shykoff *et al.* 2003), which may swamp out the effect of a higher selfing rate. Demographic studies of *G. maculatum* show that female seed

production is population specific, but can range from 1.7-2 times as much as hermaphrodites (Van Etten and Chang, unpublished). From these values, the average selfing rate of the seed pool for each population can be calculated, which depends upon the sex ratio and the relative seed production. For example, in D1, with 50% females, which on average produce 1.77 times as many seeds as hermaphrodites, 66% of the seeds produced in a population will be from outcrossed female individuals. Of the other 34% of the seeds, only 9.8% of them will be from selfing, leading to a total of 3.5% of the seeds being produced from selfing in the population. A similar calculation for D3 leads to a 9.1% selfing rate after accounting for seed production differences (Chang 2006) and the sex ratio. In both cases, this selfing rate of the entire seed pool is much less than the selfing rate found in this study. Second, the lack of an effect of the mating system may be caused by selection against selfed individuals. The selfing rate is measured on ungerminated seeds while the SGS was measured on flowering individuals. It has been shown that significant inbreeding depression exists in early life history stages of this species, with cumulative inbreeding depression measured to be 0.38-0.84 under greenhouse conditions (Chang 2007). Inbreeding depression may be even stronger under more stressful natural conditions (Armbruster & Reed 2005). In combination, the higher seed production of females and the high inbreeding depression may lead to very few selfed individuals surviving to flowering, the stage at which the SGS was measured.

At least two possibilities may explain the differences between population types in SGS: differences in the density of plants and/or differences in gene movement distances (Hamrick & Nason 1996). One way that density can influence SGS is that as a population becomes denser, seed shadows will overlap more. Thus, nearby individuals

might be from a variety of maternal lineages, leading to lower genetic structure. In a review, of five species examined, all had lower SGS in populations with higher density (Vekemans & Hardy 2004). Density could explain some of our results - two of the three dimorphic populations have much higher adult densities than the monomorphic populations and these two populations have the lowest relatedness at the shortest distance classes, as we would expect if density were the cause. However, the pattern between density and relatedness is opposite for M3, which is less dense but has low structure (Fig. 4.2).

Another possible explanation for weaker structure in dimorphic populations is increased pollen movement distances in dimorphic populations. As noted earlier, the two sexes are not randomly distributed throughout populations so it seems plausible that pollen movement distances on average might be longer in dimorphic populations because when individuals are surrounded by females, pollen would have to travel from hermaphrodites that are farther away. Indeed, several studies have suggested that females obtain pollen from further distances than hermaphrodites (Garcia *et al.* 2005; Olson *et al.* 2006). In this case, the overall pollen dispersal distance would be higher in dimorphic populations and could lead to less structure, explaining the differences in SGS between the two types of populations.

Sex ratio and genetic structure at the patch level

Our results indicate that in two of the three populations, patches with a higher proportion of females had stronger SGS. As discussed above, this could be due to differences in density and/or gene movement distances. Based on our results, if gene movement distances were the primary influence on SGS, we would expect female-biased

areas to have shorter pollen or seed dispersal distances. Given the low frequency of pollen donors in these areas, it seems unlikely that pollen moves shorter distances. Instead, it seems likely that the lowered density in female biased areas has led to stronger SGS because in the two populations with significant differences in structure between the patches, the female biased patches were less dense (Fig. 4.1, nearest neighbor distances (m): OT female-biased=0.33, hermaphrodite-biased=0.34; OTG female-biased=0.60, hermaphrodite-biased=0.57; MP female-biased=0.38, hermaphrodite-biased=0.15).

Regardless of the cause, SGS of the patches may have important repercussions for the maintenance of females because the majority of the hermaphrodites in a population will be located in genetically structured environments, which may increase the occurrence of biparental inbreeding. Pollinator observations in this species have shown that pollinators typically move to the nearest plant (Van Etten and Chang, unpublished). Since the nearest flowering plant (Table 4.1, nearest neighbor distances) is within the area in which there is significant relatedness, plants may often receive pollen from related individuals. Biparental inbreeding has been shown to incur inbreeding depression in this species in both females and hermaphrodites (Chang 2007). Thus, hermaphrodites may have reduced seed fitness due to biparental inbreeding, which may increase the chance of maintaining females.

Hierarchical differences in SGS

As this study shows, comparing only the population average SGS may obscure differences occurring within a population. The pattern between SGS and sex ratio at the population level did not match the pattern on the patch level; among populations, dimorphic populations had weaker structure while within populations female-biased

patches usually had stronger structure. If only the population level had been examined in this study, we would erroneously expect that because dimorphic populations were less structured, biparental inbreeding may not be a significant factor affecting the maintenance of females. However, investigating SGS in patches within populations showed that biparental inbreeding may indeed be influencing hermaphrodites' seed fitness and may actually increase the chances of maintaining females in a population. Our results show that in some cases a detailed within population investigation is needed to determine the actual genetic structure an individual experiences. Gynodioecious species are an excellent example of when this type of detail is needed due to the spatial sex structuring, but there are many other situations in which it may be necessary. For example, if the environment influences selfing rates in a population (reviewed in Barrett & Eckert 1990) then genetic structure may change within a population depending on the environment. This difference in genetic structure may in turn affect seed production, local adaptation, etc (Griffin & Eckert 2003). Another example would be cases where seed or pollen dispersal change drastically across a population. This could be due to many factors including differences in prevailing winds, vegetation cover (Garcia *et al.* 2005), or distance to pollinators' nests. Spatial variation in factors that affect genetic structure may be very common and thus a more detailed look at genetic structure within populations is warranted.

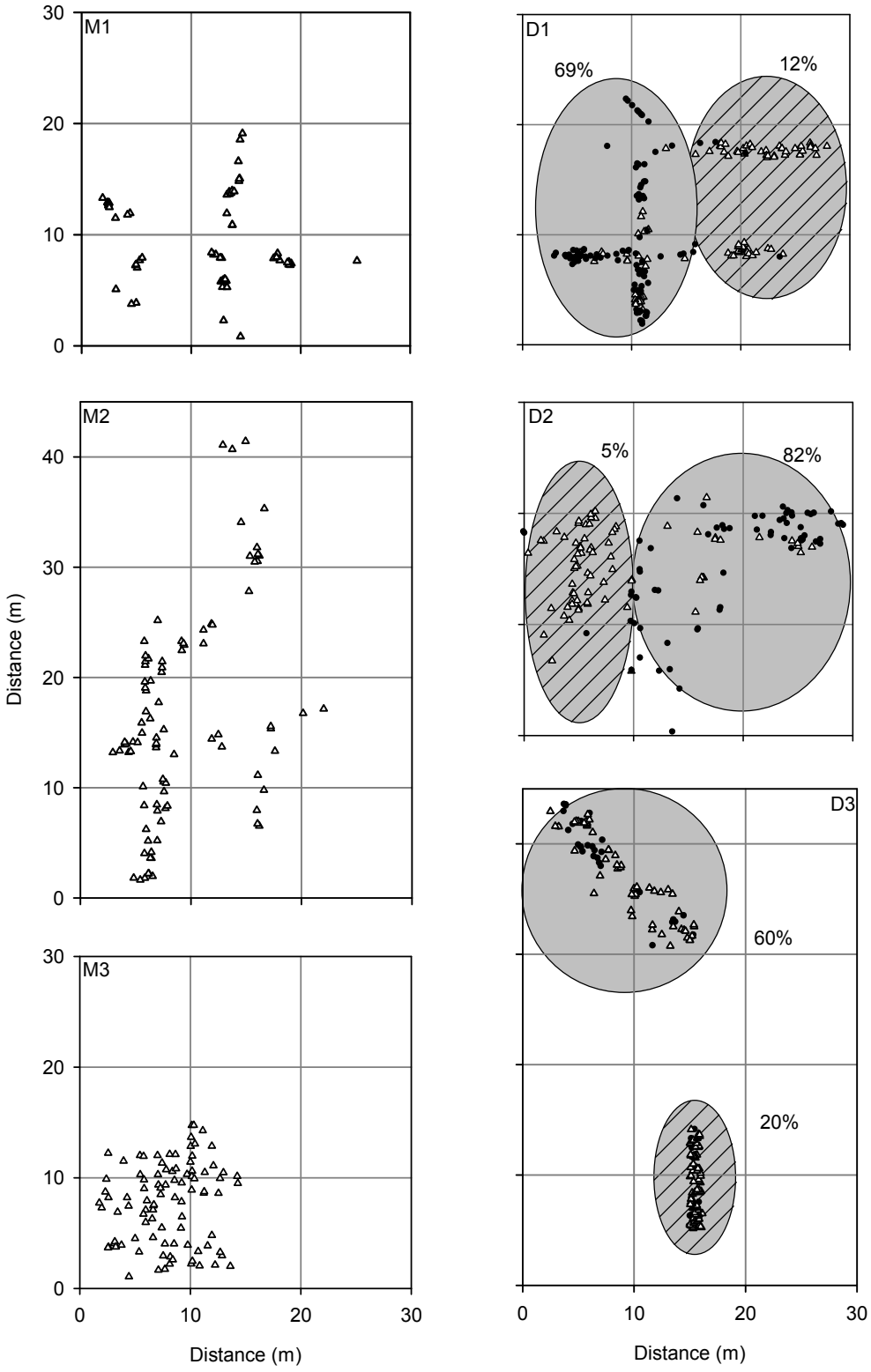

M1
D1
69%
12%
M2
D2
5%
82%
M3
D3
60%
20%
Distance (m)
Distance (m)
0
10
20
30
40

Figure 4.1 (previous page). Maps of the individuals used in the spatial genetic structure analyses in each population. Hermaphrodites are white triangles, females are black circles. Hermaphrodite-biased patches are denoted by the gray, hatched circle while female-biased patches are denoted by the gray, non-hatched circle. Percentages indicate the percentage of females in each of the patches.

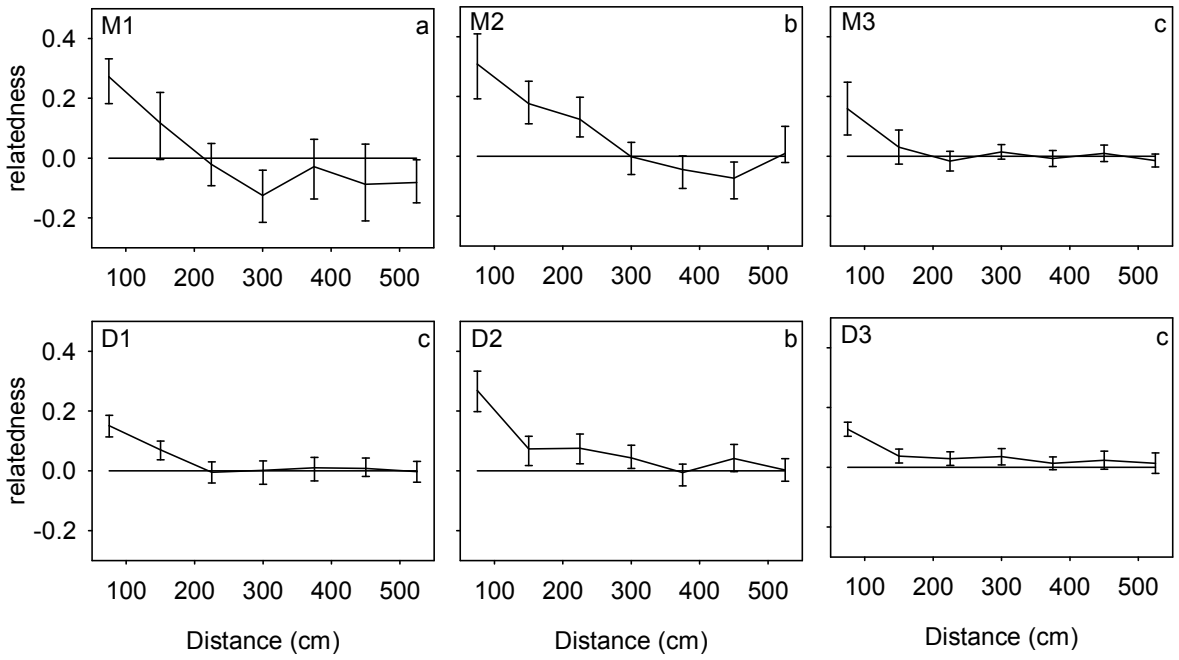


Figure 4.2. All populations show significant spatial genetic structure as shown by significant relatedness values (bootstrap confidence interval not overlapping zero). Populations sharing a letter in the upper right hand corner of the correlogram are not significantly different from each other.

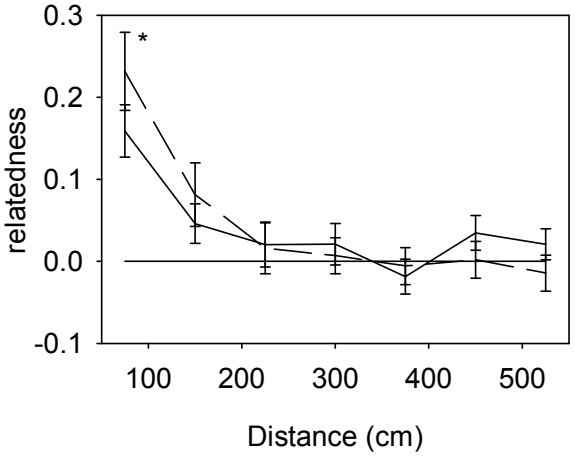


Figure 4.3. Monomorphic populations (dashed line) have higher relatedness than dimorphic populations (solid line) at the first distance class. Asterisks indicate that the relatedness at that distance class is significantly different between the population types ($P<0.05$).

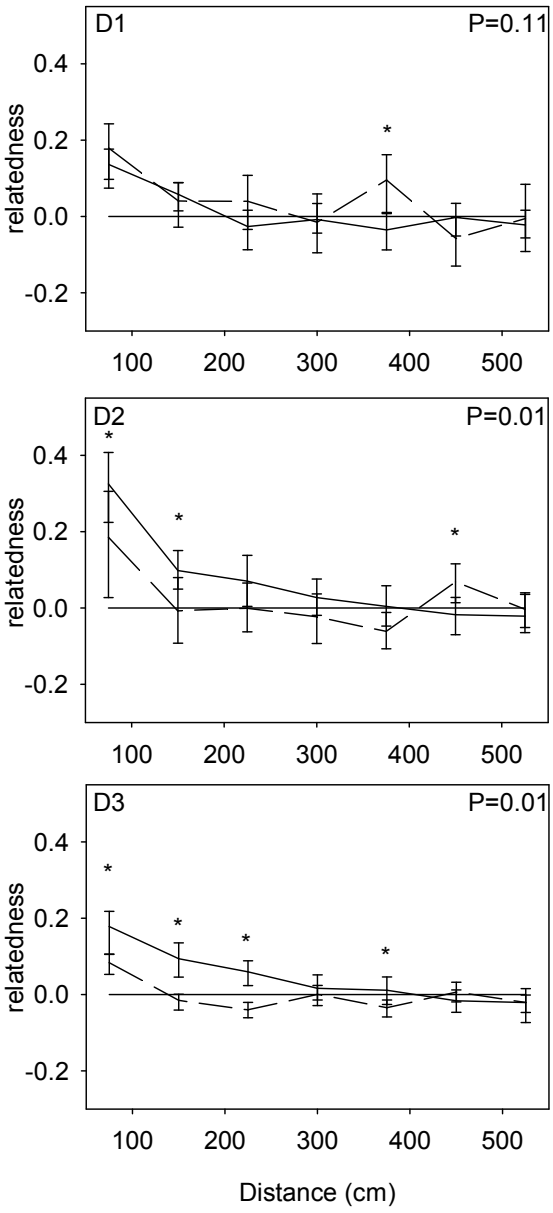


Figure 4.4. In two of the three dimorphic populations the female-biased patch (solid line) has more structure than the hermaphrodite-biased patch (dashed line). A relatedness value (r) is significantly different from zero if the bootstrap confidence interval does not overlap with zero. Asterisks denote that the patches differ in relatedness at that distance class based on the heterogeneity test ($P<0.05$). P values indicate whether the entire correlogram differs between the patches.

Table 4.1. Population descriptions including the location, percentage of females (% F), the approximate number of flowering ramets, the number of individuals sampled for the spatial genetic structure analyses (N_S) if appropriate, adult density and the average distance between neighboring plants.

Pop	Location	% F	Approx. Number of Adults	N_S	Adult Density (per m^2)	Nearest neighbor distance (m) (SE)
Monomorphic populations						
M1	Oconee Forest/Lake Herrick	0	200	96	1.343	0.256 (0.068)
M2	Botanical Gardens/ White Trail	0	150	96	0.433	0.651 (0.057)
M3	Botanical Gardens/ Callaway Building	0	300	110	0.489	0.666 (0.041)
M4	Heritage Park	0	>1500	-	-	-
Dimorphic populations						
D1	Botanical Gardens/ Orange Trail	50	>1500	200	2.27	0.200 (0.004)
D2	Botanical Gardens/ Orange Trail Gully	50	300	200	0.480	0.475 (0.041)
D3	Memorial Park	33	>1800	183	11.849	0.103 (0.002)

Table 4.2. Mating system parameters including multilocus selfing rate (s_m), single locus outcrossing rates (t_s), biparental inbreeding (t_m- t_s), correlated paternity (r_p), and mean single locus inbreeding coefficient (F). To obtain population means for dimorphic populations, means were corrected for the population sex ratio. N_{fam}=number of families used, N_{seeds}=total number of seeds genotyped.

Pop	N_{fam}	N_{seeds}	s_m (SD)	t_s (SD)	t_m- t_s (SD)	r_p	F
Monomorphic populations							
M3							
Hermaphrodites	19	244	-0.009 (0.020)	0.979 (0.021)	0.030 (0.016)	0.141 (0.034)	-0.150 (0.056)
M4							
Hermaphrodites	16	229	-0.103 (0.062)	1.083 (0.044)	0.020 (0.054)	0.069 (0.033)	-0.200 (0.001)
Dimorphic populations							
D1	50	461	0.072 (0.059)	0.863 (0.052)	0.065 (0.023)	0.170 (0.050)	-0.031 (0.109)
Hermaphrodites	19	150	0.098 (0.113)	0.801 (0.094)	0.101 (0.032)	0.194 (0.088)	-0.029 (0.173)
Females	31	311	0.046 (0.036)	0.925 (0.046)	0.028 (0.032)	0.146 (0.049)	-0.032 (0.132)
D3	34	562	0.112 (0.053)	0.854 (0.066)	0.025 (0.032)	0.159 (0.027)	-0.176 (0.057)
Hermaphrodites	17	288	0.165 (0.080)	0.812 (0.093)	0.023 (0.032)	0.173 (0.037)	-0.166 (0.057)
Females	17	274	0.008 (0.029)	0.963 (0.076)	0.029 (0.071)	0.136 (0.032)	-0.200 (0.130)

Table 4.3. Population level genetic diversity parameters including average number of alleles per loci (AP), observed heterozygosity (H_o), expected heterozygosity (H_e), Wright's inbreeding coefficient (F_{IS}), and among population structure (F_{ST}). Asterisks after the F statistics indicate significant deviation from zero.

Pop	AP	H_o	H_e	F_{IS}	F_{ST}
Monomorphic populations					
M1	2.33	0.378	0.320	-0.166*	
M2	3.00	0.429	0.447	0.018	
M3	2.89	0.361	0.352	-0.029	
Dimorphic populations					
D1	3.17	0.329	0.376	0.107	
D2	2.50	0.351	0.365	0.076	
D3	2.89	0.413	0.386	-0.087	
All					0.159

Table 4.4. Patch level genetic diversity parameters including observed heterozygosity (H_o), expected heterozygosity (H_e), Wright's inbreeding coefficient (F_{IS}), and between patches structure (F_{ST}). Asterisks after the F statistics indicate significant deviation from zero.

Patch	H_o	H_e	F_{IS}	F_{ST}
D1				
Hermaphrodite-biased	0.349	0.373	0.065	
Female-biased	0.330	0.378	0.132	0.014
D2				
Hermaphrodite-biased	0.333	0.361	0.113	
Female-biased	0.365	0.362	0.022	0.013*
D3				
Hermaphrodite-biased	0.399	0.379	-0.086	
Female-biased	0.426	0.392	-0.088	0.019*

CHAPTER 5

EFFECTS OF ENVIRONMENTAL HETEROGENEITY ON THE DISTRIBUTION OF SEXES WITHIN AND AMONG POPULATIONS IN A GYNODIOECIOUS SPECIES, *GERANIUM MACULATUM*[1]

[1] Van Etten, M.L. and S-M. Chang. 2009. *New Phytologist*. 183: 649-660. Reprinted here with permission of the publisher.

SUMMARY

- Populations containing both females and hermaphrodites (dimorphic) are generally found in drier sites than those with only hermaphrodites (monomorphic). The sex-differential plasticity hypothesis (SDP) suggests that this is caused by hermaphrodites reducing allocation to seeds in harsh environments allowing female establishment. We proposed that a similar process could explain sex distribution within populations.
- We compared light availability and soil moisture between sites of three monomorphic and three dimorphic populations and between microsites occupied by females and hermaphrodites within populations. We also correlated seed production in dimorphic populations with environmental measures.
- We found dimorphic and monomorphic populations occurred in sites with similar soil moisture but within two dimorphic populations, females occurred in drier microsites than hermaphrodites, as predicted by the SDP hypothesis. Contrary to the predictions, hermaphrodites' seed production was not influenced by the environment. Rather, females' seed production was correlated with environmental conditions in two populations, though the direction of the correlation differed between populations.
- Our results suggest that in this species, the SDP hypothesis does not explain sex distribution among or within populations. However, microsite environments may influence the distribution of sex within a population and potentially aid in maintaining gynodioecy.

KEY WORDS: sex-differential plasticity hypothesis, *Geranium maculatum*, microsite differences, environmental differences, gynodioecy

INTRODUCTION

Gynodioecy, the co-occurrence of female and hermaphroditic individuals within a population, has interested biologists since Darwin first noticed that females often produced more seeds than hermaphrodites (Darwin, 1877). Theory has shown that this seed fitness increase is necessary to allow females to invade hermaphroditic populations and coexist with their hermaphroditic counterparts (Charlesworth & Ganders, 1979; Charlesworth, 1981). Although the requirements and mechanisms of increased seed fitness in females have been frequently studied in populations containing both sexes, fitness differences in these populations speak little to the absence of females in other populations. One proposed explanation for the distribution of sexes among populations is that the frequent establishment and loss of populations does not allow populations to reach equilibrium, leading to the maintenance of females on a metapopulation level even if females have less fitness than typically required (Couvet *et al.*, 1986; Gouyon & Couvet, 1987; Belhassen *et al.*,1989; Pannell, 1997). Metapopulation dynamics have had limited support, but are more likely to apply to a select group of species with high turnover rates in natural populations (e.g. Belhassen *et al.*,1989; Olson *et al.*, 2005).

An alternative explanation for the presence or absence of females through an ecological mechanism has been proposed (Delph, 1990b; Delph, 2003) to explain the commonly observed pattern that females tend to be present in populations located in harsher environments. This hypothesis, put forth by Delph (1990b; 2003), proposes that

environmental conditions are important in determining the relative seed fitness of the sexes and the relationship between fitness and the environment can explain why we see dimorphic populations in some areas and monomorphic populations in others. More specifically, it suggests that because hermaphrodites can gain fitness through both pollen and seeds, they may have evolved a plastic reproductive strategy that decreases the emphasis placed upon seed production under harsh conditions. In contrast, because females can only gain fitness through seeds, they may have evolved to keep seed production relatively consistent regardless of the environmental conditions. Under this hypothesis (hereafter the sex-differential plasticity hypothesis or the SDP hypothesis), one expects to see that hermaphrodites have higher seed fitness in benign environments and lower seed fitness in harsh environments, leading to females being able to invade and become established in populations in harsh environments because they can better compete with the lower hermaphrodite seed production.

Several lines of evidence suggest that the scenario proposed by the SDP hypothesis might be important in determining the distribution of females among populations for some gynodioecious species. First, in a study comparing 38 environmental factors of 65 populations of *Wurmbea biglandulosa* across a large geographical scale, Vaughton and Ramsey (2005) concluded that the best predictor for the presence of females, i.e. dimorphic populations, was higher temperatures, higher radiation (light intensity), and less rainfall, conditions assumed to be harsher. Similar results have also been found in *Lobelia siphilitica* (Caruso & Case, 2007). Additionally, studies that either vary the environmental conditions directly (Barr, 2004; Dorken & Mitchard, 2008) or evaluate the environmental quality indirectly by using surrogate

measurements, such as plant size (Delph, 1990b; Delph, 1990a; Ashman, 1999; Sarkissian *et al.*, 2001), have shown that hermaphrodites are more affected by environmental conditions than females, a pattern consistent with the SDP hypothesis. Combined, these studies support the hypothesis that the sex specific relationship between the environment and seed production could be key to understanding the mechanisms behind the distribution of dimorphic and monomorphic populations in gynodioecious species.

In addition to variation in the presence or absence of females among populations, many gynodioecious species have variation in the distribution of females within populations. The sexes are often spatially aggregated within populations (Graff, 1999; Olson *et al*., 2006; this study), with patches of females interspersed with hermaphrodites. A simple explanation may lay in the genetic control of sex. In most gynodioecious species, femaleness is determined by a mitochondrial gene while male function can be restored by a nuclear gene (Budar & Pelletier, 2001). Because mitochondria are primarily inherited from the maternal individual, limited seed dispersal coupled with founder effect may lead to spatial structuring of sex (Olson & McCauley, 2002; Klaas & Olson, 2006; Olson *et al.*, 2006). Less common is a pure nuclear control of sex, which under limited pollen and seed dispersal could also lead to spatial structuring. Alternatively, the mechanism proposed by the SDP hypothesis could operate within populations, so that females persist in harsh microsites within populations where hermaphrodites produce fewer seeds. It is well documented that environmental conditions, such as soil moisture and light availability, are often quite heterogeneous among microsites within a population (e.g. Hutchison & Matt, 1977; Robertson *et al.*,

1993; Palmer, 2003). This is particularly true for forest understory habitats, where gaps created by tree fall or the forest edge effect can dramatically change the water and light availability for a particular microsite (Hutchison & Matt, 1977; Chen *et al.*, 1993; Chen *et al.*, 1995; Xu *et al.*, 1997). It has also been shown that microsites of similar quality tend to aggregate (Fortin *et al.*, 1989; Rossi *et al.*, 1992; Legendre, 1993; Clark *et al.*, 1996; Nicotra *et al.*, 1999) in natural populations. Based on the SDP hypothesis, the aggregation of sexes may reflect the underlying aggregation of microsites, with females more prevalent in harsher microsites and hermaphrodites more prevalent in less harsh microsites. The sex-differential plasticity hypothesis, thus, can potentially explain both among and within population variation in sex distribution. To our knowledge, this hypothesis has not been tested at the local scale within populations.

In this study, we tested several predictions derived from the sex-differential plasticity hypothesis at two spatially hierarchical scales: among populations and among microsites within populations of *Geranium maculatum*. Specifically, we asked 1) do sites occupied by monomorphic and dimorphic populations differ in their light availability or soil moisture, 2) within a population, are the sexes aggregated, 3) within a population do females and hermaphrodites live in different light availability or soil moisture environments, and 4) within a population does seed production vary with the environmental conditions. We predicted that if sex-differential plasticity contributes significantly to the distribution of females among populations of this species we would expect to see that populations in wetter sites would lack females because it would be more difficult for females to gain a seed fitness advantage over hermaphrodites in those populations. Similarly, we predicted that mechanisms functioning within a population

would lead to the aggregation of females in drier microsites, where they would have a seed production advantage over hermaphrodites.

Materials and Methods

Geranium maculatum L. is a gynodioecious, rhizomatous perennial ranging from the South Eastern US to Canada and west to the Great Plains (Radford *et al.*, 1968). Populations used in this study were located in Georgia and were in the forest understory near streams. *G. maculatum* leaves emerge prior to canopy tree leaves and remain until the fall (In the OT population used in this study, *G. maculatum* leaves emerged prior to 17 March 2008 while the canopy leaves did not fully emerge until approx. 29 April 2008). Flowering begins in early spring (approx. 24 March 2008 for OT), with individuals producing on average six flowers per inflorescence (Chang, 2006). Flowers are visited by generalist pollinators including bees, flies and butterflies. Hermaphrodites are self-compatible and selfing rates range from 0-17% depending on the population (Van Etten *et al.*, unpublished data). Inbreeding depression is high and variable between populations (cumulative postdispersal inbreeding depression ranges from 0.38 to 0.84, Chang, 2007). Seeds are dispersed by the elastic dehiscence of the schizocarp, to an average of 3 m from the maternal plant (Stamp & Lucas, 1983). Based on field and greenhouse observations, sex appears to be genetically determined. Preliminary data rule out the possibility that sex is controlled entirely by cytoplasmic genes, but the exact genetic control has not yet been determined (Van Etten & Chang, unpublished data). Females have small aborted anthers and smaller petals when compared to hermaphrodites (Ågren & Willson, 1991; Chang, 2006). Flower number is approximately the same

between sexes in natural populations (Ågren & Willson, 1991; Chang, 2006) though in one greenhouse study females produce slightly more flowers than hermaphrodites (Van Etten *et al.*, 2008). In natural populations, females produce more seeds than hermaphrodites (20 – 50% increase, Ågren & Willson, 1991; Chang, 2006) and seeds that have a higher germination rate (Chang, 2006). Higher seed production is probably caused by higher seed set (more seeds produced per flower, Ågren & Willson, 1991; Chang, 2006) though the ovule number is the same between sexes. Local populations around Athens, GA range in female frequency from 0-50% (Chang, 2006).

Populations

Six populations were used in this study: three monomorphic (CA:33˚54'0.84"N, 83˚23'9.24"W; WT:33˚54'30.29"N, 83˚23'53.11"W; HP:33˚45'48.56"N, 83˚26'37.96"W) and three dimorphic (OT:33˚54'4.92"N, 83˚22'47.34"W; OTG: 33˚54'4.90"N, 83˚22'44.93"W; RL:33˚45'44.34"N, 83˚15'53.58"W). CA, WT, OT and OTG were located in Athens, GA while HP and RL were in Oconee County, GA. The dimorphic populations varied in the percentage of females: OT and OTG had ~50% female, and RL had ~25% female. All populations were located in the forest understory and all but CA were within 20 m of a creek or river with continuous water flow. All populations used appear to be well established and persistent; the areas in which populations were found have been protected from major disturbance for several decades and four of the populations used in this study have been monitored for other experiments for ~7 years. Additionally, a demographic study suggests that the mortality of established plants was very low over a 3-year period (Van Etten, unpublished data). The populations chosen were within a larger group of populations that have been surveyed for

their sex ratio. The populations' sex ratio does not appear to have a spatial component; dimorphic populations are interspersed with monomorphic populations. For example, OT and OTG (dimorphic populations) are within 0.48 km of CA (a monomorphic population), while HP and CA (both monomorphic) are over 18 km apart.

To make sampling and mapping of plants easier in the dimorphic populations, permanent sampling points were placed throughout the populations. Parallel transects were laid down every three meters and on each transect a sampling point was placed every three meters, forming a grid of sampling points. This led to 49-53 sampling points per population, which were used in plant selection, mapping and measuring of the environmental conditions. All flowering plants in the dimorphic populations were mapped by stretching a meter tape down the length of a row of sampling points and then measuring the distance from the plant to the meter tape with another meter tape. Locations were then converted to x,y co-ordinates. We also recorded the sex of every plant mapped.

Environmental conditions

Light availability and soil moisture were measured in all populations. Light and moisture were chosen as environmental variables of interest because many studies have found that soil moisture affects the sex ratio (see introduction). Additionally, light availability is often the cause of soil moisture differences; areas with higher light have higher evapotranspiration and therefore lower soil moisture (Rosenberg *et al.*, 1983). Light availability measures were taken using hemispherical photos (Nikon CoolPix885 digital camera with Nikon FC-E8 fisheye Converter lens). Pictures were taken approximately 25 cm off the ground, with the top of the camera pointing north and the

camera level. Pictures were taken either on an overcast day or just after sunrise or just prior to sunset. In dimorphic populations, pictures were taken every third sampling point in the spring (20 March 2008-22 March 2008, prior to canopy leaf emergence) and at each sampling point in the fall (7 October 2008-4 November 2008, prior to significant leaf loss). In monomorphic populations, two 15 m long transects were laid down to span the population and photos taken every 3 m. For these populations, photos were only taken in the fall (16 October 2008-4 November 2008). Pictures were analyzed for percent canopy openness using Gap Light Analyzer (Frazer *et al.*, 1999).

Soil moisture was measured as percent volumetric water content (VWC) using a hand held moisture sensor (Hydrosense by Spectrum Technologies Inc., 12 cm probes). Leaf litter was removed and readings were taken by completely inserting the probes perpendicular to the soil surface. In the dimorphic populations, three soil moisture readings were taken and their average recorded at each of the sampling points. Readings for the dimorphic populations were taken twice – once in the spring of 2008 (10 May) and once in the fall of 2008 (14 October- 15 October). For the monomorphic populations, at each of the places where light photos were taken, three soil moisture readings were taken and their average recorded. Readings for the monomorphic populations were taken in the fall of 2008 (16 October –17 October). In order to get data that were comparable among populations, each set of measurements (fall or spring) were taken within four days of each other during which time there was no significant rainfall.

Kriging for the environmental conditions

To determine if hermaphrodites and females in dimorphic populations were located in different environments, we used ordinary kriging to estimate soil moisture and

light availability for each plant mapped in a population. Kriging is a method that uses the measurement of a variable taken from several sampling sites to estimate the value of that variable at unmeasured sites based on a model that takes into account the pattern of spatial correlation of that variable (Rossi *et al.*, 1992; Wackernagel, 2003; Schabenberger & Gotway, 2005). This method was developed primarily for mining purposes aiming to predict the location of desirable ores and has been adopted by ecologists to predict variation in natural populations (Rossi *et al.*, 1992). Environmental conditions, like ore deposits, are not randomly located in an environment, but are aggregated in space. This method takes advantage of such aggregation and uses it to predict, for example, ore concentrations or light availability, by creating mathematical models relating the variable of interest with a given location. To parameterize these models, several methods are typically used, including maximum likelihood, restricted maximum likelihood (REML), least square methods and Bayesian approaches. We used these methods to obtain parameters for models describing each environmental variable separately (light availability and soil moisture) and compared the models with non-spatial models using the Akaike Information Criterion (AIC, Akaike, 1974) values to determine if the environmental variables were spatially structured. The non-spatial model outperformed the spatial model in two cases and thus kriging was not used for those measures: for the spring light measures, there was insufficient data to construct an appropriate model; in OTG for spring soil moisture, the non-spatial model was better. For the other variables, parameters estimated using REML yielded the best-fit models and were used to estimate the light availability and soil moisture for each of the individuals mapped in the

dimorphic populations. Models and estimations were calculated using the geoR package in R (Ribeiro Jr & Diggle, 2001).

Demographic traits

To determine if seed production was correlated with either of the environmental conditions measured, ~ 50 plants per sex per population were selected and data recorded in 2006-2008 for OT and in 2007-2008 for OTG and RL. In OT and OTG, at each of the sampling points, the closest female and hermaphrodite were selected. In RL, because females were at a lower density, female plants were chosen throughout the population as well as the nearest hermaphrodite within 1 m.

For each year observed, the number of flowers, fruits and seeds were recorded for each of the sampled plants. All seeds and fruits were collected by covering the inflorescence with a bridal veil bag until maturation to prevent seed dispersal. From these measures, we calculated the cumulative seed production as the total number of seeds produced over the 2-3 year study period.

Statistical Analysis

Environmental differences between monomorphic and dimorphic populations were tested using the average measured environmental data for each population as the response variable and the population type as the predictor variable in an ANOVA.

To determine if the sexes were spatially aggregated, we used a spatial autocorrelation method often used to determine if there is fine-scale genetic structure. In the context of genetic structure, this analysis measures the genetic similarity between pairs of individuals separated by a particular distance (Sokal & Oden, 1978a; Ennos, 2000) and calculates relatedness using an autocorrelation coefficient, r, described in

Smouse and Peakall (1999). Though the coefficient r is normally used when analyzing genotypes from multiple genetic marker loci, it can be adapted to other types of variables. In our case, we use sex (female or hermaphrodite) as the "genotypes" to calculate the r coefficient. This coefficient is bounded by one and negative one, with positive values indicating that individuals are more similar than expected by chance (in our case the same sex) and negative values indicating individuals are less similar than expected by chance (in our case the opposite sex). The significance test for r-values was carried out against the null hypothesis that the sexes were randomly distributed throughout the population, corresponding to an r-value of zero. For each distance class, a mean similarity between pairs of individuals at that distance class is calculated, and tested to see if it is significantly different from zero by bootstrapping of the "genotypes". Significant positive r-values would indicate that plants of the same sex tend to aggregate with each other while significant negative r-values would indicate that opposite sexes tend to aggregate together. In the case where the r-values are significantly different from zero at small distances but not at large distances, the distance at which the r-value changes from significantly to not significantly different from zero approximates the size of sex patches. This analysis was performed using GenAlEx V6.2b (Peakall & Smouse, 2006).

To determine if females and hermaphrodites were located in significantly different environmental conditions, the estimated soil moisture and light availability values from kriging for each mapped individual were used in an ANOVA. To validate the results from the kriging estimates, we carried out two other analyses. First, each mapped individual was assigned the measured value (light availability or soil moisture) from the nearest sampling point, which was never more than 1.5 m away. These values

were then used in an ANOVA with the environmental measure as the response variable and sex as the predictor variable to determine if the sexes differed in their environment. Second, the sex ratio within a 3 m radius circle of each measuring point was determined and was regressed against light availability and soil moisture using Proc Reg in SAS. In this analysis, the spring light availability measures were used in addition to those used in the kriging analysis. We expected to see a significant influence of environmental factors on the sex ratios in this analysis if females and hermaphrodites differ significantly in their preference for light availability or soil moisture.

To determine the relationship between seed production and the microsite environment, the estimated light availability and soil moisture around plants for which seed production data were collected were regressed with cumulative seed number. Additionally, an ANCOVA including cumulative seed number as the response variable and sex, environmental measures and their interaction as the predicting variables was used to test if the slope of the regression was significantly different between females and hermaphrodites, indicated by a significant sex by environment interaction. Levene's test for equality of variance was used to test if variation in seed fitness differed between females and hermaphrodites. To determine which factor(s) were most important for seed production, we compared models containing one or more of several predicting variables, sex (dummy coded as a one or a zero), light availability, soil moisture, the density of pollen donors within 3 m radius circle, the density of flowering plants within a 3 m radius circle and the sex ratio (% females) within a 3m radius circle using the AIC option in Proc Reg in SAS to determine the best fit model. Statistical tests were performed in SAS 9.2.

RESULTS

Does the environment differ between monomorphic and dimorphic populations?

Average light availability but not soil moisture differed significantly between sites occupied by monomorphic and dimorphic populations. Monomorphic populations were located in significantly brighter sites than dimorphic populations (Monomorphic mean=13.08% canopy openness, Dimorphic mean=10.51%; P=0.004, d.f.=1,4, Fig. 5.1a). The brightest site was CA with 13.7% average canopy openness, probably because it is parallel to a trail, while the shadiest was OT with 10.2% average canopy openness. Although the average soil moisture did not differ significantly between sites occupied by monomorphic and dimorphic populations (P=0.16, d.f.=1,4, Fig. 5.1b), there was variation among populations, with RL being particularly wet. RL had an average of 22.18% VWC in the fall compared to 16.8% and 18.2% in the other dimorphic populations sites (Fig. 5.1b). The within-population range of microsite soil moisture was also highest in RL; ranging from 14-42% VWC in the spring and 14-40% in the fall, whereas the ranges in the other populations spanned between 12% and 30% in the spring and 11% and 23% in the fall.

Are the sexes aggregated within a population?

All populations showed significant spatial structure of sex (Fig. 5.2, 5.3). However, the populations differed in the strength and spatial range of the structure. OTG had the highest initial r-value (r at 1 m =0.744, Fig. 5.2b), indicating that, compared to OT and RL, individuals in this population are surrounded by individuals of the same sex more often. OT and RL had lower initial r-values (r=0.387, Fig. 5.2a and r=0.459, Fig. 5.2c, respectively) indicating that there is slightly more mixing of the sexes at small

distances than OTG, although individuals are usually still surrounded by the same sex. In OT, the sex structure was significant for a farther distance, until about 11 m, than the other two populations. The other populations had shorter distances before r reached zero (OTG 7 m and RL 4 m), indicating that sex patches were smaller. These results can also be seen in the population maps (Fig. 5.3): in OT, there are large areas with primarily one of the sexes. In addition, in both OT and OTG, r becomes significantly negative at large distances indicating that at large distances individuals are of the opposite sex (Fig. 5.2a, b). This, too, is evident in the population maps where there are distinct female patches and hermaphrodite patches (Fig. 5.3). In RL, however, the sex patches are more intermixed leading to a non-negative r-value at long distances.

Are hermaphrodites and females located in different environments within a population?

Hermaphrodites and females were located in significantly different environments within a population. In all three populations, females were in significantly brighter microsites than hermaphrodites (Fig. 5.3, 5.4a, OT $P<0.0001$, d.f.=1,1282; OTG $P<0.0001$, d.f.=1,166; RL $P=0.038$. d.f.=1,196). In two of the populations (OT and OTG), females were also in drier environments although in RL they were in significantly wetter environments (Fig. 5.3, 5.4b, OT $P<0.0001$, d.f.=1,1282; OTG $P<0.0001$, d.f.=1,166; RL $P<0.0001$, d.f.=1,196).

Results were qualitatively similar in the analysis using the values from the nearest measuring point, with the exception that some results were not significant (OT, d.f.=1,1282: light $P<0.0001$, soil spring $P=0.0004$, soil fall $P<0.0001$; OTG d.f.=1,166: light $P=0.02$, soil spring $P=0.23$, soil fall $P<0.0001$; RL d.f.=1,196: light $P=0.10$,soil spring $P<0.0001$, soil fall $P<0.0001$). Similar results were also found when using the sex

ratio within a 3 m radius circle around each measuring point (Table 5.1). In RL, wetter environments tended to be associated with a higher percentage of females (spring P=0.034, and fall P=0.0022), as was found in the kriging analysis. In contrast to RL, in OT and OTG drier environments tended to be associated with higher percentage of females (OT: spring P=0.077 and fall P=0.052; OTG: spring P=0.667 and fall P=0.082) and in OTG in brighter environments (spring P=0.165 and fall P<0.0001). Though some of the results were only marginally significant (with 0.1<P<0.05), they are nonetheless consistent with results from kriging estimates.

Is seed production correlated with the environmental conditions?

Using cumulative seed number as a measure of seed production, we found contrasting trends among populations. In OT, plants in brighter sites had higher seed production than ones in darker sites regardless of their sex (P=0.002, Table 5.2). Conversely, in RL, plants in darker environments tended to have higher seed production (P=0.04, and P=0.03, respectively, Table 5.2) although sex and local sex ratio also influenced seed production. Further analysis shows that in this population, the sexes differed in the strength of their response to soil moisture (P=0.01, d.f.=1,42, Fig. 5.5e,h). In RL, for both spring and fall soil moisture, females had higher seed production in drier areas (spring P=0.03; fall P=0.01; Fig. 5.5e,h), while hermaphrodites' seed production did not vary with soil moisture (Fig. 5.5e,h). Higher female seed production in drier sites in this population was probably caused by females producing fewer fruits (Female slope in spring = -0.035, P=0.03; Female slope in fall = -0.69, P=0.02) and seeds (Female slope in spring = -0.18, P=0.05) in wet environments compared to dry environments. In OTG, although the model was not significant (P=0.066, d.f.=3,39), the environmental variables

did not affect seed production but the local density of pollen donors, local density of flowering plants and the local sex ratio did affect seed production. The local sex ratio was in the best model for two of the populations (RL and OTG) and in both cases the higher the percentage of females the lower the seed production (Table 5.2).

Hermaphrodites did not have more variation in their seed production nor were they more strongly influenced by the environment than females, hence, providing no support for the sex-differential plasticity hypothesis. More specifically, the variance in cumulative seed production did not differ between genders in any population (RL: females σ^2=174.72, hermaphrodites σ^2=106.74, F=1.52, P=0.22, d.f.=1,45; OTG: females σ^2=28.10, hermaphrodites σ^2=26.39, F=0.01, P=0.94, d.f.=1,66; OT: females σ^2=73.44, hermaphrodites σ^2=40.68, F=1.68, P=0.20, d.f=1,95). Additionally, females' seed production in OT and RL was significantly correlated with light and light and soil moisture, respectively while there was no significant correlation for hermaphrodites (Fig. 5.5).

DISCUSSION

This study investigated the relationship between the environment and the distribution of sex both within and among populations. Among populations, previous studies in other species had found that dimorphic populations were in drier environments than monomorphic populations. However, we found that the soil moisture was similar for both types of populations but that the dimorphic populations were in darker environments than the monomorphic populations. Contrary to this result, we found the expected pattern of females being located in drier microsites within two of the dimorphic

populations. This expectation was based on the sex-differential plasticity (SDP) hypothesis, which suggests that hermaphrodites' seed fitness would be negatively affected in drier microsites, giving females an advantage under these conditions. We found no indication that this had occurred in the populations we studied. We also did not find evidence that hermaphrodites' seed production was more strongly influenced by the environment than females'. Our results, thus, suggest that while the sexes may be associated with different microsites within populations, the SDP hypothesis does not provide a satisfactory mechanism to explain this pattern. This hypothesis is, hence, unable to explain the sex distribution patterns of females either within or among populations in this species.

Among population environmental differences

Previous studies have shown that soil moisture may play an important role in large-scale patterns of female distribution, with dimorphic populations found more often in drier areas (Vaughton & Ramsey, 2005) and a higher frequency of females in sites with drier soils (Wolfe & Shmida, 1997; Alonso & Herrera, 2001; Asikainen & Mutikainen, 2003; Case & Barrett, 2004; but see Alonso *et al.*, 2007; Caruso & Case, 2007). One of our main goals in this study was to test whether the same pattern occurred at smaller scales. We compared populations with and without females located within approx. 19 km of each other. Our results showed quite a different picture. We found that dimorphic populations, on average, tended to be in darker environments but in similar soil moisture environments. Contrary to this pattern, at the within population level, we found that females tended to be located in brighter microsites, consistent with what we expected (discussed further in next section). One explanation for these contrasting results

could be that different selective and stochastic processes might be responsible for patterns at different geographical scales. Contrasting results depending on the spatial scale were also found in two previous studies that examined ecological correlations with female frequencies in natural populations of *Daphne laureola* at different geographical scales (Alonso & Herrera, 2001; Alonso *et al.*, 2007). Comparisons among populations separated at most by 600 km showed that drier populations had a lower female frequency (Alonso *et al.*, 2007) while a subset of more local populations (within 60 km) had previously shown the opposite (Alonso & Herrera, 2001). Alonso and colleagues suggested that site-specific plant traits and mating system variation could potentially lead to the contrasting results at different spatial scales though supporting evidence remained absent. It is possible that characteristics such as founder effect and mating systems are more important for the presence of females at this spatial scale than the ecological conditions such as soil moisture and light availability measured in this study. Future studies that increase the number of populations and the geographical spatial scale will allow a more direct comparison for our system and the *D. laureola* studies.

Locations of sexes within populations

Contrary to the among population patterns, the results from the within population analysis were consistent in two populations with our original prediction that females would be more prevalent in sites with lower soil moisture and higher light availability. Females tended to grow in drier and brighter areas than hermaphrodites with the exception of RL where females were located in wetter sites. The aggregation of the sexes into specific microsites could be influenced by several factors. First, founder effects could cause the aggregation of the sexes such that females were originally introduced into

a particular area and have not disperses throughout the population. Founder effects may be especially important if sex is under cyto-nuclear control. With this type of genetic control, male sterility is caused by a gene in the mitochondria, which is usually passed on by the seed parent (but see McCauley *et al.*, 2005; McCauley *et al.*, 2007; Pearl *et al.*, 2009). Combined with limited seed dispersal, these two factors could cause a patchy distribution of sex that happens to coincide with different light and soil environments. Though our data do not allow us to completely rule out this possibility, it seems unlikely to be the sole reason for the observed spatial structure because genetic structure analyses on several populations have shown that genetic structure for neutral nuclear genes extends only to approx. 2-3 meters in this species (Van Etten & Chang, unpublished data). Cytoplasmic genes would likely have more structure than nuclear markers because of their uniparental inheritance (Hu & Ennos, 1997). Thus, regardless of the type of genetic control of sex in this species, sex structure in all populations we studied extends past the genetic structure, indicating that structuring of neutral genes does not fully explain the pattern we see in sex distribution.

Another possible cause for the observed sex distribution is that the sexes may have different seed fitness in different environments, such as suggested by the SDP hypothesis. If females produce relatively more seeds and/or produce seeds better able to establish in harsher environments, their frequency could increase in these environments. We found support for this pattern in terms of seed production in RL but not in the other two populations (see more discussion in next section). Beyond seed production, in other gynodioecious species, females have been found to produce larger and better provisioned seeds (reviewed in Shykoff *et al.*, 2003), and thus may be able to better germinate or

survive in harsher sites. In *G. maculatum*, female seeds are slightly heavier (Chang, 2006) and germinate at a higher rate in the greenhouse. However, whether germination or survival rates differ between the sexes in natural environments is unknown for this species.

The observed sex distribution could also result from the indirect effect that environmental conditions may have on seed fitness through its influence on mating systems. It has been suggested that the selfing rate may vary with the environment (Vaughton & Ramsey, 2005), which would differentially affect the sexes. In dry areas, the selfing rate of hermaphrodites may be higher than in wet areas, possibly because flower size in some species decreases in drier areas leading to more selfing (Barrett & Eckert, 1990; Jonas & Geber, 1999; Herrera, 2005; Lambrecht & Dawson, 2007). If this occurs in *G. maculatum*, the strong inbreeding depression found in both pre and post seed dispersal traits (Chang, 2007) could lead to the reduction in hermaphrodites' seed fitness to a level low enough for females to have an advantage and be maintained in those areas. A mating system analysis taking into account the environment of the individuals would help determine if the selfing rate within a population depends upon the environment. Though we do not know the mechanism(s) behind the association of the sexes with different microsites, this association may aid in maintaining both sexes. Manipulative studies are needed to clarify the influence of light availability on the sexes in order to determine if heterogeneous environments may be aiding in maintaining both sexes.

Correlations between seed production and environmental gradients

The SDP hypothesis suggests that in harsh environments hermaphrodites reduce allocation to seed production, while females' seed production remains comparatively

constant, boosting females' relative seed production in these areas. We predicted that if this were occurring in *G. maculatum*, hermaphrodites' seed production should be more dependent upon the environment than females', leading to a steeper decline for hermaphrodites than females in seed fitness as the environment became harsher. Our results do not show this trend – females' seed production generally was more affected by the environment than hermaphrodites'. In addition, the sign of the slopes differed among populations (Fig. 5.5) suggesting that there is not a consistent trend across populations in the type of environmental conditions that could lead to higher seed fitness. Instead, it appears that the relationships we found between the environmental factors and seed production might be specific to each population. Our results, thus, provide no support for the power of the SDP hypothesis as a general mechanism in explaining the sex distribution at the local level for this species. The influence of other mechanisms, such as pollen limitation, on seed production may simply outweigh the importance of seed production plasticity across soil moisture or light gradients.

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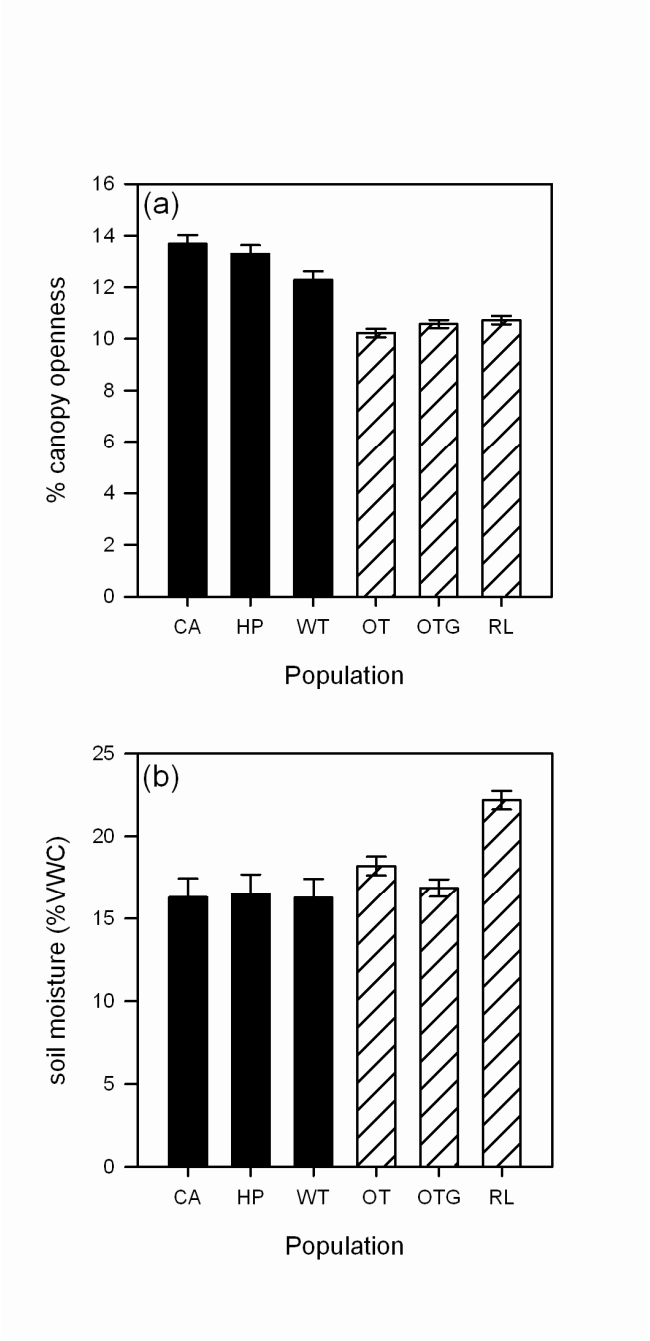


Figure 5.1. Mean and SE of light availability (a) and fall soil moisture (b) for monomorphic (solid bars) and dimorphic populations (hatched bars). VWC=volumetric water content.

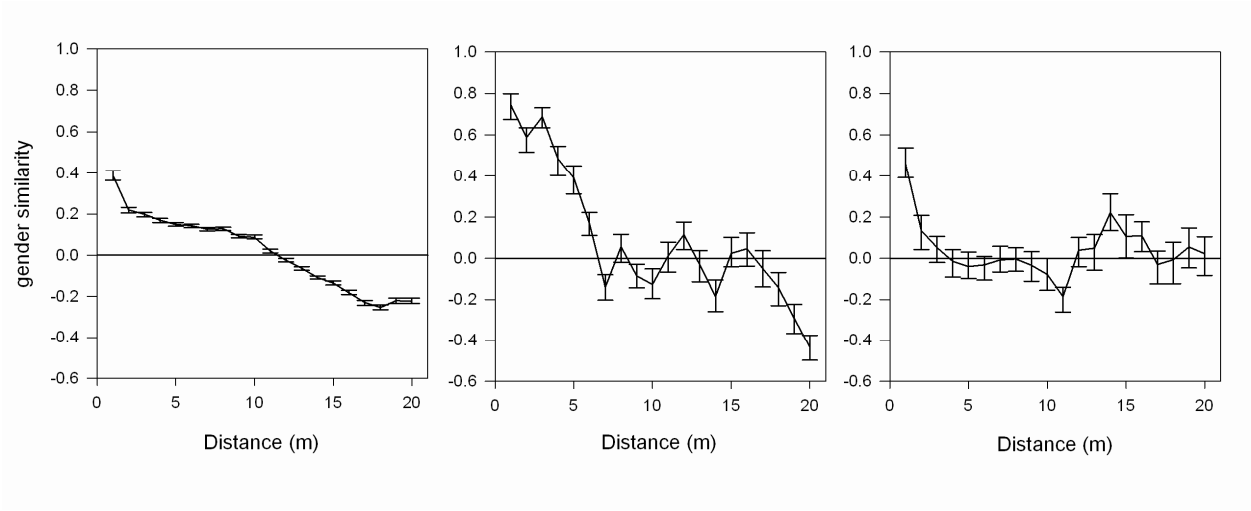


Figure 5.2. Autocorrelograms showing sex structure in OT (a), OTG (b) and RL (c). Positive similarity indicates individuals are surrounded by the same sex while negative values indicate they are surrounded by the opposite sex. Error bars are obtained from bootstrapping.

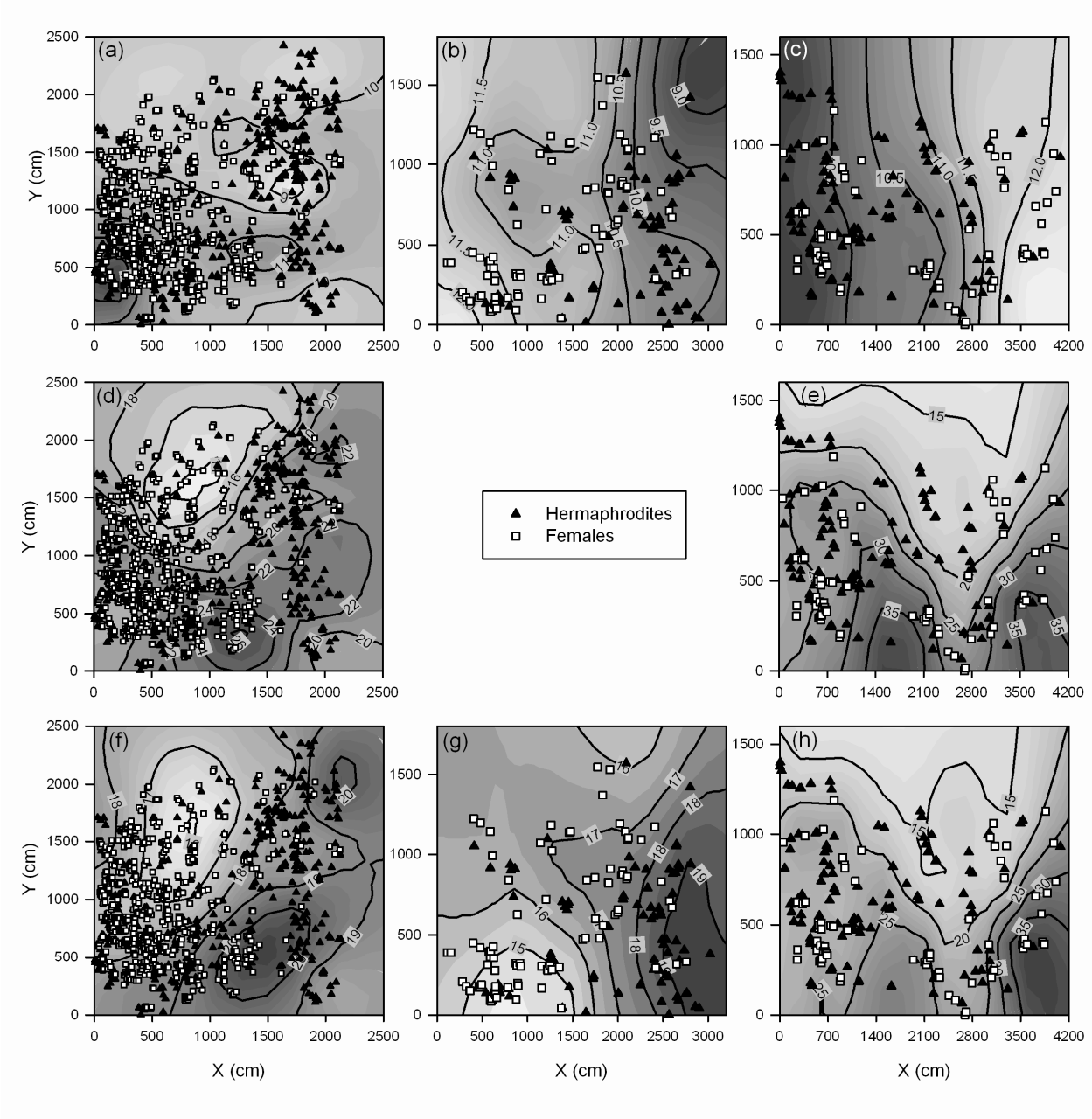


Figure 5.3. Maps of flowering individuals in each population (OT - a,d,f; OTG - b,g; RL - c,e,h) on fall light availability (a-c), spring soil moisture (d-e) and fall soil moisture (f-h). Females are white squares, hermaphrodites black triangles. The shade of grey indicates the level of light availability or soil moisture, with lighter grey being brighter or drier.

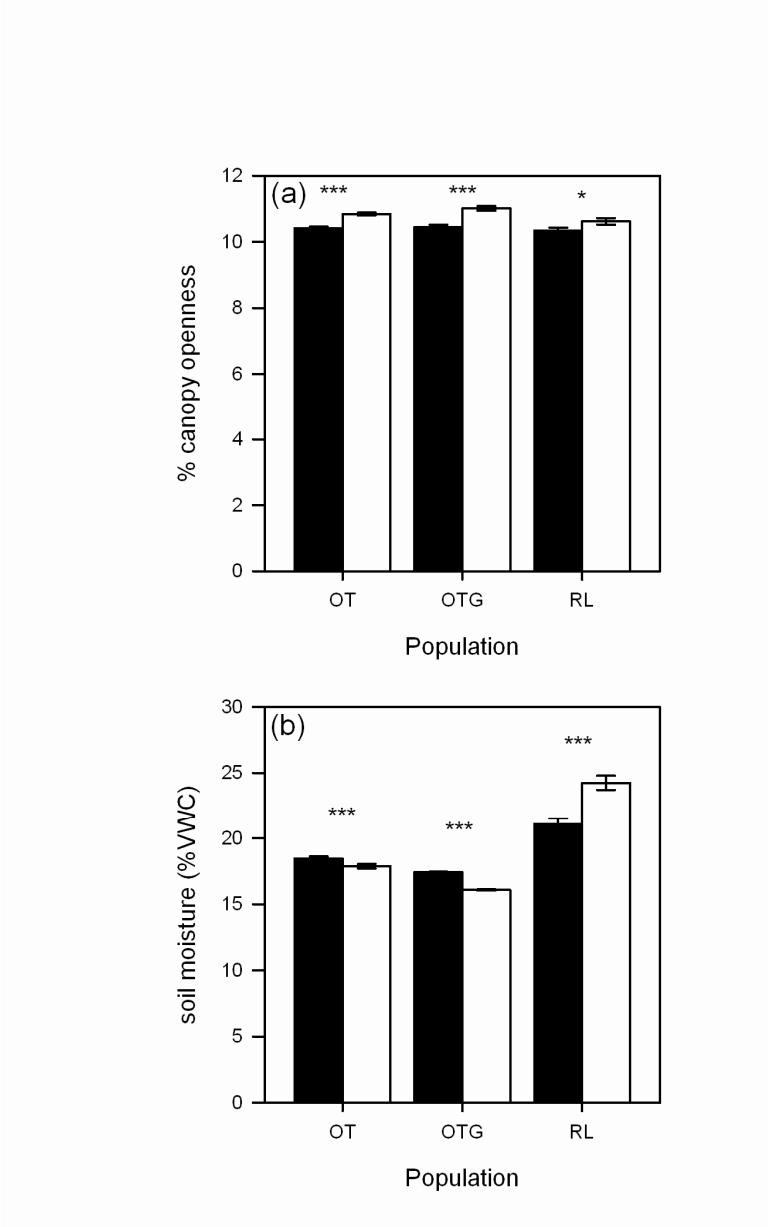


Figure 5.4. Mean and SE of light availability (a) and fall soil moisture (b) for hermaphrodites (solid bars) and females (open bars). VWC=volumetric water content.

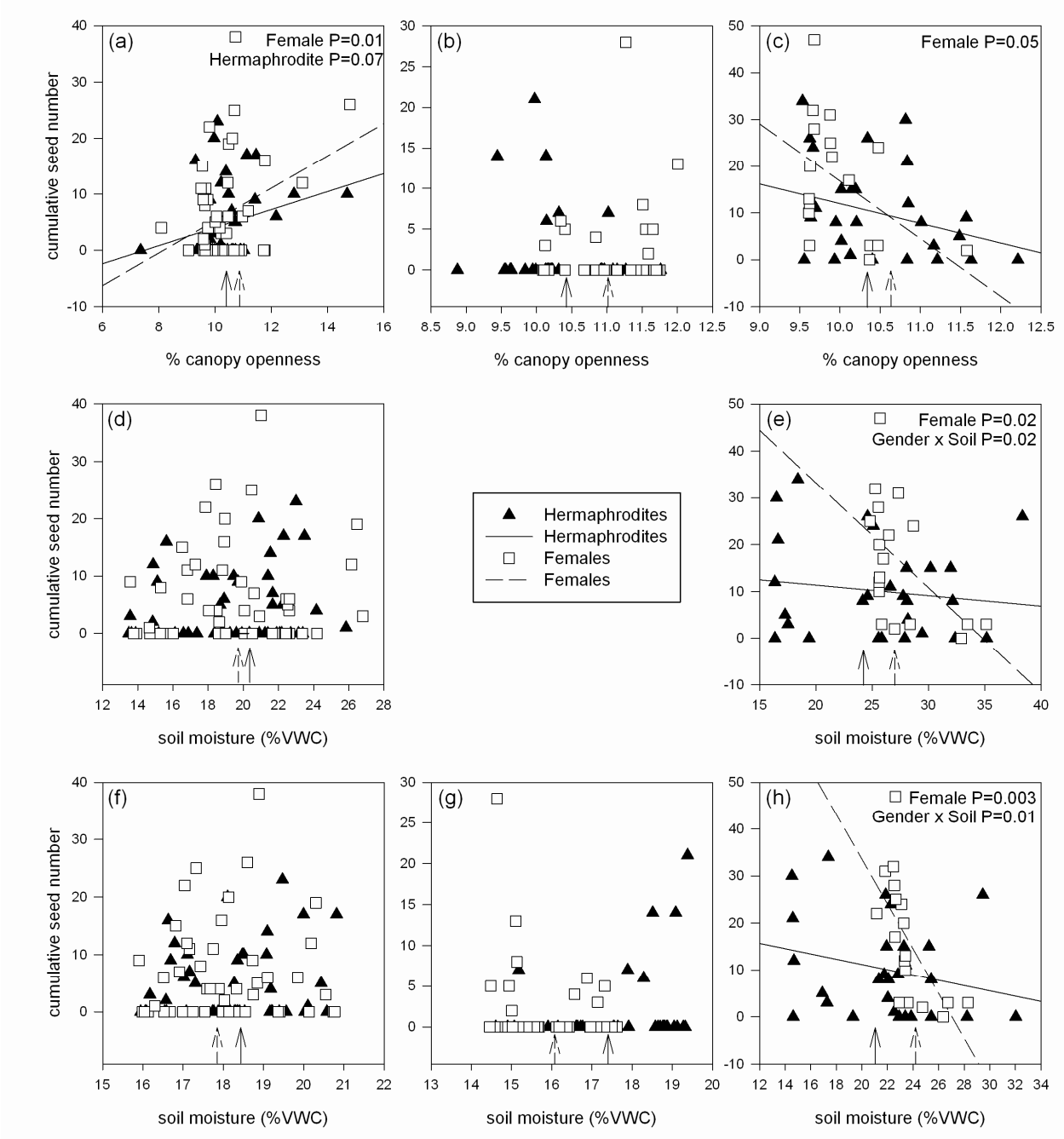


Figure 5.5. Regressions between cumulative seed production and fall light availability, (a-c), spring soil moisture (d-e), and fall soil moisture (f-h) for hermaphrodites (triangles and solid lines) and females (squares and dashed lines) for each dimorphic population

(OT: a,d,f ; OTG: b,g; RL: c,e,h). Arrows indicate the average environment for each sex from kriging analysis. VWC=volumetric water content.

Table 5.1. Slope, intercept and significance of regressions between environmental variables and sex ratio (% females) within a 3 m radius of each measuring point in each population. Light=% canopy openness, soil=% volumetric water content, N=number of observations, [b]=P<0.09, [a]=P<0.06, *=P<0.05, **=P<0.01, ***P<0.0001

	Light Spring			Light Fall			Soil Spring			Soil Fall		
Pop	N	slope	intercept	N	slope	intercept	N	slope	intercept	N	slope	intercept
OT	14	0.010	0.165	48	0.032	0.175	47	-0.019[b]	0.879	48	-0.033*	1.113
OTG	17	0.081	-2.241	54	0.201***	-1.683	53	0.006	0.307	54	-0.032[b]	0.989
RL	16	-0.040	1.406	51	0.032	0.001	48	0.011*	0.076	51	0.018**	-0.056

Table 5.2. Parameter estimates (t-values) of the top model predicting cumulative seed number for each population. Soil moisture=% volumetric water content, light availability=% canopy openness, hermaphrodite density=number of hermaphrodites in 3 m radius circle, adult density= number of flowering plants in 3 m radius circle, sex ratio=% females in 3 m radius circle, df1 and df2 are the numerator and denominator degrees of freedom for the model, respectively. [b]=P<0.09, [a]=P<0.06, *=P<0.05, **=P<0.01, ***P<0.0001

Pop	Sex	Soil moisture	Light Availability	Hermaphrodite density	Adult density	Sex ratio	df1,df2	Model F	Model P
OT			2.24 (3.10)**				1,95	9.59	0.002
OTG				-1.37 (-2.26)*	0.33 (1.53)	-17.22 (-2.60)*	3,39	2.59	0.066
RL	7.93 (2.27)*		-6.75 (-2.85)**			-16.72 (-2.57) *	3,43	5.55	0.0026

CHAPTER 6

MAINTAINING FEMALES: THE ROLE OF POLLINATORS IN *GERANIUM MACULATUM*[1]

[1] Van Etten, M.L. and S-M. Chang. To be submitted to *Journal of Ecology*.

ABSTRACT

One of the major transitions in flowering plants is from hermaphroditism to separate sexes. Gynodioecy, the co-occurrence of female and hermaphroditic individuals, is thought to be an intermediate step between these two extremes. Retaining females in a population requires that females have a seed fitness increase to compensate for the lack of pollen fitness. However, female seed compensation may be negatively influenced by the often documented pollinator discrimination against them. The cause and consequences of pollinator discrimination were explored using experimental populations with a variety of sex ratios. We found that females received fewer visits than hermaphrodites, which decreased their seed production to a level similar to hermaphrodites. Additionally, females were most discriminated against when rare, causing a further decrease in their seed production to below that of hermaphrodites. These results suggest that pollinator discrimination may decrease the chances of both the initial invasion of females and their later maintenance in populations.

INTRODUCTION

Plants, unlike animals, exhibit a wide array of breeding systems, ranging from both male and female functions within the same flower to separating sexual functions on different individuals (Richards 1986). This variation makes plants highly useful in studying the context of transitions between these breeding systems. One of the major transitions in flowering plants is from hermaphroditism (where both sexual functions are housed within a single flower, approx. 72% of plant species), to dioecy (sexual functions on separate plants, approx. 4% of species, Richards 1986). Gynodioecy, the co-

occurrence of female and hermaphroditic individuals, is thought to be the most likely intermediate step between these two extremes (e.g. Charlesworth and Charlesworth 1978). Hence, characterizing the dynamics of this initial evolutionary step from hermaphroditism to dioecy aids in understanding one of the most important evolutionary transitions in plant breeding systems.

In addition to being an intermediate breeding system, gynodioecy has interested evolutionary biologists since Darwin (1877) because of the apparent paradox of why selection would favor individuals that lose half of their potential fitness. Because females cannot be pollen donors for any seeds, their loss of pollen fitness must be compensated for through higher seed fitness to remain in populations. The amount of increase in seed fitness needed for females to be maintained in populations ranges from just slightly more to twice as much as hermaphrodites' seed production, depending upon the type of genetic control (Charlesworth and Charlesworth 1978; Charlesworth 1981; Charlesworth 1999; Lloyd 1974). Though compensation in seed fitness has been studied in many species (reviewed in Shykoff et al. 2003), the factors contributing to compensation are less well studied (Ashman 1992; Delph and Mutikainen 2003; Shykoff 1988; Stevens and Van Damme 1988). Thus, determining factors that influence seed production is key to understanding the evolutionary trajectory of gynodioecious species.

Pollinators are one factor that may greatly influence seed production. Many gynodioecious species are animal-pollinated and thus pollinator visitation may play an important role in seed production in many gynodioecious species. Generally, females receive less pollinator service than hermaphrodites, likely due to females being less attractive or by having fewer rewards (reviewed in Delph 1996). Females usually have

smaller flowers or fewer flowers than hermaphrodites (Delph 1996; Shykoff et al. 2003), both of which pollinators may assume indicate poorer rewards. In fact, females often do provide fewer rewards than hermaphrodites since they lack pollen and in some cases produce less nectar as well (reviewed in Delph 1996; Diaz and Cocucci 2003; Molano-Flores 2002; Talavera et al. 1996). Regardless of the cause, pollinator discrimination against females may influence the evolutionary dynamics of gynodioecy in many species because it could potentially decrease the relative seed fitness of females, making it harder for females to persist in populations.

The importance of pollinator discrimination, in part, depends upon its strength, which may be influenced by the relative frequency of females to hermaphrodites. It has been found that pollinators tend to discriminate against plants that are at low frequencies (Brittain and Newton 1933; Faegri and Van Der Pijl 1971; Free 1970; Levin 1972). This minority disadvantage has also been found to occur between morphs of the same species (Epperson and Clegg 1987; Levin 1972; Rausher et al. 1993; Smithson 2001; Smithson and Macnair 1996; Smithson and MacNair 1997). For example, a study placing two morphs of *Phlox drummondii* differing in floral shape at different frequencies found that pollinators visited the rare morph less often than expected given the ratio of the two morphs, despite both morphs producing the same rewards (Levin 1972). This minority disadvantage may be especially important in gynodioecious species because females are usually less common than hermaphrodites, especially during the initial establishment of females.

The population sex ratio may also influence the composition and abundance of visiting pollinators. Pollinator behavior has been shown to follow optimal foraging

theory (Charnov 1976; Goulson 2000) and patches with low resources are likely to be discriminated against. In gynodioecious species, females provide fewer rewards so populations with more females may attract fewer pollinators (Ashman and Diefenderfer 2001). While lower visitation rates may influence both females' and hermaphrodites' seed fitness, females may be at more of a disadvantage because they may receive fewer visits and because hermaphrodites can potentially compensate for lower pollinator abundance by self pollinating. Additionally, the types of pollinators may change with the frequency of females depending upon the energetic needs of the pollinators or the types of rewards they collect. Changes in the composition of the pollinator pool may influence discrimination against females but may also have population wide repercussions including changing the amount of geitonogamous pollination and gene flow (Goulson and Wright 1998; Goulson et al. 1998; Wolfe and Barrett 1987). Understanding the impact of sex ratio on pollinator discrimination, abundance and composition is important in determining the significance of pollinators to the evolutionary dynamics of gynodioecy.

Although many studies have investigated pollinator discrimination against females (reviewed in Delph 1996), few have carefully examined the influence of sex ratio on pollinator behavior. Among studies that have used experimental populations to investigate the effect of sex ratio, the results lack consistent trends. For example, in *Kallstroemia grandiflora*, the sex ratio did not affect pollinator visitation, fruit set, seed set or seed mass (Cuevas et al. 2008). In contrast, Ashman and Diefenderfer (2001) found that increasing the frequency of females decreased the discrimination against females, but increased the degree of pollen limitation leading to lower fruit production.

Other studies that also found that higher female frequency in the population reduces seed production generally assumed that it was due to less pollen available at higher female frequencies and rarely tested pollinator discrimination as a possible mechanism for this result (e.g. McCauley and Brock 1998; Miyake and Olson 2009). More studies specifically investigating both pollinator discrimination and pollen limitation are needed to determine the relative importance of these two mechanisms on the interaction between the population sex ratio and the relative fitness of the sexes.

In this paper, we address the effect of the sex ratio on pollinator discrimination and on seed fitness by manipulating the sex ratio in experimental populations. Specifically, we answer the following questions: 1) do pollinators discriminate against females and what traits is this discrimination based upon, 2) does pollinator discrimination affect seed fitness and 3) does sex ratio affect pollinator behavior and the relative fitness of the sexes. We expect that, similar to most other species, females will be discriminated against because of their smaller petals and fewer floral resources. We also expect that females will be more discriminated against when at lower frequencies and, as a result, females will have lower seed fitness when at lower frequencies than when at higher frequencies.

METHODS

Geranium maculatum L. is a gynodioecious, rhizomatous perennial ranging from the south eastern US to Canada and west to the Great Plains (Radford et al. 1968). Flowering in natural populations begins in early spring, with individuals producing on average six flowers per inflorescence (Chang 2006). Flowers are visited by generalist

pollinators including bees, flies and butterflies. Flowers are protanderous, with 2 sets of 5 anthers dehiscing prior to the stigma lobes reflexing and becoming receptive. This process takes a total of approx. 2-3 days under greenhouse conditions (Willson et al. 1979, Van Etten, personal observation). The stigma remains receptive for several days, however once pollinated the stigma lobes close and the petals fall off soon after (Van Etten, personal observation). Hermaphrodites are self-compatible and average selfing rates range from 0-17% depending on the population (Van Etten et al., Chapter 4). Seeds are dispersed by the elastic dehiscence of the schizocarp, to an average of 3 m from the maternal plant (Stamp and Lucas 1983). Based on field and greenhouse observations, sex is genetically determined. Preliminary data rule out the possibility that sex is controlled entirely by cytoplasmic genes, but the exact genetic control has not yet been determined (Van Etten and Chang, unpublished data). Females have small aborted anthers and smaller petals when compared to hermaphrodites (Ågren and Willson 1991; Chang 2006). Flower number per inflorescence is approximately the same between sexes in natural populations (Ågren and Willson 1991; Chang 2006) although in one greenhouse study females produce slightly more flowers than hermaphrodites (Van Etten et al. 2008). In natural populations, females produce more seeds than hermaphrodites (20 – 50% increase, Ågren and Willson 1991; Chang 2006) and seeds that have a higher germination rate (Chang 2006). Local populations around Athens, GA range from 0-50% female (Chang 2006).

Seeds were collected from female and hermaphroditic plants in two populations in Athens, GA, in 2003 and were germinated and grown in the greenhouse. They were later placed outdoors in a pollinator exclusion enclosure for a total of 2 years. Plants were

watered and fertilized regularly. Individuals with more than 7 buds were selected for use and all open flowers were removed before the experiment.

Experimental arrays were constructed in three locations within the Georgia State Botanical Gardens (Athens, GA). Locations for the arrays were selected to mimic where natural populations are found but were at least 50 m away from natural populations. In each location, a grid was created such that each plant would be 0.5 m from each of its neighbors, with 6-7 plants per row for a total of 31 plants per location.

Plants were randomly selected from the pool of available plants to obtain the sex ratios of 13% (4F, 27H), 26% (8F, 23H), and 42% females (13F, 18H), hereafter low, intermediate and high female frequency, respectively. These ratios were selected to span the range of sex ratios in local populations. Locations of the plants were randomized for each array. We used a Latin Squares design so that for each time period, each sex ratio was represented in one of the three locations. After at least 3 pollinator observation days were complete, the sex ratios were rotated among locations so that at the end of the experiment each location had housed all the sex ratios. Plants were re-randomized when sex ratios were rotated.

For each array, we observed pollinators simultaneously at each location twice a day - once in the morning (between 10 am -12:30 pm) and once in the afternoon (3-5pm). For each observation bout, we observed pollinators for two hours and only observed on non-cloudy days. For each pollinator observed, we recorded its type (see below), which plants it visited and the time on each flower. Pollinators were followed until they left an array. Pollinators were broadly grouped into size classes because size is likely to determine their effectiveness in pollinating *G. maculatum* flowers. Small bees were

~0.75 cm long, and where most likely small sweat bees. Medium bees were between 0.75 and 1.3 cm long and consisted of honey bees and green and black sweat bees. Flies (primarily syphrid flies) were the same size as the medium bees but were grouped separately because a pilot study suggested they behaved differently than bees. Large bees were anything greater than 1.3 cm and included primarily carpenter bees, but also bumblebees and large black bees (most likely carpenter bees). Other pollinators seen were butterflies, wasps and beetles but were not included in our analysis because of their very low frequency (5% of the total visits).

For each observation bout, we also recorded the number of open flowers on each plant. Because some plants were used in multiple arrays of different sex ratios, for each flower, the lower surface of the sepal was dotted with DecoColor paint, with each day and location a different color. The use of DecoColor had been tested in our pilot studies and shown not to affect the seed maturation in this species. To determine the amount of pollen deposition, stigmas were collected on average 22 days after the flower first entered the female phase. We avoided collecting stigmas too early to avoid any potentially detrimental effects on fruit development but preliminary results showed that the time until stigma collection did not affect pollen deposition in this study ($F_{1,824}$=2.32, P=0.12). All stigmas from females (N=374) and a randomly selected subset from hermaphrodites (N=529) were examined under a dissecting microscope and the number of pollen grains counted. Fruits were collected when mature, approximately 4 weeks after pollination. Seeds were counted and a randomly selected subset weighed to the nearest mg (Female N=211, Hermaphrodite N=462).

A variety of plant traits were measured to determine which traits pollinators prefer. To measure floral traits, we collected two fully expanded petals from different flowers per plant and two pre-dehiscent anthers (hermaphrodites only) for pollen production. Petals were scanned into the computer and their area measured using ImageJ (Abramoff et al. 2004). For pollen production, pollen was removed from the anthers using fuschin jelly which was then melted to form a semi-permanent slide (Kearns and Inouye 1993). Pictures of these slides were taken using a digital camera attached to a dissecting microscope and the pollen grains counted using ImageJ. Each image was visually inspected to include grains missed by ImageJ. To obtain a measure of plant resources, after the completion of the experiment rhizomes were weighed to the nearest mg after removing the soil. Rhizome weight was thought to be a better indicator of available resources than leaf number because *G. maculatum* individuals overwinter as rhizomes and store large amounts of energy and nutrients in the rhizome throughout the growing season (Van Etten, pers. obs.).

In a separate experiment, to determine whether nectar production differs between sexes, nectar was collected from plants grown in the greenhouse. Plants used were of similar age and progression into flowering. At approx. 9:00 am**,** flowers were collected from females and hermaphrodites and the phase of the flower noted. For hermaphrodites the phases were: (1) prior to anther dehiscence, (2) 5 anthers dehisced, (3) 10 anthers dehisced, (4) stigma lobes open, or (5) petals wrinkling. For females the phases were: (1) prefemale, (2) stigma lobes open, or (3) petals wrinkling. Because of the very small amount of nectar in flowers, flowers were centrifuged to remove nectar (Heinrich 1983). This was done by first removing the petals and anthers, then placing the remaining flower

into a 1.5mL microcentrifuge tube in which a piece of sturdy plastic with a hole in the center was placed, which allowed the nectar but not the remaining floral tissue to move to the bottom of the tube. Flowers were briefly centrifuged (6 seconds) and the nectar collected using a 5μL micropipette with fine tips. Nectar volume was estimated by measuring the length of the pipette-tips containing nectar to the nearest mm using a dissecting microscope.

To determine whether hermaphrodites receive a substantial amount of self pollen, we carried out a small experiment to compare pollen deposition from non-emasculated and emasculated hermaphrodites in a natural population near one of our experimental sites (population sex ratio of approx. 50% females). The experiment was done twice: 4/3/07-4/9/07 and 4/18/07-4/25/07. Focal hermaphrodite plants (N_{time1}=17, N_{time2}=17) were chosen throughout the population and their flowers emasculated daily for one week. The same treatment was imposed on nearby females (N_1=18, N_2=11). A second hermaphrodite plant nearby was selected as the non-emasculated control (N_1=18, N_2=15). A focal flower with a non-receptive stigma was selected on each plant and marked using a DecoColor paint pen. After a week, stigmas from each focal flower were collected and pollen was counted as described previously.

Data analysis

To satisfy the normality assumptions of the following analyses, measures of visitation rate, pollen deposition and seed production were transformed. Pollen deposition, visit duration per flower, visits per hour, and seed set were log transformed; seed number was square root transformed; fruit set was arcsine square root transformed; the box-cox transformation was used for flower number (λ=0.25), visits per flower

(λ=0.25), the number of flowers visited per plant (λ=1.5), the proportion of flowers visited (λ=0.5) and the visit duration per plant (λ=0.5).

As applicable, two sets of measures were calculated, one set on a whole plant basis and one on a per flower basis (e.g. visits per plant or visits per flower). Visits per plant were calculated as the total number of visits to flowers on a plant per observation hour. Visits per flower were calculated as the visits per hour divided by the number of flowers on the plant. Similarly we also calculated: the average duration of a visit per plant and per flower; the average number of flowers visited on a plant per visit and the average proportion of the total number of flowers visited per visit; and the seeds produced per day and per fruit. Because each observation time is not independent, an average over the days within the sex ratio treatment was obtained for each plant for each of the above measures.

To examine visitation differences between the sexes (question 1) and the effect of sex ratio on visitation (question 3), we performed ANOVAs on several characters. The response variables tested were: flower number per day, visits per plant, visits per flower, flowers visited per plant, proportion of flowers visited per plant, visit duration per plant, visit duration per flower, small bee visits per plant and flower, medium bee visits per plant and flower, large bee visits per plant and flower, fly visits per plant and flower, pollen deposition per stigma, seed number per day, seeds per fruit, fruits per flower and seed weight. The ANOVAs used sex, week, location, sex ratio and sex*sex ratio as predictors and rhizome size as a covariate using proc glm in SAS (SAS Inc. 2000).

Because females produced fewer flowers than hermaphrodites in this experiment, the sex ratio based on the number of female or hermaphrodite flowers often differed from

the sex ratio based on the number of female or hermaphrodite plants (average flower sex ratio for each array: Low(plant sex ratio of 13%F)=6.2, 5.6, 4.0%; Int(26%F)=30, 11, 16.1%; High(42%F)=29, 28, 33.6%). This could influence the sex ratio perceived by the pollinators and the amount of pollen available for seed production (Case and Ashman 2009). Thus, the average flower sex ratio for each array was also tested in place of the sex ratio in the above analyses. Results only differed in one case (visits per plant), which is noted in the results section.

To further investigate discrimination differences (question 1), we also determined whether pollinators were constant to a particular sex. To do this, we calculated the types of transitions between plants for foraging bouts that included multiple plants. Because most pollinator movements were not random but occurred between nearest plants (63% moved to the next closest plant), we chose to compare the expected and observed transitions between near neighbors. The sex ratio based on the plants within 0.5 m was calculated for each plant. This sex ratio was then assigned to each observed pollinator transition between neighboring plants. The expected transitions were calculated as the average sex ratio around females (for the female to female and female to hermaphrodite transitions) and hermaphrodites (for the hermaphrodite to hermaphrodite and hermaphrodite to female transitions) for each array. This was then compared to the observed transitions within 0.5 m using a chi-squared test in proc freq in SAS.

To determine which of the floral traits are attractive to pollinators (question 1), we regressed visitation rates with several morphological features. A regression was used to determine the effect of flower number, petal size, pollen production (in hermaphrodites) and plant size on visitation rates for each type of pollinator using proc reg in SAS. To

examine which plant traits best predicted the observed visitation rates, we selected the model with the lowest AIC value, a measure of the fit between the model and the data (Akaike 1974), which could have included sex, flower number and/or petal size. To determine whether the sexes differed in these morphological features, we tested for the effect of sex on petal size, flower number and nectar amount. Petal size and rhizome size were tested using a t-test. Flower number was tested using an ANOVA with sex, week, location, sex ratio and sex*sex ratio as predictor variables and rhizome size as a covariate using proc glm in SAS. Nectar amount was tested using an ANOVA with nectar quantity as the response variable and sex, phase and sex*phase as predictors using proc glm in SAS.

To determine if the visitation rate affected seed fitness (question 2) we compared the ANOVAs of seed production described above to a model with the same predictors but with visitation rate as a second covariate. If including visitation rate in the model leads to a decrease in the difference between sexes then it suggests that the sex differences were caused by the visitation rate differences. To examine the relationship between visitation rates, pollen deposition and seed set, regressions among these variables were performed using proc reg in SAS. Quadratic relationships between variables were also tested as were differences between the sexes in the relationship (using the sex*variable term from proc glm).

To examine which pollinators most affected pollen deposition and seed number (question 2), we determined which model best fit the data for each sex using proc glm with the aic option in SAS and selecting for the model with the lowest AIC score. The possible predictors were the visits per plant for each type of pollinator. To determine if

the increase in pollen deposition on hermaphrodites was due to self-pollen transfer, the pollen deposition from the non-emasculated and emasculated plants was analyzed using an ANOVA with pollen deposition as the response variable and flower treatment and location within population as predictors using proc glm in SAS.

RESULTS

Morphological differences

Females produced about half as many flowers and about 40% smaller flowers than hermaphrodites (Table 6.1, both $P<0.0001$), despite similar rhizome sizes ($P=0.22$). Females and hermaphrodites produced the same amount of nectar once the phase of the flower was taken into account (stigma lobes open flowers: $F_{mean}=1.83\mu L$, $H_{mean}=1.84$; wrinkled petals flowers: $F_{mean}= 1.64$, $H_{mean}=1.69$; sex: $F_{1,44}=0.11$, $P=0.74$; phase: $F_{4,44}=3.24$, $P=0.02$; sex*phase: $F_{2,44}=0.11$, $P=0.90$). Hermaphrodites on average produced 512 pollen grains per anther (S.E.=9.25) for an estimated 5,120 pollen grains per flower.

Pollinator discrimination

Every type of pollinators we observed discriminated against females in several ways. Females consistently received fewer visits both at the plant level and the flower level across the sex ratios (both $P<0.0001$, Fig. 6.1A, Table 6.1). There was a higher than expected transition rate from hermaphrodite to hermaphrodite (Table 6.2, HH vs HF) and a trend for higher than expected transition to hermaphrodites after visiting a female (Table 6.2, FH vs. FF). Pollinators on average visited fewer ($P=0.01$) though a larger proportion ($P=0.002$) of flowers on females than those on hermaphrodites (Table 6.1).

However, these differences can be accounted for by flower number differences between the sexes; in a model including flower number as a covariate, sex does not significantly affect the number of flowers visited per plant ($F_{1,256}$=0.38, P=0.54) or the proportion of flower visited ($F_{1,256}$=0.46, P=0.50). On the plant level, females were discriminated against by all pollinator types (Fig. 6.2A, Table 6.1). Similarly, on the flower level females were discriminated against by all but the small bees (Fig. 6.2B, Table 6.1).

This discrimination could be caused by the flower number differences found between the sexes because all classes of pollinators also preferred plants with more flowers (Table 6.3). However, for the same number of flowers, females received fewer visits per plant (Fig. 6.3A, visits/plant: sex $F_{1,289}$=29.43 P<0.0001, flower number $F_{1,289}$=207.57 P<0.0001, sex*flower number $F_{1,289}$=3.51, P=0.06; visits/flower: sex $F_{1,287}$=37.64 P<0.0001, flower number $F_{1,287}$=0.13, $F_{1,287}$=2.32, P=0.13), suggesting flower number differences do not completely explain the discrimination. Another cause for discrimination could be sex differences in petal size. However, for the same petal size, females received fewer visits per plant and per flower (Fig. 6.3B; visits/plant: sex $F_{1,113}$=0.82, petal size $F_{1,113}$=0.65, sex*petal size $F_{1,113}$=5.21, P=0.02; visits/flower: sex $F_{1,112}$=0.51, petal size $F_{1,112}$=2.26, sex*petal size $F_{1,112}$=0.41, P=0.52, female slope P=0.71, hermaphrodite slope P=0.008), suggesting petal size differences alone do not account for the discrimination. A model including sex, flower number and petal size best explains visitation per plant and explains ~58% of the variation (sex: t=2.15, P=0.03; flower number: t=15.21, P<0.0001; petal size: t=4.16, P<0.0001; r^2=0.58, model P<0.0001).

Effect of discrimination

Discrimination against females was associated with lower pollen deposition on females' flowers. Females received significantly fewer pollen grains than hermaphrodites (F_{lsmean}=25.5, H_{lsmean}=40.5, P=0.03, Table 6.1, Fig. 6.1B). When visitation rates were included in the model as a covariate, it significantly affected pollen deposition but the sex differences remained (F_{lsmean}=35.7 pollen grains, H_{lsmean}=40.7; sex: $F_{1,216}$=6.10, P=0.01; squared visits/flower: $F_{1,216}$=13.38, P=0.0003; sex*squared visits/flower: $F_{1,216}$=4.42, P=0.04). There was also a difference between the sexes in the relationship between visits per flower and pollen deposition (P=0.04), with hermaphrodites having a broader peak (Fig. 6.4A). To examine whether the increased pollen deposition on hermaphrodites was due to self-pollen transfer, we compared pollen deposition from non-emasculated to emasculated hermaphrodites. There was no significant effect of emasculation (non-emasculated=52.4 pollen grains, emasculated=48.4 pollen grains, $F_{1,35}$=0.10, P=0.75), suggesting little self pollen transfer.

Discrimination against females was also associated with decreased seed production. Without accounting for differences in visitation rates, there was no difference between females and hermaphrodites in seed number (Table 6.1, Fig. 6.1C, P=0.95), seed set (Table 6.1, P=0.10) or fruit set (Table 6.1, P=0.54). However, including visitation rates in the model as a covariate led to a significant difference between the sexes in seed set (squared visits pre flower $F_{1,272}$=6.83, P=0.01, F_{lsmean}=0.31 seeds/fruit, H_{lsmean}=0.17, P=0.02). This suggests that without pollinator discrimination, females would have higher seed set than hermaphrodites. To further investigate this, the relationships between pollen number, seed set and visitation were examined. There was a

significant quadratic relationship between pollen number and seed set, with the highest point at about 2.5 seeds/fruit at about 130 pollen grains, after which point it plateaued (Fig. 6.4B, squared pollen number: $F_{1,827}$=20.66, P<0.0001; sex: $F_{1,827}$=4.45, P=0.04; sex*pollen number: $F_{1,827}$=0.00, P=0.99). Similarly, there was a quadratic relationship between visits per flower and seed set, but females had a narrower peak than hermaphrodites (P=0.03, Fig. 6.4C). For both sexes, the average pollen deposition and visits per flower falls below the peak seed set, suggesting pollen limitation.

The sexes differed in which types of pollinators were most important to pollen deposition and seed production (Table 6.4). For females, although medium bees discriminated against females very highly, they were important for pollen deposition and seed number (P=0.002, P<0.0001, respectively), as were small bees (P=0.005). For hermaphrodites, medium bees were most important for pollen deposition (P=0.0002) and small, medium and large bees were most important for seed production (P=0.03, P<0.0001, P=0.09, respectively).

Effect of sex ratio

Sex ratio affected both the total pollinator visitation rate and the relative contribution by pollinator types. Overall, the highest female frequency had the highest visits per plant and flower (P=0.0005 and P<0.0001, respectively, Fig. 6.5A, Table 6.1). This result seems to be caused primarily by an increase in the visitation rates of medium and small bees at the highest female frequency (Fig. 6.5B, medium: visits/flower P<0.0001; small: P=0.003). Opposite of the other bees, large bees had the highest visitation rates at the lowest female frequencies (Fig. 6.5B, P=0.003). Flies had the highest visits/plant at the intermediate female frequency (P=0.01). Concurrent with the decreased visitation rate at

the lowest female frequency, it also had lower seed production (seed number: P=0.02; seed set: P=0.05; and fruit set: P=0.04) than the other female frequencies (Fig. 6.1C, Table 6.1).

The sex ratio also slightly affected the discrimination against females. Females received an increasingly higher proportion of the visits as the sex ratio increased (Fig. 6.5C, sex*flower sex ratio: $F_{1,292}$=9.46, P=0.002). Despite the higher discrimination at low female frequencies, there were no significant interactions between sex and sex-ratio on pollen deposition, seed number, seed set or fruit set (Table 6.1, sex*sex ratio effects). However, females on average produced significantly fewer seeds at the lowest female frequency than females at the other frequencies (low vs int: P=0.05, low vs high: P=0.04, Fig. 6.1C).

DISCUSSION

In this study, we examined the importance of pollinators in determining the seed fitness of females and hermaphrodites, which is a major factor influencing the evolution and maintenance of gynodioecy. We found that all types of pollinators preferred hermaphrodites but the preference was the strongest when females were rare. Due to this discrimination, females' seed production was significantly reduced at the lowest female frequency but was similar to hermaphrodites in the other sex ratios. These results show that pollinators play a role in determining the relative seed fitness of the sexes and that their importance is influenced by the context of population sex ratio. Consequently, pollinators and their preferences could influence both the evolution and maintenance of gynodioecy.

Pollinator discrimination

We found that all types of pollinators discriminated against females. Several studies have found similar results (Bell 1985; Delph and Lively 1992) and most have attributed the discrimination to flower size or number differences. As in *G. maculatum*, hermaphrodites usually have larger flowers (Delph 1996; Shykoff et al. 2003), which pollinators may use to make foraging decisions. However, preference for larger petals does not completely explain differences in visitation rates seen in this study because for a given petal size, hermaphrodites still receive more visits than females. Instead of being attracted to individual flower size, pollinators have also been known to use flower number as a cue (Eckhart 1991; Galloway et al. 2002; Klinkhamer and De Jong 1990; Mitchell 1994; Schmid-Hermpel and Speiser 1988). In this study, hermaphrodites had larger display sizes, which may account for the discrimination against females. However, this alone also does not completely explain the discrimination because for a given display size, hermaphrodites receive more visits than females. The model best describing visitation rates includes sex as well as both flower size and floral display, suggesting that other sex specific traits are important in pollinator discrimination.

In addition to petal size and floral display, pollinators could be attracted to some other floral trait like pollen, nectar, or UV reflectance. Because many pollinators collect pollen for food, the presence or absence of pollen may affect visitation. For example, in *Fragaria virginiana*, odor extracts were presented to pollinators, which were attracted by the scent of the anthers (Ashman et al. 2005). The presence of pollen may have less of an effect in *G. maculatum* because only the large bees, which were not the major contributors to pollen transfer and seed production, were seen actively collecting pollen.

However, further manipulative studies are needed to determine the importance of the presence of pollen. Nectar resources could also affect the visitation since all the pollinators in this study were observed drinking nectar. Nonetheless, we did not find any evidence for discrimination caused by nectar resources since sexes produced similar amount of nectar. Further studies examining the quality (sugar concentration) or the rate of nectar replenishment are needed to confirm that the sexes are similar in these other aspects of nectar production. Lastly, UV reflectance, which is seen by pollinators but not measured in this study, may be different between sexes. Studies investigating sex differences in the UV reflectance patterns are needed.

Pollinators effect on seed fitness

Despite receiving fewer visits and less pollen, females produced similar numbers of seeds as hermaphrodites. If, however, females had the same visitation rate as hermaphrodites, females would produce more seeds than hermaphrodites. This suggests that females are pollen limited due to pollinator discrimination. Pollen limitation has been found in other gynodioecious species, but primarily at higher female frequencies (Ashman and Diefenderfer 2001 80%F; McCauley and Brock 1998 40%; Molinafreaner and Jain 1992 30%; Widen and Widen 1999 78%). It has been considered that this is caused by the lower frequency of the hermaphroditic pollen donors. Our results suggest that pollinator discrimination may also cause pollen limitation. Such pollen limitation may select for females with traits that increase pollinator attractiveness. Indeed, this may explain why females exhibit floral displays and nectar production similar to hermaphrodites, which is relatively uncommon in other gynodioecious species (reviewed in Delph 1996; Diaz and Cocucci 2003; Molano-Flores 2002; Talavera et al. 1996). For

example, in *Hebe strictissima*, females produce the same number of flowers but produced about four times less nectar than hermaphrodites (Delph and Lively 1992). Similarly, females of *Sidalcea oregano* produced more dilute nectar than hermaphrodites, which was correlated with petal size (Ashman and Stanton 1991).

The lack of higher seed production for females may have important consequences to the maintenance of females because regardless of the type of genetic control of male sterility, females must have higher seed fitness than hermaphrodites in order to remain in populations (Charlesworth and Charlesworth 1978; Charlesworth 1999; Lloyd 1974). There are at least two other possible ways that females could still be maintained. First, females could produce better seeds than hermaphrodites. In this study we found that females produced slightly heavier seeds than hermaphrodites (Fig. 6.1D, P=0.02). A previous study on *G. maculatum* showed a positive influence of seed weight on germination (Chang 2006), suggesting that seed weight may be correlated with progeny quality. Thus, females may produce seeds that germinate and grow better, thus increasing their long-term seed fitness. Second, because this is a perennial species, our short term, one-year measurements of seed production may not fully represent the plants lifetime seed production. For example, females could be producing slightly more seeds per year (which we found in this study), producing seeds more consistently, or producing seeds over a longer time span. Indeed, observations over 3-4 years have shown that females reproduce more often, thereby increasing their seed production relative to hermaphrodites (Van Etten and Chang, Chapter 3).

The effect of sex ratio

The array sex ratio affected pollinator visitation rates, discrimination against females and the relative seed fitness of the sexes. Large bees were more frequent at the low female frequency. These pollinators seemed to be more interested in pollen collecting than other pollinators; therefore, it is not surprising that they would prefer arrays with more pollen producing plants. On the other hand, medium and small bees had the highest visitation rates at the highest female frequency. From our observations, these pollinators appeared to be interested in the nectar rather than in collecting pollen. Because females and hermaphrodites produce the same amount of nectar, it seems reasonable that they would be prevalent at all female frequencies. One possible explanation for their higher abundance at the higher female frequencies may be competition between pollinators. It is possible that larger pollinators may be depleting the floral resources at the lower female frequency thereby leaving fewer resources for the smaller pollinators (Heinrich 1983). A similar change in the composition/abundance of pollinators was found in *Fragaria virginiana* by Ashman and Diefenderfer (2001): as the frequency of females declined, the abundance of pollen collecting pollinators increased. Changes in pollinator compositions, as seen in *G. maculatum* and *F. virginiana,* may have important impacts on selfing rates, gene movement and gene flow if the different types of pollinators have different foraging patterns (Goulson and Wright 1998; Wolfe and Barrett 1987).

Sex ratio also affected the discrimination against females. At the lowest female frequency, females were more discriminated against. As their frequency increased, so did the proportion of visits they received. This minority disadvantage has been found in other species where different morphs provide similar rewards. For example, in *Phlox*

drummondii, pollinators prefer the more frequent of two floral types (Levin 1972). A minority disadvantage has also been found in *Fragaria virginiana*, a gynodioecious species, where females received more visits as their frequency increased (Ashman and Diefenderfer 2001). However, even when females were the majority (80% female), they received significantly fewer visits than the less common hermaphrodites. Thus, it appears that in *F. virginiana* there is more at work than just the minority disadvantage. Consistent with the *F. virginana* study, our results suggest that minority disadvantage does not completely explain visitation rate differences in *G. maculatum*. For females, increasing in frequency increased their visitation rates, but for hermaphrodites, visitation rates were not influenced by their frequency. However, in our study, hermaphrodites were never a minority so to test fully the frequency dependence of visitations to both sexes, arrays in which hermaphrodites are the rare morph should be tested.

Sex ratio also affected the relative seed fitness of the sexes. At the lowest female frequency, females had about half the seed production of hermaphrodites while at higher female frequencies they produced about 1.1-1.5 times more seeds. Interestingly, a similar positive trend between female frequency and relative seed fitness has been found in natural populations of several gynodioecious species (Ashman 1999; Delph 1990c; Delph and Carroll 2001; Williams et al. 2000). Traditionally, seed production has been thought to be the cause of the sex ratio; when females have more of an advantage in seed production, they increase in frequency leading to a positive correlation. However, through artificially manipulating the sex ratio, our study suggests that the sex ratio may also be influencing the seed production through changes in pollinator discrimination. This may have important consequences for the initial invasion of females because when

females first invade a population they will be at low frequencies. If females are discriminated against more strongly at low frequencies, as we found in this study, females may be pollen limited leading to a decrease in their relative seed fitness. Thus, to successfully become established, other mechanisms increasing females' seed fitness must counteract this decrease in seed production.

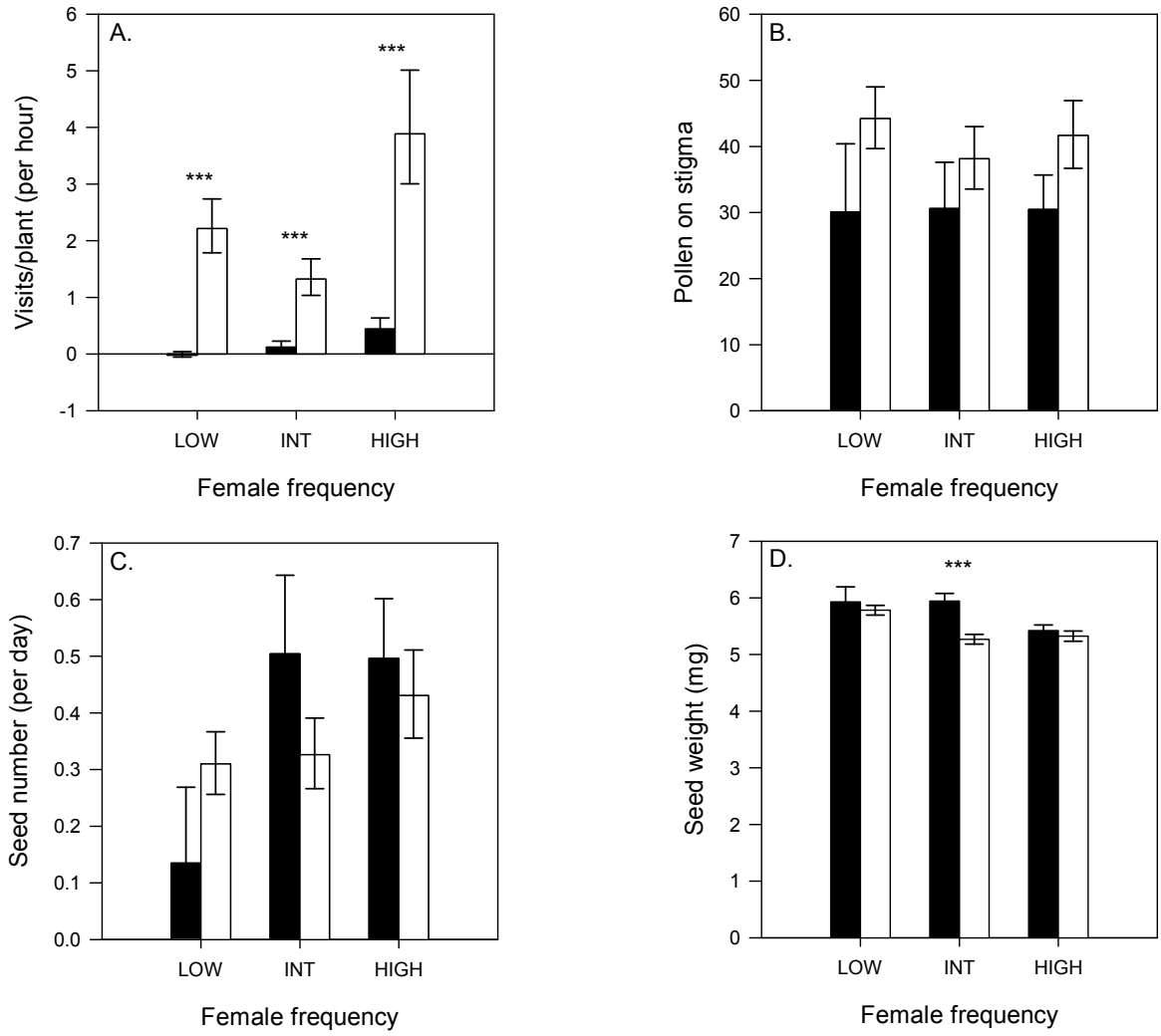


Figure 6.1. Backtransformed lsmeans of the visits per plant (A), pollen deposition (B), seed number (C) and seed weight (D) for females (solid bars) and hermaphrodites (empty bars) at each sex ratio. Error bars indicate ±1 SE. Asterisks denote a significant difference between the sexes. *** P<0.0001, **P<0.01, *P<0.05.

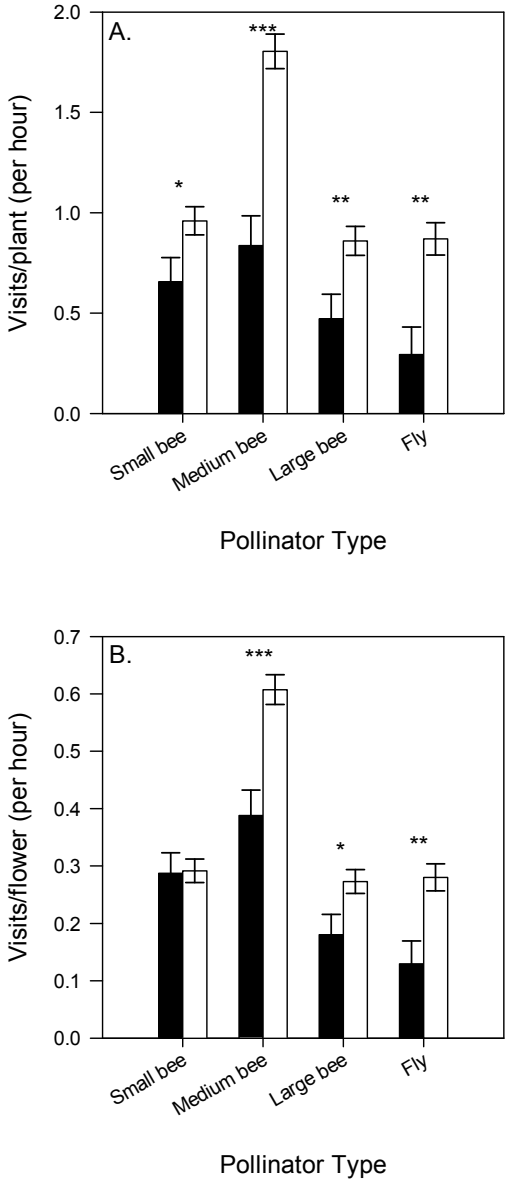


Figure 6.2. Backtransformed lsmeans of the visits per plant (A) and visits per flower (B) for each pollinator type by sex (females=solid bars, hermaphrodites=empty bars). Error bars indicate ±1 SE. Asterisks denote a significant difference between the sexes. *** P<0.0001, **P<0.01, *P<0.05.

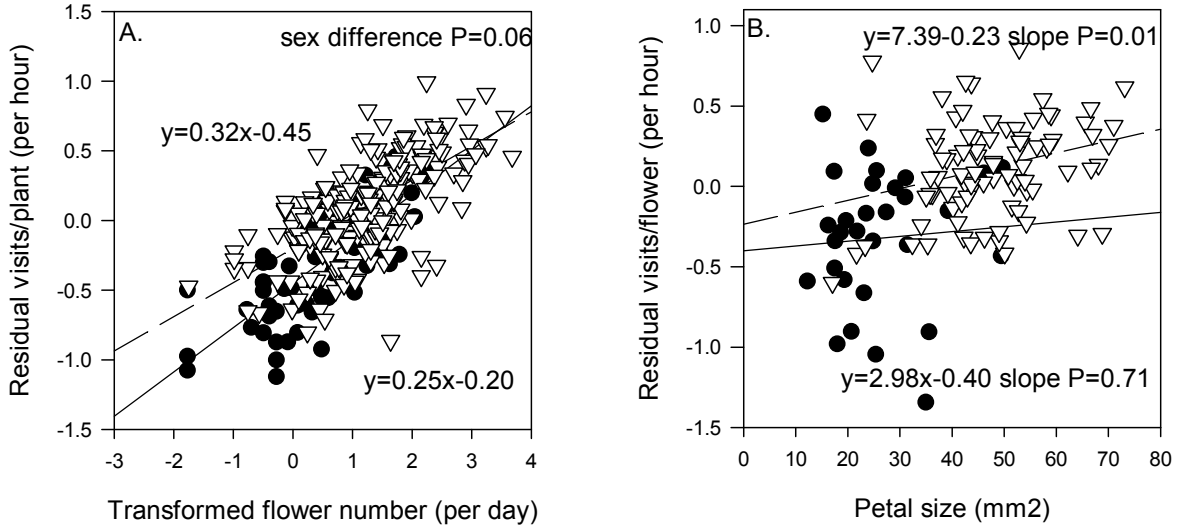


Figure 6.3. Regressions between flower number and visits per plant (A) and petal size and visits per flower (B) for females (solid circles and solid line) and hermaphrodites (white triangles and dashed line).

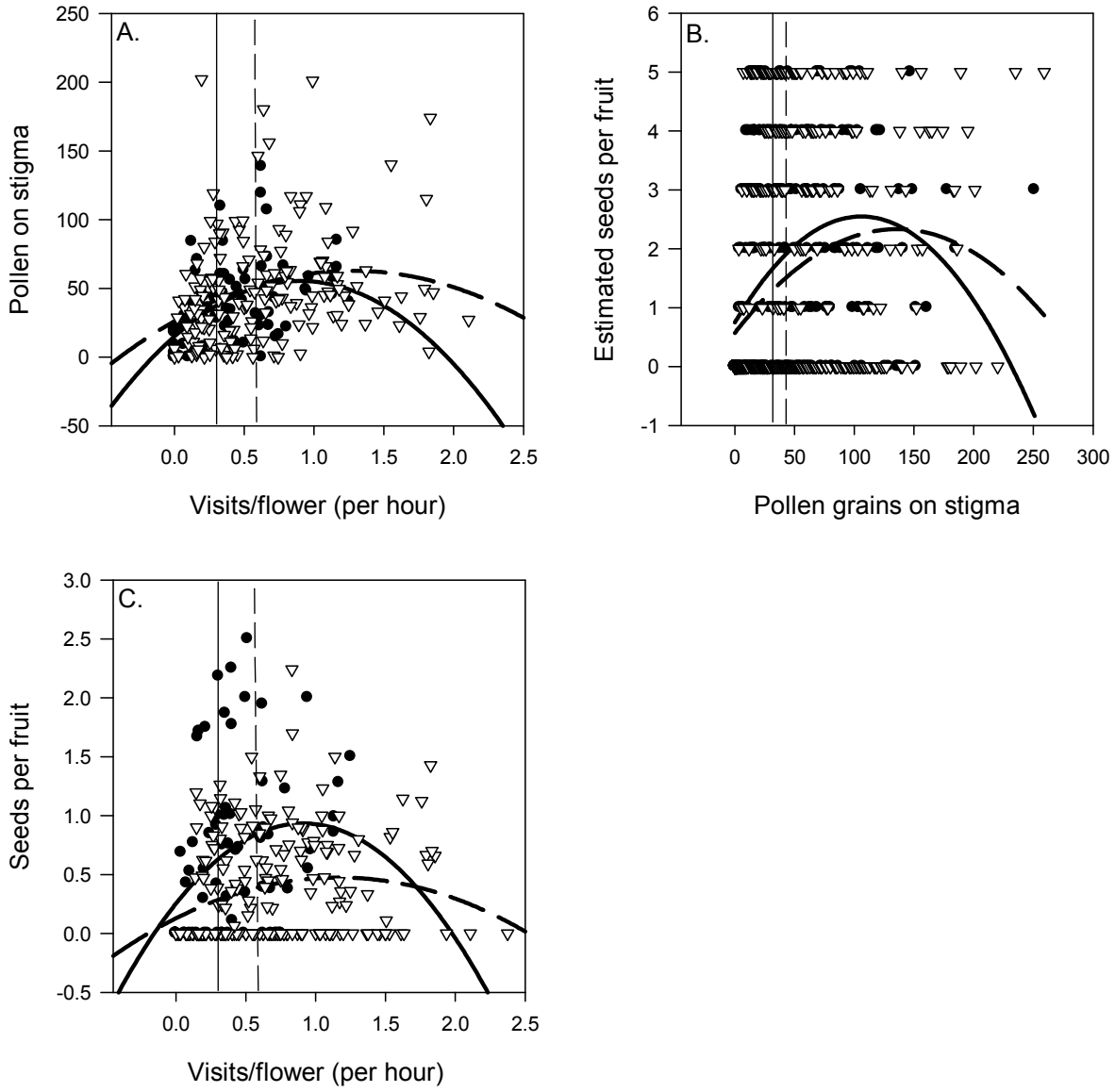


Figure 6.4. Regressions between visits/flower and pollen deposition (A), pollen deposition and seed set (B), and visits/flower and seed set (C) for females (solid circles and solid line) and hermaphrodites (white triangles and dashed line). Vertical lines indicate means for each sex.

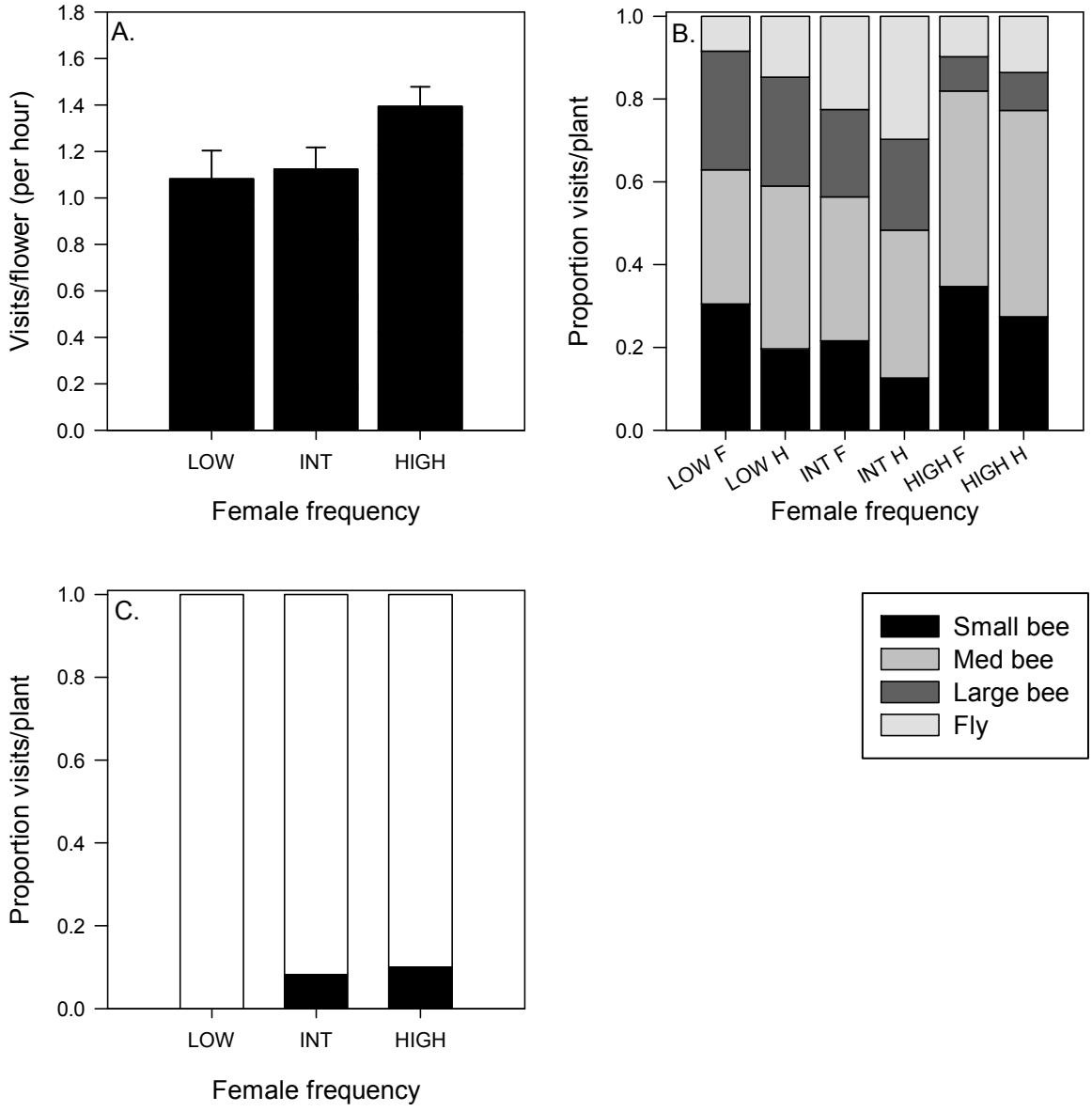


Figure 6.5. The sex ratio influences the visitation rates (A) and the relative abundance of pollinator types (B) and discrimination against females (C).

Table 6.1. Means for sexes and F values for morphological traits, visitation and seed production (d.f.). *** P<0.0001, **P<0.01, *P<0.05.

	Female mean	Herm mean	Sex (1)	Sex ratio (2)	Sex *Sex ratio (2)	Location (2)	Week (2)	Plant size (1)	Error d.f.
Flower number	1.60	2.88	30.77***	0.38	2.93*	2.94*	1.79	27.63***	282
Petal size (mm^2)	27.70	48.12	181.22***						288
Rhizome size (g)	122.73	131.12	1.19						291
Visits/plant	0.41	1.34	70.49***	8.18***	2.23	19.49***	27.22***	16.54***	282
Visits/flower	0.30	0.58	48.40***	15.33***	0.84	58.13***	60.38***	0.07	280
Flowers/visit	1.29	1.43	4.53*	0.91	0.56	0.86	2.06	9.23**	257
Prop. flowers visited	0.62	0.50	7.81**	1.47	3.49*	2.09	4.53**	22.94***	257
Visit duration/plant (sec)	12.12	15.97	4.41*	0.44	1.64	13.38***	17.99***	0.13	257
Visit duration/flower (sec)	8.32	10.4	4.23*	2.42	2.82	10.72***	15.78***	0.29	268
Small bee visits/plant	0.61	0.96	4.91*	5.93**	1.11	20.14***	6.42**	1.73	282
Small bee visits/flower	0.27	0.29	0.26	12.24***	0.17	42.17***	5.46**	5.22*	278
Med bee visits/plant	0.82	1.81	27.42***	10.46***	1.10	10.18***	14.99***	14.86***	282
Med bee visits/flower	0.37	0.61	17.18***	12.06***	0.08	13.32***	31.20***	0.85	278
Large bee visits/plant	0.41	0.85	7.54**	4.14*	0.64	36.33***	5.50**	3.81*	282
Large bee visist/flower	0.17	0.27	4.73*	6.21**	0.61	53.92***	10.23***	1.82	280
Fly visits/plant	0.27	0.86	11.30***	2.21	0.98	9.72***	1.19	4.53*	282
Fly visits/flower	0.12	0.28	9.07**	3.34*	1.31	12.86***	3.78*	0.76	281
Pollen deposition	30.36	41.28	4.33*	0.07	0.11	1.90	14.26***	0.24	219
Seed number (per day)	0.36	0.35	0	3.00*	1.50	1.75	12.04***	5.21*	282
Seed set (seeds/fruit)	0.25	0.18	2.33	3.38*	0.18	3.22*	5.17**	0.12	276
Fruit set (fruits/flower)	0.24	0.19	0.54	3.59*	1.75	5.52**	18.56***	0.62	260
Seed weight (mg)	5.76	5.46	7.24**	5.72**	4.15*	0.71	1.21	31.30***	638

Table 6.2. Observed percent of transitions (expected) to the nearest neighbors for each array. FF=female to female, FH=female to hermaphrodite, HF=hermaphrodite to female, HH=hermaphrodite to hermaphrodite. Asterisks indicate significant chi-squared values meaning the observed values deviate from expected values. *P<0.05, **P<0.01, ***P<0.0001.

	Transition					
Female frequency	FF	FH	χ^2 (d.f.=1)	HF	HH	χ^2 (d.f.=1)
Low	0.11 (0.30)	0.89 (0.70)	1.53	0.04 (0.09)	0.96 (0.91)	3.56
Low	0.00 (0.00)	1.00 (1.00)	-	0.04 (0.09)	0.96 (0.91)	9.36**
Low	0.00 (0.17)	1.00 (0.83)	-	0.02 (0.14)	0.98 (0.86)	8.22**
Int	0.18 (0.26)	0.82 (0.74)	1.13	0.18 (0.27)	0.82 (0.73)	5.76*
Int	0.00 (0.06)	1.00 (0.94)	-	0.08 (0.21)	0.92 (0.79)	12.95**
Int	0.18 (0.35)	0.82 (0.65)	2.25	0.08 (0.16)	0.92 (0.84)	6.86**
High	0.26 (0.45)	0.74 (0.55)	5.26*	0.20 (0.33)	0.80 (0.67)	11.27*
High	0.21 (0.29)	0.79 (0.71)	3.11	0.23 (0.37)	0.77 (0.63)	16.83***
High	0.26 (0.29)	0.74 (0.71)	0.28	0.23 (0.37)	0.77 (0.63)	14.67***

Table 6.3. Regression coefficients between visits per plant for each pollinator type and plant traits for females and hermaphrodites. Asterisks indicate significant regression coefficients. *P<0.05, **P<0.01, ***P<0.0001.

	Females			Hermaphrodites			
	Flower number	Petal size	Plant size	Flower number	Petal size	Pollen production	Plant size
Small bee	0.68***	0.19	-0.12	0.93***	0.25*	-0.04	-0.22
Medium bee	0.39***	0.02	0.1	0.55***	0.12	-0.08	0.01
Large bee	0.29*	0.47**	0.02	0.86***	0.02	0.11	0.16
Fly	0.33	-0.27	0.28	0.83***	0.08	-0.06	0.08

Table 6.4. Regression coefficients for the best model predicting pollen deposition and seed number. Asterisks indicate significant regression coefficients. *P<0.05, **P<0.01, ***P<0.0001.

	Females					Hermaphrodites				
	Small bee	Medium bee	Large bee	Fly	Model P	Small bee	Medium bee	Large bee	Fly	Model P
Pollen deposition		1.03**			0.002		0.52***			0.0002
Seed number	0.13**	0.23***			<0.0001	0.05*	0.10***	0.03		<0.0001

Chapter 7

Selection Through Both Pollen and Seed Fitness and the Effect of Sex Ratio in a Gynodioecious Species[1]

[1] Van Etten, M.L. and S-M. Chang. To be submitted to *Evolution*.

Abstract

While most plants gain fitness through both pollen and seed fitness, research has primarily focused only on seed fitness. However, pollen fitness plays a key role in theories about the evolution of mating strategies, including the evolution of separate sexes through gynodioecy, a breeding system with separate female and hermaphroditic individuals. Theory suggests that as the population female frequency increases, selection will favor increasing pollen fitness in hermaphrodites, leading to the evolution of male individuals. In this study, we examined the "maleness" of hermaphrodites, selection pressures through pollen and seed fitness and the effect of female frequency. Fitness gained through seed production and seed siring was evaluated in experimental arrays with varying sex ratios and used in a selection analysis. Several lines of evidence suggest that increased maleness is unlikely to evolve in this species. First, we found that although hermaphrodites gained ~70% of their fitness through pollen donation, plants with equal seed and pollen fitness had the highest total fitness. Second, similar traits were selected upon through pollen and seed fitness. Third, we found no significant effect of sex ratio on the strength of selection on "male" traits. These results suggest that hermaphrodites are not selected upon to become more male, rather, selection favors equal fitness gained through pollen and seed fitness, even at high female frequencies.

Introduction

At their core, evolutionary studies entail understanding how traits influence fitness. For hermaphroditic species, as are 70% of plants (Yampolsky and Yampolsky 1922), fitness includes that gained through pollen donation and through seed

development. Because most plants should gain about half their fitness through pollen and half through seed fitness, pollen fitness plays a large part in many theories of floral and mating system evolution (e.g. Bell 1985; Campbell 2000). However, due to the lack of proper and easy tools to track pollen fitness, research has focused primarily on seed fitness. Recently, technological advances have made it possible to empirically estimate pollen fitness in ecological studies (e.g. Morgan and Conner 2001; Smouse et al. 1999; van Kleunen and Ritland 2004). Surprisingly, the results from this research do not always conform to theoretical expectations. For example, it is commonly thought that floral traits are primarily shaped by selection through pollen fitness rather than seed fitness (Bell 1985). However, most studies have found selection on traits like flower number and size through both pollen and seed fitness and some have found no evidence of selection through pollen fitness (reviewed in Ashman and Morgan 2004). These results suggest that our knowledge of the contribution of pollen fitness to evolutionary processes is sorely lacking (Ashman and Morgan 2004; Burd and Callahan 2000).

One of the theories in which pollen fitness plays an important role is the evolution of separate sexes, a major evolutionary transition in plants. Gynodioecy, the co-occurrence of female and hermaphroditic individuals, is thought to be an intermediate stage in this evolutionary process. In this breeding system the two types of individuals gain fitness in different ways; females have lost the male component of fitness and gain fitness only through seed production while hermaphrodites can gain fitness through both pollen and seed fitness. Gynodioecy is unique because unlike most hermaphroditic species, hermaphrodites in populations with females are not expected to gain fitness equally through male and female function. This is because the addition of females into a

population increases the number of available ovules but not the number of pollen donors. Therefore, with an increase in the frequency of females in a population, the number of ovules sired by a hermaphrodite will increase while their seed production remains the same, leading to a larger fraction of their total fitness gained through pollen fitness (Lloyd 1976). The proportion of pollen fitness gained should therefore depend upon the frequency of females, such that hermaphrodites are expected to gain a larger proportion of their fitness through pollen fitness as the frequency of females increases.

The frequency dependant nature of the contribution of pollen fitness to hermaphrodites' fitness has been suggested to be a key factor in the evolution of male plants (Charlesworth and Charlesworth 1978; Maurice et al. 1994; Seger and Eckhart 1996). This is because as the frequency of females increases, selection should increasingly favor traits (such as more pollen production) that increase pollen fitness. Increasing male function should be selected for, even if it results in less seed fitness, because hermaphrodites will be gaining a larger portion of their total fitness through pollen donation. If there is a trade-off between pollen and seed fitness, this could eventually lead to pure male individuals, creating a dioecious population. Theory has suggested that this predicted pathway could occur regardless of the genetic control of gynodioecy (Charlesworth and Charlesworth 1978; Jacobs and Wade 2003; Maurice et al. 1993), and phylogenetic analysis has shown that dioecious species seem to have evolved from both nuclear and cyto-nuclear control of sex (Desfeux et al. 1996; Maurice et al. 1993).

Although the predicted pathway from gynodioecy to dioecy has some indirect support, no research has directly investigated pollen fitness or selection on "male" traits

in a gynodioecious species. As expected from the predicted pathway to dioecy, a range of "maleness" of hermaphrodites across species has been found and has been correlated with the population sex ratio. For example, in *Echium vulgare*, while all hermaphrodites produced some seeds, many of their flowers did not produce fruit, suggesting that much of hermaphrodites' fitness is derived from pollen donation rather than seed production (Klinkhamer et al. 1994). The predicted correlation between the "functional maleness", a trait estimated as the relative reproductive success as a pollen or seed parent in hermaphrodites, and the sex ratio is also found in some species. For example, in *Geranium sylvaticum*, hermaphrodites had lower seed production in populations with a higher frequency of females (Asikainen and Mutikainen 2003). Similar results have been found in *Silene acaulis* (Delph and Carroll 2001), *Fragaria virginiana* (Ashman 1999), *Hebe strictissima* (Delph 1990c) and *Ochradenus baccatus* (Wolfe and Shmida 1997). While these studies indirectly support theoretical predictions that hermaphrodites should be more functionally male as the frequency of females increases, none have directly investigated male fitness and how it changes with the sex ratio.

To better test the predicted increase in "maleness", we directly examined seed and pollen fitness and how they are affected by the sex ratio by experimentally varying the sex ratio in experimental arrays and measuring the fitness gained through seed production and pollen donation. From theory, there are several predictions involved in the evolution of males from hermaphrodites, each of which we investigated. First, for hermaphrodites to evolve into pure males, there must be a trade-off between male traits (such as pollen number, flower number, flower size) and female traits (such as seed or fruit production) or pollen and seed fitness. Second, the "functional maleness" of hermaphrodites should

increase with the female frequency. Third, selection should favor "male" traits through pollen fitness and selection should be increasingly strong as the frequency of females increases.

METHODS

Geranium maculatum L. is a gynodioecious, rhizomatous perennial ranging from the South Eastern US to Canada and west to the Great Plains (Radford et al. 1968). Flowering in natural populations begins in early spring, with individuals producing on average six flowers per inflorescence (Chang 2006). Flowers are visited by generalist pollinators including bees, flies and butterflies (Martin 1965). Flowers are protanderous, with 2 sets of 5 anthers dehiscing prior to the stigma lobes reflexing and becoming receptive, taking a total of approx. 2-3 days under greenhouse conditions (Willson et al. 1979, Van Etten, personal observation). The stigma remains receptive for several days, however once pollinated the stigma lobes close and petals fall off within the following day (Van Etten, personal observation). Hermaphrodites are self-compatible and average selfing rates range from 0-17% depending on the population (Van Etten et al., Chapter 4). At most, five seeds per fruit can be produced, which are dispersed by the elastic dehiscence of the schizocarp, to an average of 3 m from the maternal plant (Stamp and Lucas 1983). Based on field and greenhouse observations, sex is genetically determined. Preliminary data rule out the possibility that sex is controlled entirely by cytoplasmic genes, but the exact genetic control has not yet been determined (Van Etten and Chang, Chapter 2). Females have small aborted anthers and smaller petals when compared to hermaphrodites (Ågren and Willson 1991; Chang 2006). Flower number per

inflorescence is approximately the same between sexes in natural populations (Ågren and Willson 1991; Chang 2006) although in one greenhouse study females produced slightly more flowers than hermaphrodites (Van Etten et al. 2008). In natural populations, females produced more seeds than hermaphrodites (20 – 50% increase, Ågren and Willson 1991; Chang 2006) and seeds that had a higher germination rate (Chang 2006). Local populations around Athens, GA range from 0-50% female (Chang 2006).

To test how the sex ratio affected seed and pollen fitness, experimental arrays were constructed. Details of the experimental design and data collection can be found in Van Etten and Chang (Chapter 6). Briefly, arrays with three sex ratios were created: 13% (4F, 27H), 26% (8F, 23H), and 42% females (13F, 18H), hereafter low, intermediate and high female frequency, respectively. We used a Latin Squares design so that for each week, each sex ratio was represented in one of three locations. After a week, the sex ratios were rotated among locations so that at the end of the experiment each location had housed each of the sex ratios. Plants were re-randomized when sex ratios were rotated. The flower number per day, petal size, pollen production, plant size, pollinator visitation rate (per plant and per flower), pollen deposition, seed number and fruit number were measured.

Genotyping

To obtain genotypes from each of the experimental plants, newly expanded leaves were collected from each plant used in the arrays. To obtain progeny genotypes, up to 5 seeds per plant per array were randomly selected for the analysis. When possible, seeds were taken from different fruits. Seeds were scarified, soaked in water for several days, and their seed coat removed. DNA was extracted from leaves and seeds using a CTAB

protocol (modified from Doyle and Doyle 1990). Six microsattelite loci were amplified for each individual (contact authors for primer sequences and PCR conditions). Genotypes were determined using the ABI Prism 3730 at the Sequencing and Synthesis Facility at the University of Georgia and the software TracI (available from VIB Genetics) was used to score the genotypes.

Paternity Analysis

To determine the paternity of progeny, we used fractional paternity assignment using Cervus (Kalinowski et al. 2007). This program uses allele frequencies to determine the likelihood cutoff used for determining the most likely pollen parent. For a given seed, each hermaphrodite was given a likelihood score based on their genotype. If this score was above the cutoff, then that hermaphrodite is highly likely to be the actual father. In some cases where no hermaphrodites fell above the cutoff or there were multiple possible pollen parents for that progeny, a fraction of a progeny was assigned to each of the potential pollen parents in proportion to their likelihood compared to the other potential fathers. In this way, 467 of the progeny were assigned to one (176) or more fathers (291, average of 3.9 potential fathers). There were 59 progeny that did not have a likely father within the array.

Data analysis

Seed fitness was simply calculated as the number of seeds produced. Pollen fitness was estimated in two steps. First, the total number of assayed seed fathered was calculated (number of seed fathered by a particular hermaphrodite). Second, because we only genotyped a subset of the seeds produced in each array, to make the comparison between the values for pollen and seed fitness meaningful, we need to factor in the

subsampling process during paternity analysis. To do so, we multiplied the estimated male fitness (# of seeds fathered by a particular hermaphrodite among the assayed seeds) by the inverse of the proportion of the total seeds assayed (total seeds produced in that array/ # seeds sampled for genotyping) to obtain the "actual" number of seeds fathered. Total fitness was calculated as the number of seed produced (seed fitness) plus the actual number of seeds fathered (pollen fitness).

We tested the trade-offs between pollen and seed fitness (question 1) using a subset of the data. We used only those plants around the mean plant size (rhizome size 89-160 mg). This was done because a positive correlation between two traits may be found simply because larger plants are producing larger or more of both traits, even if there is a tradeoff between the traits. Thus, comparisons between traits should be done on plants of nearly the same size (Ashman 2003; Campbell 2000). We examined the correlation between pollen and seed fitness using Spearman's rank correlation, because neither could be transformed to normality. We also correlated "male" traits (pollen production, petal size and flower number) with seed production measures (seed set, fruit set and seed number) to examine tradeoffs between "male" and "female" traits. These analyses were performed using Proc Corr with the spearman option in SAS.

To examine the functional gender of hermaphrodites (question 2), we calculated the ratio of seed fitness to the total fitness as the observed functional gender. Values of 1 indicate that all fitness gained was through seed production while a value of 0 means that all fitness was gained through male function. This observed functional gender was then compared to the expected functional gender based on the available ovules in each array. Because the number of ovules available per pollen donor differs between arrays of

different sex ratios, we calculated the expected functional gender for each hermaphrodite in each array. This was calculated by first calculating the expected seed production for a hermaphrodite=number of flowers it produced and expected seeds sired=(number of flowers it produced /total number of pollen bearing flowers in the array)*(total number of flowers in array). The first term in the expected seeds sired represents the relative contribution of a particular hermaphrodite to the pollen pool and the second term represents the number of ovules available in the array. The expected functional gender was then calculated as: expected seed production/(expected seed production + expected seeds sired). This calculation assumes similar seed production between hermaphrodites and females. On average, this gives values in each sex ratio of Low=0.465, Intermediate=0.446 and High=0.367. The deviation between the expected and the actual functional gender was calculated as expected-observed, so that positive values mean that a hermaphrodite is more male-biased and negative values mean that a hermaphrodite is more female-biased in their fitness gain than expected.

To test how functional gender relates to total fitness, a regression was done between functional gender and total fitness. To test how the functional gender changes with the sex ratio, an ANOVA was done with functional gender as the response and week, location and sex ratio as predictor values in SAS using proc glm.

To determine the pattern of natural selection on the traits we measured (question 3), we used a multivariate phenotypic selection analysis (Lande and Arnold 1983). Trait values (flower number, petal size, pollen production and plant size) were first normalized, then standardized (mean 0, standard deviation 1) and fitness was divided by the mean value in an array to get a relative fitness. We carried out the selection analyses

using seed and pollen fitness in addition to the total fitness in order to determine whether traits measured were under different selection through male and female functions. For analyzing directional selection, which is essentially a multiple regression, we used the proc reg function in SAS. For analyzing quadratic selection, in which quadratic terms for each of the traits are also included in the multiple regression, we used the proc rsreg function in SAS, and significant quadratic gradients were doubled (Stinchcombe et al. 2008).

To further examine how selection may be acting on traits (question 3), we also conducted a path analysis. Path analyses determine the interaction among different traits and allow one to calculate both the direct and indirect effects of these traits on fitness. For seed fitness, the model included petal size, flower number, pollen production and plant size as exogenous variables (variables with no explicit causes in the model, only correlations with other exogenous variables) and visits per hour, visits per flower, visit duration, pollen deposition, seed set, fruit set and seed number as endogenous variables (variables that are causally affected by other variables in the model; all calculated relative to the array mean to remove the effect of array location and week; see Fig. 7.4 for pathways). For pollen fitness, the model included pollen production, petal size, flower number and plant size as exogenous variables and visits per hour, visits per flower, visit duration and pollen fitness as endogenous variables (all relative to the array mean; see Fig. 7.3 for specified pathways). For total fitness, the model was the same as for seed fitness, but total fitness was added as an endogenous variable. Path analyses were conducted using proc calis in SAS.

The effect of the sex ratio on selection (question 3) was tested for each trait using an ANOVA with fitness (seed, pollen or total) as the response and pollen production, flower number, petal size, seed set, fruit set, rhizome weight, sex ratio and sex ratio*trait as the predictors. A significant interaction term between sex ratio and a trait indicates that the relationship between fitness and the trait differs across the sex ratios. This was done using proc glm in SAS.

RESULTS

Trade-offs:

There was no evidence for a trade-off between pollen and seed fitness. Rather, plants with high pollen fitness also had high seed fitness, even when controlling for plant size (Spearman's coefficient=0.350, $P<0.0001$). There was a negative correlation between pollen production and seed number ($P=0.01$), seed set ($P=0.003$) and fruit set ($P=0.003$). However, there were no relationships between petal size and seed production (seed number: $P=0.21$, fruit set: $P=0.11$, seed set: $P=0.52$) or between flower number and seed production (seed set: $P=0.15$, fruit set: $P=0.54$).

Functional gender:

Averaged over all the sex ratios, hermaphrodites gained 70.8% of their fitness through pollen donation. Some hermaphrodites did not make any seeds and exclusively gained fitness through pollen donation while others only gained fitness through seed production (Fig. 7.1A). Plants that had the highest number of seeds sired gained about 62% of their fitness through pollen fitness (quadratic effect d.f.=1, $P<0.0001$) while plants with the highest total fitness gained about 47% of their fitness through pollen

fitness (quadratic effect d.f.=1, P=0.02, Fig. 7.1C). Most hermaphrodites (74%) gained more of their fitness through pollen fitness than was expected given the array sex ratio (Fig. 7.1B). Although most hermaphrodites were more male-biased than expected, those with little deviation from the expected functional gender based on the assumptions that each hermaphroditic flower contributed equally to the outcrossing pollen pool had the highest total fitness (quadratic effect d.f.=1, P<0.0001).

Sex ratio affected the deviation from the expected functional gender. The observed functional gender was not affected by the sex ratio (Low=0.29, Intermediate=0.29, High=0.30, d.f.=2, P=0.97). Because hermaphrodites at the lowest female frequency are expected to be less male-biased, the deviation was greater for hermaphrodites at the lowest female frequency (Low=0.18, High=0.07, d.f.=1, P=0.057), with the intermediate female frequency being in between (Intermediate=0.16).

Selection:

We found significant selection through pollen fitness on two traits (Table 7.1). There was significant directional selection for more flowers (d.f.=1, P=0.0002) and disruptive selection on petal size (d.f.=1, P=0.04). To better understand what is causing this selection we modeled the data using a path analysis (Fig. 7.2). Consistent with results from the selection analysis, the trait that most influenced pollen fitness was flower number (total effect=0.23, which is essentially the slope of the regression between flower number and pollen fitness after accounting for the effect of other traits). This effect was primarily due to flower number increasing the total number of pollinator visits (indirect affect=0.13), as well as the total pollen production (direct affect=0.11). As was found in

the selection analysis, there was little effect of pollen production per anther, either directly (direct= -0.08) or indirectly through pollinator visitation (indirect=0.001).

We found significant selection through seed fitness on three traits (Table 7.1). There was significant directional selection for more flowers, higher seed set and higher fruit set (all d.f.=1, $P<0.0001$). There was also significant stabilizing selection for fruit set (d.f=1, P=0.02). In contrast to fitness gained through pollen production, flower number had a larger direct effect than indirect effect on seed fitness (direct=0.29, indirect=0.16, Fig. 7.3). Pollen deposition had some effect on seed and fruit set (0.20 and 0.15, respectively). Interestingly, visitation rate had a larger effect on fruit set (0.35) than on seeds sired (0.18).

We found significant selection through total fitness on three traits (Table 7.1). There was directional selection for more flowers, higher seed set and higher fruit set (d.f.=1, P=0.006, $P<0.001$, P=0.0006, respectively). There was no significant selection on pollen production or petal size. Although the path analysis for total fitness is similar to the separate analyses of pollen and seed fitness, there are several interesting results (Table 7.2). First, of the traits measured, flower number has the largest effect on total fitness (total effect=0.41), because of its large contributions to both pollen and seed fitness. Second, flower number has a greater effect on seed fitness than it does on pollen fitness (0.45, 0.23, respectively), suggesting that an increase in flower number would increase seed fitness more than it would pollen fitness.

Effect of sex ratio on selection:

Selection pressures only differed significantly among sex ratios for two traits. The strength of selection on seed set decreased with increasing female frequency through

seed fitness (d.f=2, P=0.001, Fig. 7.4B) and total fitness (d.f.=2, P=0.02, Fig. 7.4C). There was also increasingly strong selection for more pollen production as the frequency of females increased through total fitness (P=0.04, Fig. 7.4C). In addition, selection through pollen fitness on pollen production tended to increase with the female frequency (P=0.11).

DISCUSSION

Much theory about the evolution of separate sexes involves selection on pollen fitness (e.g. Bell 1985; Campbell 2000), yet no studies have directly examined pollen fitness in this context. Our results show that in *Geranium maculatum*, the conditions predicted by theory to lead to the evolution of male individuals do not occur in the sex ratios examined. First, we found no indication of the expected trade-off between pollen and seed fitness; individuals with high pollen fitness also had high seed fitness, even when accounting for plant size. Second, although most hermaphrodites were male biased, those with the highest total fitness had approximately equal pollen and seed fitness, which should select for retaining female sexual function. Third, we found little selection on "male" traits through pollen fitness, except for higher flower number, which also was selected for through seed fitness. Flower number increased the number of visits, which increased pollen fitness, and increased the number of ovules, which increased seed fitness. Thus, selection appears to be acting on a trait that increases both male and female function. Fourth, we found that the sex ratio did not affect selection in the predicted way. Theoretically, as the female frequency increases, selection is expected to increase on traits that increase pollen fitness. Selection on "male" traits did not vary significantly

with the sex ratio. Together, these results strongly suggest that there is little selection for hermaphrodites to become males under the conditions we tested.

We did not find the expected trade-off between pollen and seed fitness. This trade-off is key to not only the evolution of dioecy, but for many theories about floral evolution (Campbell 2000). Most studies of gynodioecious species have found some kind of trade-off, but it depends upon which traits are used (Ashman 2003; Atlan et al. 1992; Dykstra et al. 2009; Ehlers and Thompson 2004; Koelewijn 2003; but see Koelewijn and Hunscheid 2000). For example, in *Silene vulgaris*, there is a trade-off between anther size and ovule size but not between anther size and ovule number (Dykstra et al. 2009). In *Fragaria virginiana*, there is a trade-off between pollen production and fruit set, but a positive correlation between pollen per flower and ovules per flower (reviewed in Ashman 2003). In addition to trade-offs being trait specific, they may also differ with the resource environment. If plants are not resource limited, any trade-off that occurs under natural conditions may be masked when using plants with plenty of resources. Indeed, there is a trend that studies using greenhouse grown plants do not find a trade-off while ones that used plants grown under field conditions do (reviewed in Ashman 2003). Thus, our results of no trade-off between male and female fitness may be due to the abundant resources available to the experimental plants. Further research that manipulates resource levels will help to verify this possibility.

Even though most hermaphrodites gain approximately 70% of their fitness through pollen donation, hermaphrodites with the highest total fitness were those that gained approximately equal fitness through seed and pollen fitness. This suggests that selection favors the maintenance of both functions in hermaphrodites. Additionally, we

found that functional gender remained relatively constant across array sex ratios, despite the changes in the ratio of ovule donors to pollen donors, which contradicts theoretical predictions (Delph 2003; Delph and Wolf 2005; Lewis 1941; Lloyd 1976). Two factors may cause this deviation from the expected functional gender: lower than expected seed production or higher than expected number of seeds sired. By comparing the expected seed production to the actual seed production, it appears that hermaphrodites produce fewer seeds than expected at the lowest female frequency. This is most likely caused by pollinator limitation at this frequency decreasing seed production (Van Etten and Chang, Chapter 6).

Similar to finding no selection for increased functional "maleness", we found little selection on "male" floral traits. Theory suggests that in order for pure males to evolve, selection acting to increase traits that increase pollen fitness, such as pollen production, flower number or petal size, is required (Bell 1985). We did not find directional selection on these traits through pollen fitness, except for flower number. Interestingly, flower number increased both seed and pollen fitness, although through different routes. Flower number seemed to influence pollen fitness through increasing both the total number of visits and pollen production. In contrast, it increased seed fitness by increasing the number of ovules available to fertilize. There are several other examples from hermaphroditic species where there is little selection through pollen fitness. In *Raphanus raphanistrum*, Conner et al. (1996) found no selection on floral traits after the effect of distance between mates had been removed. In *Silene latifolia*, there was only weak selection on floral traits through pollen fitness (Wright and Meagher 2004). However, strong selection on floral traits through pollen fitness, even after

accounting for interplant distances, was found in *Narcissus triandrus* (Hodgins and Barrett 2008). The different patterns found in the few studies that focused on male fitness suggest that more studies are needed to determine how strong a role selection through pollen fitness may play in floral evolution not only in gynodioecious species but also in the more common, hermaphroditic species, as well.

Based on theories (e.g. Charlesworth and Charlesworth 1978; Jacobs and Wade 2003), we expected that as the female frequency increased, so would selection on "male" traits because hermaphrodites would be getting a larger proportion of their fitness through pollen donation. However, we found that selection on "male" traits did not vary significantly with the sex ratio, although there was a trend for stronger selection for more pollen production at higher female frequencies. One reason for these results may be that the effect of the sex ratio on functional gender was not great enough in this study. On average, hermaphrodites at the lowest female frequency should get ~54% of their total fitness through pollen donation and at the highest female frequency ~63% through pollen donation. Thus, the 9% difference between the two extreme treatments may not be enough of a difference to significantly affect selection in this species. Expected functional gender is based not only on the sex ratio but also on the relative seed production of the sexes (Delph and Wolf 2005); higher ovule production by females (or lower production by hermaphrodites) further skews the ovule:pollen production in the population, which results in higher expected functional maleness of hermaphrodites. However, because of the similarity in flower and seed production between the two sexes in *G. maculatum* (Ågren and Willson 1991; Chang 2006), the expected functional gender of hermaphrodites only changes slightly with the sex ratio. If females produced more

ovules than hermaphrodites, or could have higher seed set, then hermaphrodites' expected functional gender would be more male biased and therefore selection should more strongly select for increased "maleness". Thus, it appears that the similar flower and seed production between the sexes in *G. maculatum* may act as a constraint to the evolution of more male-biased traits.

Together, our results suggest that males are not likely to evolve in this species. We do not find selection for increased male function, in part because there is not a trade-off between pollen and seed fitness. Rather, our results suggest that any increase in "maleness" would lead to lower total fitness, making it difficult for the evolution of more male-biased individuals. We also don't find stronger selection for "male" traits at higher female frequencies as predicted by some theories. Thus, it appears that under the conditions used in this experiment, hermaphrodites will not be selected upon to become pure male individuals.

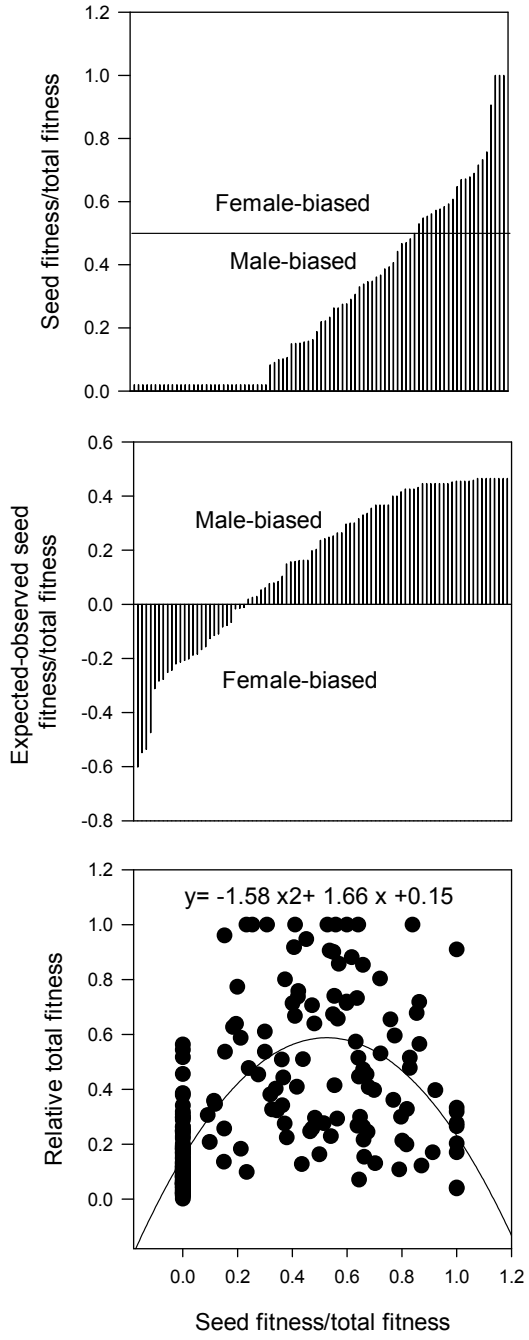
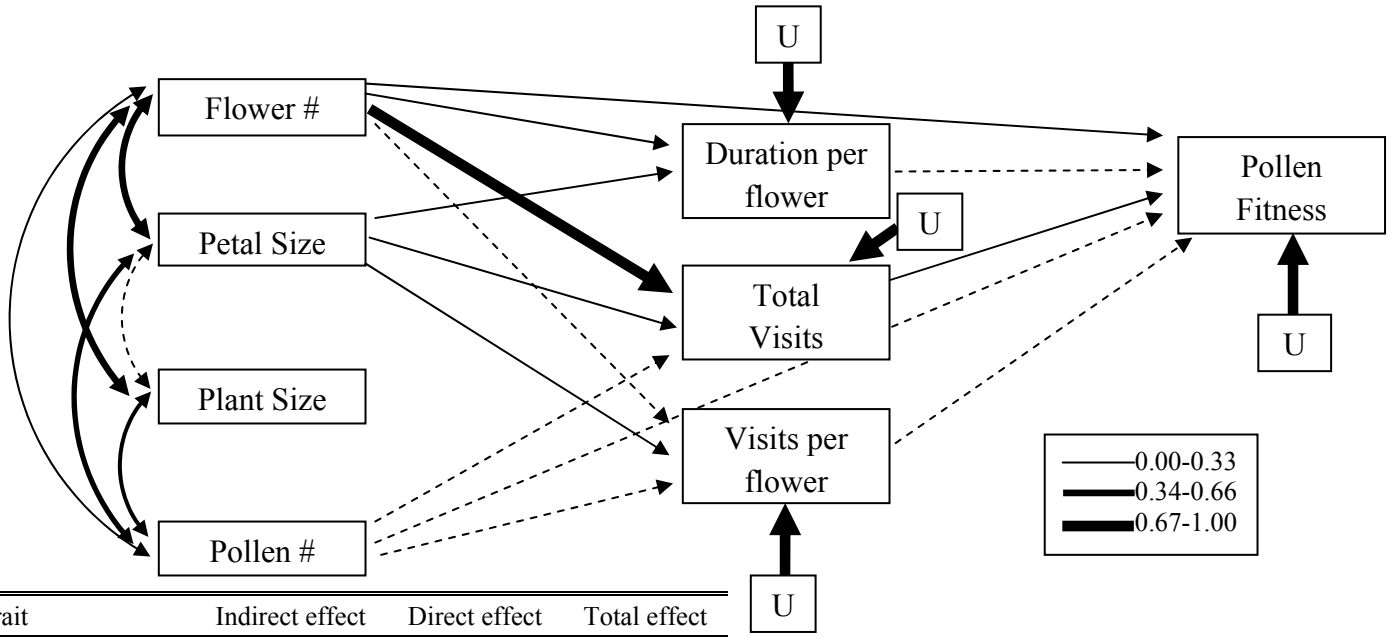
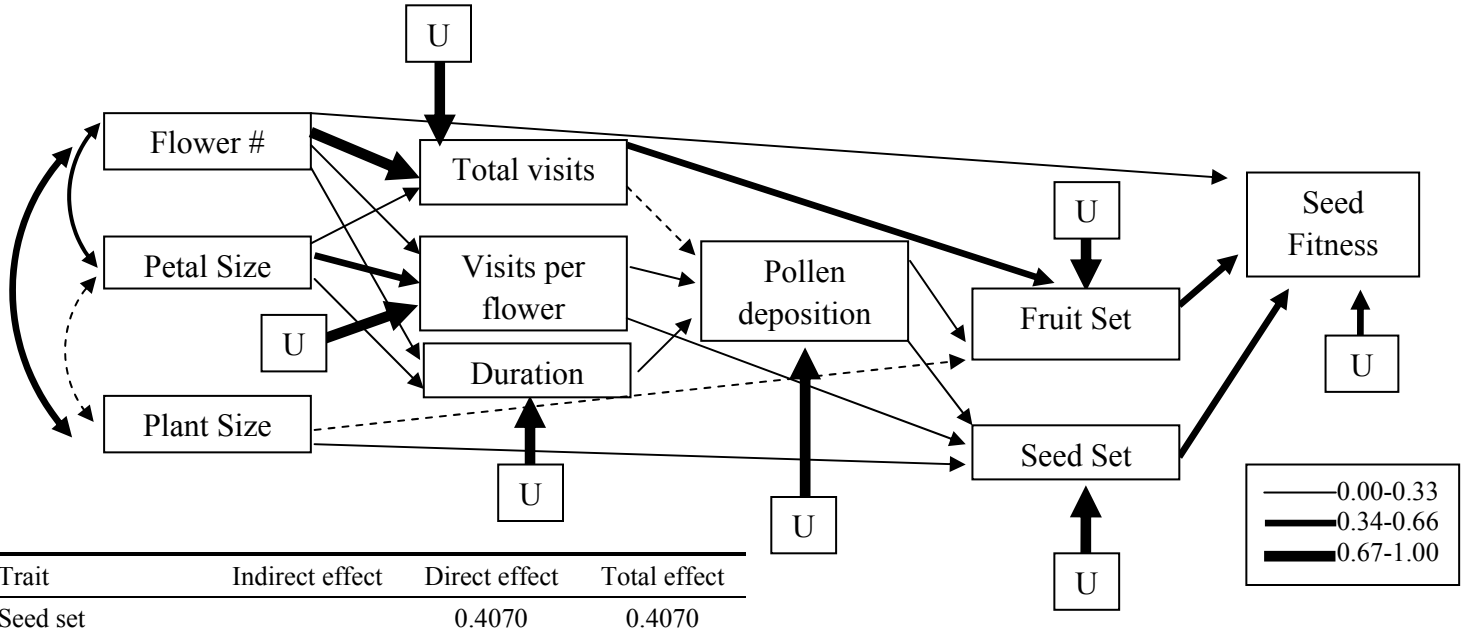


Figure 7.1. The functional gender of hermaphrodites. A) Most hermaphrodites (each bar a hermaphrodite) were male biased (functional gender <0.5). B) Most hermaphrodites (each bar a hermaphrodite) were more male biased than expected given the sex ratio (difference >0, see methods for the calculation of expected functional gender). C) The highest total fitness was obtained by hermaphrodites with a functional gender ~0.5.



Trait	Indirect effect	Direct effect	Total effect
Total visits		0.1785	0.1785
Visits per flower		-0.0990	-0.0990
Duration		-0.0508	-0.0508
Pollen production	0.0013	-0.0809	-0.0796
Flower number	0.1286	0.1054	0.2340
Petal size	0.0016		0.0016

Figure 7.2. Path analysis for fitness gained through pollen donation. Dashed lines indicate a negative effect, solid lines indicate a positive effect. The thickness of the line shows the standardized effect size. Table shows indirect and direct effect sizes for traits.



Trait	Indirect effect	Direct effect	Total effect
Seed set		0.4070	0.4070
Fruit set		0.6393	0.6393
Pollen deposition	0.1745		0.1745
Total visits	0.2214		0.2214
Visits per flower	0.1245		0.1245
Duration	0.0157		0.0157
Flower number	0.1605	0.2859	0.4464
Petal size	0.1034		0.1034
Plant size	-0.0265		-0.0265

Figure 7.3. Path analysis for fitness gained through seed production. Dashed lines indicate a negative effect, solid lines indicate a positive effect. The thickness of the line shows the standardized effect size. Table shows indirect and direct effect sizes for traits.

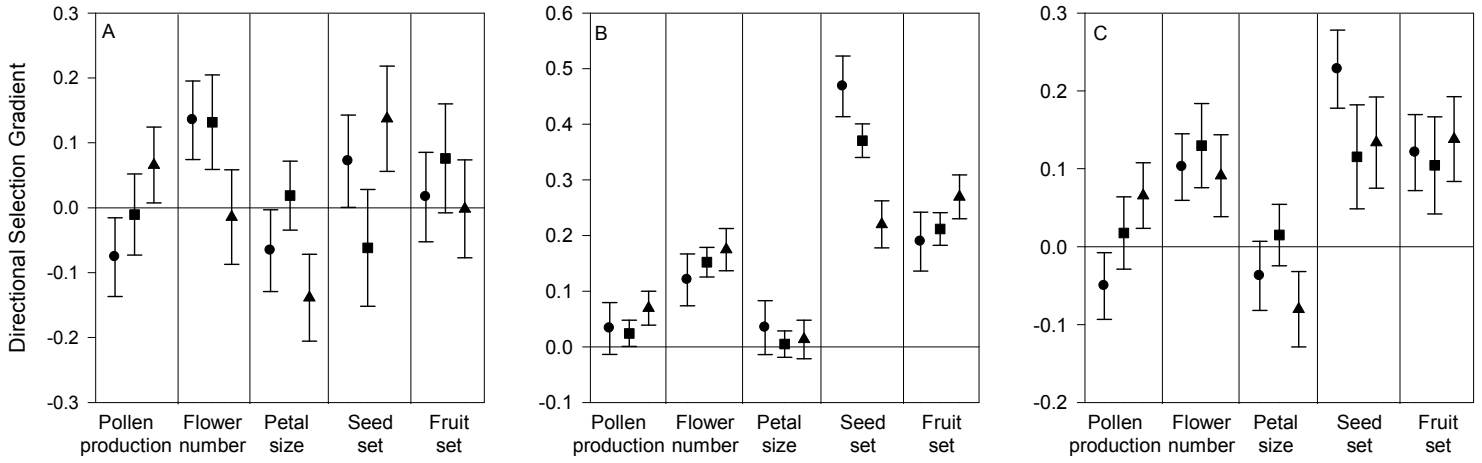


Figure 7.4. Directional selection gradients for each sex ratio (low=circle, intermediate=square, high=triangle) for fitness gained through pollen donation (A), seed production (B) and both (C).

Table 7.1. Directional and quadratic selection gradients for fitness gained through pollen, seed and total fitness. *P<0.05, ** P<0.01, *** P<0.0001.

Trait	Directional Selection Gradient (d.f.=1)	Quadratic Selection Gradient (d.f.=1)
Pollen fitness		
Pollen production	-0.003	
Flower number	0.093**	
Petal size	-0.042	0.104*
Seed set	0.062	
Fruit set	0.017	
Seed fitness		
Pollen production	0.024	
Flower number	0.152***	
Petal size	0.005	
Seed set	0.370***	
Fruit set	0.212***	-0.090*
Total fitness		
Pollen production	0.004	
Flower number	0.100**	
Petal size	-0.025	
Seed set	0.172***	
Fruit set	0.110***	

Table 7.2. Direct and indirect effects from path analysis using total fitness.

Trait	Indirect effect	Direct effect	Total effect
Seeds sired		0.6757	0.6757
Seeds produced		0.5720	0.5720
Seed set	0.2331		0.2331
Fruit set	0.3665		0.3665
Pollen deposition	0.1100		0.1100
Total visits	0.1472		0.1472
Visits per flower	0.0379		0.0379
Duration	-0.0427		-0.0427
Flower number	0.4108		0.4108
Petal size	0.0494		0.0494
Pollen production	-0.0186		-0.0186
Plant size	-0.0165		-0.0165

Chapter 8

Conclusion

Gynodioecy is thought to be the most common transition from hermaphroditism to dioecy. There are three major stages in this transition: the initial invasion of females, the maintenance of females, and the masculinization of hermaphrodites. The research presented here addresses parts of each stage to examine this process in *Geranium maculatum*, an herbaceous perennial. I found that the initial invasion requires a large seed fitness increase by females. This is partly due to the genetic control of gender, which requires at least a 2x female seed fitness advantage, and to increased discrimination against females when they are rare. The maintenance and frequency of females is similarly influenced by seed fitness differences. Due primarily to differences in seed production and flowering frequency, I found that females are expected to be maintained in all populations examined. This seed production increase in part may be due to lower selfing and biparental inbreeding or different environments. However, pollinator discrimination may decrease seed production, lowering females' relative fitness. The last step to dioecy, the masculinazation of hermaphrodites, does not appear to occur in this species due to selection favoring more flowers, which increases both cpollen and seed fitness. Thus, without a tradeoff between pollen and seed fitness, it seems unlikely that dioecy will evolve in *G. maculatum*.

Initial Invasion

The type of genetic control and pollinator discrimination decrease the likelihood that females will be able to successfully invade a population. The type of genetic control sets the minimum seed fitness increase needed for female invasion and maintenance (Charlesworth and Charlesworth 1978; Charlesworth 1981; Lloyd 1974). In *G. maculatum*, sex is most likely under nuclear control (Chapter 2), which requires at least a two fold increase in seed fitness for females to successfully invade and be maintained (Charlesworth and Charlesworth 1978; Lloyd 1974). If sex was under cyto-nuclear control, less of an increase would be needed for females to invade. Pollinator discrimination also may decrease the ability of females to successfully invade a population. When females initially invade, they will be at low frequencies. Pollinator discrimination is especially bad when females are at low frequencies, which can decrease their seed production to lower than hermaphrodites' (Chapter 6). Thus, for females to invade, the decrease in seed production due to pollinator discrimination must be compensated. This may lead to females being able to invade under very specific circumstances.

There are several differences between populations with females and those without, which may help identify what conditions favor female invasion. First, females are found in populations with higher selfing rates in hermaphrodites (Chapter 4). This may assist female invasion because females will have a greater advantage due to their lack of selfing. For example, in the two populations with no females, hermaphrodites did not self. In these populations, if sex is under nuclear control, females must have a two fold increase in seed production to successfully invade. In the populations with females,

the selfing rate was on average 13%, in which case females only need a 1.8 fold increase to successfully invade (using Charlesworth and Charlesworth 1978 model). Second, females are found in populations in darker environments (Chapter 5). This environment may negatively affect seed production in hermaphrodites, making it easier for females to have higher seed fitness. Although this decrease was not found within natural populations, in a manipulative study in the greenhouse, there was a trend for hermaphrodites to have lower seed number, seed set and plant size under darker conditions, while females were generally less affected (Van Etten et al. 2008). Together these results suggest that population characteristics may influence the potential for invasion by females. However, more extensive sampling of a range of populations is needed to better determine whether population differences are the cause or the effect of female invasion.

MAINTENANCE

The maintenance of females, when sex is under nuclear control, is primarily determined by their seed fitness; as the relative seed fitness increases, females should increase in frequency (Charlesworth and Charlesworth 1978). I found that females in natural populations produce between 1.6-2.1x as many seeds as hermaphrodites, in part due to more frequent flowering (Chapter 3). This increase in females seed production could be a result of many different factors, several of which I investigated. First, females may be able to garner more resources or allocate them differently to produce more seeds. Physiological measures in the greenhouse show that females have similar biomass and do not have higher photosynthetic rates than hermaphrodites, suggesting that resource

acquisition and allocation do not account for higher seed production (Van Etten et al. 2008). Similarly, females do not seem to have the capability of producing more seeds than hermaphrodites, because after accounting for flower number differences females and hermaphrodites had the same seed production when receiving outcross pollen in the greenhouse (Van Etten et al. 2008).

Second, females may produce more or better seeds because they experience lower inbreeding depression. However, we found that the amount of selfing is very low within populations (Chapter 4), and thus, the fitness increase we find appears to not be entirely due to the lack of inbreeding depression in females. Alternatively, hermaphrodites may be mating with relatives (biparental inbreeding) because they live in genetically structured areas (Chapter 4) and biparental inbreeding has been shown to incur inbreeding depression in this species (Chang 2007). Although females also occur in structured areas, because of the spatial distribution of the sexes, females are generally surrounded by females and must get pollen from further away, which will decrease the relatedness between the pollen donor and the ovule donor. Thus, females may be experiencing lower biparental inbreeding and therefore lower inbreeding depression. To determine frequency and therefore the importance biparental inbreeding to relative fitness, more variable markers are needed to aid in discriminating between selfed progeny and biparentally inbred progeny in a mating system analysis.

Third, the relative fitness of the sexes may be influenced by their environment because females are found in different microsites than hermaphrodites (Chapter 5). Within a population, females usually occurred in microsites that were drier and brighter, both of which influenced females' seed production. However, different populations had

different relationships between female seed production and the environment, making it difficult to determine if females' relative seed fitness is positively or negatively influenced by the environment differences. For example, when considering the effect of the environment, along with other factors, such as the local density of pollen donors, on seed production, light availability was only important in two of the populations and the sign of the effect differed. Thus, while it is clear that the environment affects female seed production, the importance of environmental differences is not clear and warrants further research.

While physiological differences, the lack of inbreeding depression and environmental differences have the potential to increase females' seed fitness, pollinator discrimination has the potential to decrease it. Regardless of their frequency, females were discriminated against by pollinators, which may decrease their seed production (Chapter 6). This discrimination appeared to be due to both lower flower number, smaller flower size and some other sex specific factor. The importance of discrimination depends on females being pollen limited, i.e. they could produce more seeds if they received more pollen. On average, females did appear to be pollen limited, as were hermaphrodites. This is surprising because visitation rates were higher in the experiment than are usually found under natural conditions (AC Deen, personal communication). Thus, under natural conditions, pollinator discrimination may be even more important than we found in our experiment. Indeed, pollen limitation does seem to occur in some natural populations, where plants surrounded by a large proportion of females have lower seed production (Van Etten and Chang 2009). To better understand how pollen limitation affects female seed production, pollen additions should be done in natural environments.

Together, these results paint a complex picture of the factors influencing relative fitness. Traditionally researchers have suggested that seed production differences are due to the lack of selfing and/or from saved resources (Ashman 1994; Chang 2007; Darwin 1877; Eckhart 1992a; Glaettli and Goudet 2006; Schultz and Ganders 1996). The research presented here suggests that in *G. maculatum* neither of these mechanisms account for the higher seed production of females. Rather, it shows that even within a single species there are multiple mechanisms and are not limited to the lack of selfing or saved resources. Thus, future research in gynodioecious species should investigate a wider variety of mechanisms to better understand the conditions under which females can be maintained.

MASCULINIZATION

The final step in the evolution of dioecy is the masculinization of hermaphrodites. Despite earlier views that species under nuclear control should proceed to dioecy (Ross 1978), I found little evidence for selection for male-biased hermaphrodites. In natural populations, hermaphrodites still produce a large proportion of seeds (Chapter 3) and under greenhouse conditions can produce as many as females (Van Etten et al. 2008). However, when comparing seed fitness to pollen fitness, hermaphrodites obtained about 70% of their fitness through pollen donation (Chapter 7). Theoretically this should select for traits increasing male fitness, even at the expense of female fitness (Charlesworth and Charlesworth 1978; Maurice et al. 1994; Seger and Eckhart 1996). However, there was no trade-off between male and female function because good pollen donors were also good seed producers. Because of the lack of a trade-off between male and female

function, selection seems to be favoring retaining both functions. Thus, it seems unlikely in *G. maculatum* that pure male individuals will evolve and be successful.

Overall, the research presented here indicates that the conditions allowing the successful invasion of hermaphroditic populations by females are quite stringent, but once in a population it appears that gynodioecy can be maintained rather than evolve towards dioecy. This is one of the first sets of research that investigates each step in the evolution of gynodioecy. Surprisingly, this research contradicts several theories about how gynodioecious species are thought to evolve. First, theory suggests that females can invade populations if there are high selfing rates and inbreeding depression (Chang 2007; Darwin 1877; Glaettli and Goudet 2006; Schultz and Ganders 1996) and/or females reallocate resources to produce more seeds (Ashman 1994; Darwin 1877; Eckhart 1992a). However, our results suggest that neither of these processes account for higher seed production in females. This indicates that a wider variety of explanations for increased females' seed production need to be incorporated into how and when it is thought gynodioecious species evolve. Second, the results also contradict the idea that if sex is under nuclear control that increased maleness should evolve (Ross 1978). This presumption is based on a trade-off between male and female function, which is not seen in *G. maculatum*. This is further evidence that traditional models need to be expanded to more completely understand the evolution of breeding systems.

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