The role of sex ratio as a context for selection in *Silene vulgaris*

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A Dissertation presented to the Graduate Faculty
of the University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

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University of Virginia
July, 2016

ABSTRACT

When the sex ratio varies among populations, it is likely that the fitness consequences of sexual phenotypes will also differ among those populations. Sex ratio can also vary at a fine scale within populations, and so there may be multiple, potentially overlapping scales over which sex ratio can affect the evolutionary process. I explored the fitness consequences of sex ratio variation in *Silene vulgaris*, a perennial herb that maintains a sexual polymorphism between plants that produce both pollen and seed, and plants that are only capable of producing seed. My results demonstrate that there is variance in the local sex ratio individuals experience within wild populations, which creates an opportunity for selection to drive differences in fitness among individuals at a fine scale. Further, I show that nocturnal insects, which are the effective pollinators of this species, do not exhibit preferences for the sex of individuals or the sex ratio of groups. This lack of preference in pollinator behavior means that pollinator service should be uniform among individuals regardless of sex or sex ratio, and so fine-scale variation in pollen availability is likely to generate heterogeneous fitness consequences for sexual phenotypes. Finally, I use experimental populations to show that both sexes have the highest fitness when they are rare, and that the direction and magnitude of selection on floral traits is substantially altered by sex ratio. Thus, the sex ratio an individual experiences is a critical context in which selection on sexual phenotypes occurs, and should have dramatic effects on the evolution of those phenotypes at multiple scales within and among populations.

ACKNOWLEDGEMENTS

The work that I report in this dissertation would not have been possible without the boundless support I was incredibly fortunate to receive from many people. First and foremost, my advisor Butch Brodie, who exemplifies everything I hope to accomplish both personally and professionally. Butch has always supported me emotionally, intellectually, and financially, from my early floundering as a lost graduate student through my development as a scientist capable of carrying out the research that I report in this dissertation. He consistently pulled me back from my tendencies to myopically focus on minutiae and forced me to consider the broader context in which my work exists, which I think dramatically improved both the scope of my research as well as the quality of my writing. Through his direct mentorship, as well as my exposure to his work as a teacher and director of Mountain Lake, he provided me with the knowledge, experience, and confidence I need to pursue a career in academic science. I hope to be able to live up to the example that he set for me.

Each member of my dissertation committee also provided invaluable support in the development of these projects. My first reader, Laura Galloway, provided very thoughtful feedback on each chapter of this dissertation, as well as my successful DDIG application. She was always willing to sit down and discuss key aspects of experimental design, and I always appreciated the practicality of her feedback. Doug Taylor's enthusiasm and willingness to explore big picture ideas was immensely helpful whenever I struggled with real or imagined setbacks. Janis Antonovics provided a much needed skepticism, which forced me to make important connections with the literature that I

would otherwise have missed. Dave Carr provided great insight into the design of my experiments, as well as considerations about sample size and power that I believe saved me from some pitfalls I wouldn’t have avoided on my own.

I am also deeply indebted to the members of the Brodie lab past and present, especially Vince Formica, Corlett Wood, and Malcolm Augat. My development as a scientist is directly tied to my interactions with these three people, through our reading groups and our wrestling with the vagaries of writing and data analysis together. Vince was the first person to get excited about my burgeoning research project, and he knew well before I did that my project was really about social selection. Corlett always inspired me to be better and work harder, directly through her unfaltering support and willingness to talk about my work, and indirectly through her own hard work and diligent scholarship. Malcolm was especially critical to all of the field work I describe here—the genesis of all of these projects and the rigor with which they were conducted are all thanks to the deeply rewarding collaboration we had over the summers that we worked together.

The generosity and support of the EEBIO community at UVA was what most attracted me to this program when I was interviewing, and my interactions with the fine people here over the past six years were crucial in my development as a scientist. I want to especially thank Dan Sloan, who mentored my rotation project when I first started. He set an intimidating example, and helped me to understand what was involved in pursuing independent research. The Taylor lab was my first home at UVA, and Peter Fields, Andrea Berardi, and Hilary Edgington were my constant companions who provided inestimable emotional and intellectual support. Bob Cox, Christian Cox, and Ariel Kahrl provided excellent critical feedback on drafts of grants and the first chapter of this dissertation. Ray Watson's boundless enthusiasm and curiosity always helped to bolster my spirits and my desire to know more.

Mountain Lake Biological Station was my home every summer over the past six years, and I feel incredibly fortunate to have been a part of that amazing community. Henry and Becky Wilbur are the heart and soul of Mountain Lake. Their deep knowledge of natural history and their scientific wisdom were invaluable, and I was deeply touched by their friendship and support over the years. The staff at Mountain Lake, especially Jaime Jones, and Tom McNamara, facilitated all of my research and were incredibly patient, kind, and supportive throughout the unpredictability of progress that is natural to field work. Eric Nagy gave me the opportunity to work with extremely talented undergraduate students, and my work with Michelle McCauley, Sarah Leichter, Amelia Brumbaugh, and Anita Enriquez not only added to the results of my dissertation work, but also helped me to develop and mature in my role as the principle investigator of a research project.

I must also acknowledge the contributions of my family to the development of this dissertation. My parents Jim and Therese, and my sister Bridget, have always offered unquestioned and unconditional love and support in all of my personal and professional endeavors, and this support was a huge contributor to the self-confidence that I needed to push these projects through many rough patches.

I also want to thank Dana Schroeder and Brittany Watson, for their friendship and support. It’s easy to lose sight of the fact that there is still a world outside of academic science that goes on without us, and Dana and Brittany always helped to keep me positive, grounded, and sane.

Financial support for this dissertation was provided by a NSF Doctoral Dissertation Improvement Grant, the NSF REU program at MLBS, MLBS Margaret Walton Summer Research Fellowships, and the UVA Department of Biology.

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INTRODUCTION

Developing a functional understanding of how traits evolve in natural settings is complicated by the fact that selection does not act on traits independently, but rather through the complex multivariate relationship between an organism's phenotypes and its fitness, as well as interactions among individuals within a population. A key breakthrough in the study of phenotypic evolution in the wild was that selection can be estimated as the covariance between an organism's relative fitness and its trait values (Robertson 1966; Price 1970, 1972), and that those measurements could be extended to multivariate suites of correlated characters (Pearson 1903; Lande & Arnold 1983). This led to a proliferation of study of the evolution of multivariate, correlated phenotypic characters in the wild across many taxa (Kingsolver et al. 2001; Kingsolver & Pfennig 2007). What is less well appreciated is how interactions among individuals within populations affect trait evolution. The covariance approach to measuring multivariate selection directly on the trait values of individuals can be extended to include the effects of interactions among organisms, and as a result we can quantify how intraspecific interactions shape phenotypic evolution (Heisler & Damuth 1987; Frank 1997; Wolf et al. 1999).

The fitness of organisms that co-occur in populations is influenced directly and indirectly by the presence of their neighbors, and so the social context of a population can contribute to the evolutionary process. All organisms in nature interact with conspecifics to some degree, whether through competition for shared resources, or directly by engaging in social or sexual behaviors (West-Eberhard 1979). The evolution of traits involved in these interactions between organisms is unique because selection can occur not just as the relationship between the organism's phenotype and its fitness, but also as the relationship between the phenotypes of its social partners and its fitness. In this way, the traits involved in social or sexual interactions act as both agents and targets of selection, because the trait values of social partners become the context in which selection is occurring (Moore et al. 1997; Wolf et al. 1999).

For sexually-reproducing organisms, the sex ratio of the population is a critical social context in which selection occurs. In the first edition of *The Descent of Man*, Darwin addressed the observation that across myriad species the ratio of males to females is almost always equal. He proposed that natural selection should bring about an equilibrium sex ratio that is balanced, because a balanced sex ratio should lead to the greatest productivity of the population. However, this argument was famously abandoned in the second edition as a problem "so intricate that it is safer to leave its solution to the future" (Darwin 1871; Darwin 1874; Edwards 1998). In his *Genetical Theory of Natural Selection*, Fisher popularized the idea that, because every offspring has both a sire and a dam, so long as the reproductive investment required to produce sons and daughters is equal, natural selection should lead to an equilibrium 1:1 ratio of males and females (pp. 142, Fisher 1930). This form of frequency-dependent stabilizing selection should drive not just the equalization of sex ratio among populations, but also the fitness consequences of sexual phenotypes for individuals within those populations.

Although Fisher's principle spurred the study of sex allocation, and is so familiar to most evolutionary biologists as to seem obvious, it relies on several assumptions that are not universally applicable. Fisherian sex ratio evolution depends on biparentalism, discrete sexes, Mendelian sex determination, and random mating in unstructured populations, among other assumptions (Bull & Charnov 1988). Each of these assumptions are violated in the majority of plant species, which makes those species particularly compelling models for the evolution of sexual phenotypes. Angiosperms have a remarkable diversity of mating systems, including many species that feature intermediate forms between hermaphroditism and dioecy. In plant species that are hermaphroditic or partially hermaphroditic, individuals vary in their allocation of resources to the male or female components of fitness. In such species, sex can be considered a quantitative, rather than discrete trait, and the principle of equal parental investment should then apply not only to variation among dams and sires, but also between competing opportunities for fitness within an individual (Lloyd 1980a). Furthermore, populations of sexually polymorphic plants tend to exhibit large variation in sex ratio.

These fundamental differences between most plant and animal mating systems highlight the fact that plant mating systems present excellent opportunities to understand how the sex ratio of a population drives selection on sexual phenotypes. Mating systems in plants range from hermaphrodites, in which flowers on an individual plant can produce both pollen and seed, to dioecy, in which individual plants produce only one or the other gamete. The majority of angiosperm species are hermaphroditic, with only a minority of species (4-6%) that have distinct male and female individuals (dioecy; Spigler & Ashman 2012). The evolution of dioecy is thought to proceed from hermaphroditism, through intermediate mating systems in which hermaphrodites, males, and/or females co-occur (Lewis 1942; Lloyd 1980b), as well as species that feature intra-sexual polymorphisms that force incompatibilities through pollination syndromes such as heterostyly (Lloyd 1979). The best studied of these mating systems in the context of mating system evolution is gynodioecy: species that maintain a sexual polymorphism between hermaphrodites and females. Gynodioecy is the most common form of partial hermaphroditism in plants, and the majority of clades that contain dioecious species also contain gynodioecious species (Lewis 1942).

The persistence of female plants in gynodioecious species has been extensively studied theoretically and empirically for decades. Because females have half of the opportunities for fitness compared with hermaphrodites, they should exhibit at least a two-fold fitness advantage through seed in order to be maintained in populations (Charlesworth & Charlesworth 1978). This pattern of female fitness advantage can arise because female plants produce more and better seed, and/or because hermaphrodite plants exhibit some form of a physiological cost of bearing restorer genes (Bailey & Delph 2007). Regardless of whether females are intrinsically better at seed production, or hermaphrodites are worse, the presence of female plants is thought to drive selection on hermaphrodites to be more “male-like,” potentially resulting in the evolution of a dioecious mating system with separate male and female plants (Spigler & Ashman 2012). Furthermore, populations of gynodioecious species tend to exhibit large variation in sex ratio, which makes them ideal for studying the effects of sex ratio on the fitness of, and selection upon, individuals (Spigler & Ashman 2012).

In addition to variation in sex ratio among populations that is characteristic of gynodioecious species, there is likely to be variation in sex ratio at a fine scale within populations. Populations are structured non-randomly in space, which can create variation in local interactions within populations. These spatial relationships will be especially important in sessile organisms, such as most plants, because the sexual interactions available to an individual will be determined by their physical proximity to compatible mates. When seed is passively distributed, individuals are likely to co-occur with similar individuals, such that the subjective frequency of sex that an individual experiences will be biased toward their own sex (McCauley & Taylor 1997). In such a circumstance, there may be large variation among individuals within the population in the local sex ratio they experience, which creates an opportunity for selection to drive differences in fitness among individuals at a fine spatial scale.

The purpose of my dissertation is to understand whether and how selection arising from the sex of neighboring plants affects the evolution of sexual phenotypes in populations of the flowering plant *Silene vulgaris*. *S. vulgaris* (Caryophyllaceae) is a perennial, gynodioecious herb that is native to Europe and broadly distributed through its introduced range in North America (Taylor & Keller 2007). Sex determination is the result of cytoplasmic male sterility (Charlesworth & Laporte 1998; Taylor et al. 2001). For many species with CMS, mutations in the mitochondrial or chloroplast genome cause the abortion of anther development and result in functionally female plants, while nuclear-encoded proteins restore normal cytoplasmic function and result in hermaphrodite plants (Frank 1989). Populations of *S. vulgaris* exhibit extreme variation in sex ratio, from 30-100% hermaphrodite (McCauley et al. 2000). The species is pollinated by both diurnal (syrphid flies and bumble bees) and nocturnal insects (noctuid and sphingid moths) (Marsden-Jones & Turril 1957; Stone 2013). Seeds are passively dispersed as fruits dehisce, leading to a high degree of genetic and phenotypic structuring of populations (Olson et al. 2006).

In Chapter 1, I demonstrate that the distribution of plants in wild populations of *S. vulgaris* creates an opportunity for social selection to drive differences in fitness among individuals at a fine spatial scale. Hermaphrodites and females experience a subjective frequency that is biased toward their own sex at local scales, under 1.5 meters, while the subjective frequency becomes homogenized among individuals at larger spatial scales. This study demonstrates that sex ratio can create continuously varying, and potentially overlapping, social contexts within populations. I discuss what this variation means in terms of the opportunity for social selection when pollinators move among nearest neighbors.

In Chapter 2, I use experimental populations of *S. vulgaris* that vary in sex ratio to show that although diurnal pollinators prefer patches with high frequencies of hermaphrodites, nocturnal pollinators do not exhibit preferences for the sex of individual plants, or the sex ratio of aggregations of plants. I also show that only nocturnal pollinators effect fruit set in this species. Taken together, this demonstrates that variation in sex ratio among or within populations is unlikely to result differences in pollinator service. This suggests that differences in fitness due to sex ratio will only arise as the result of heterogeneity in the availability of pollen, and not through behavioral responses of pollinators. Thus, the spatial structure of populations is likely to result in fine-scale variation in fitness and selection within populations.

Finally, in Chapter 3, I quantify the fitness effects of sex ratio as well as the strength and direction of selection arising from sex ratio in experimental populations. I show that both hermaphrodites and females have the highest fitness when they are the rare sex. The direction and strength of selection also differed in a context-dependent manner. Selection on floral traits through fruit set was strongest at the extremes of sex ratio variation, and weakest when the sexes were in equal proportions. Further, the direction of selection reversed across sex ratio treatment: plants with the largest flowers were the most fit when hermaphrodites were rare, whereas plants with the smallest flowers were the most fit when hermaphrodites were common. These results demonstrate that the fitness consequences, and the resulting phenotypic selection upon sexual phenotypes depends strongly on the social context in which they are expressed.

Taken together, the results of my dissertation illustrate the importance of understanding how selection arising from interactions with social and sexual partners affects the evolution of sexual phenotypes. By combining demographic, behavioral, and microevolutionary studies, these results provide insight into how a population-level characteristic such as sex ratio can affect the fitness of individuals and phenotypic selection. The spatial structure of wild *S. vulgaris* populations generates a significant opportunity for the patterns of social selection that I quantified to drive large heterogeneity in the fitness consequences of sexual phenotypes.

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CHAPTER ONE:

Scale dependence of sex ratio in wild plant populations: implications for social selection[1]

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[1] As published: Sanderson, B. J., Augat, M. E., Taylor D. R., & Brodie III, E. D. (2016). Scale dependence of sex ratio in wild plant populations: Implications for social selection. *Ecology and Evolution*, **6,** 1411-1499.

Abstract

Social context refers to the composition of an individual's social interactants, including potential mates. In spatially structured populations, social context can vary among individuals within populations, generating the opportunity for social selection to drive differences in fitness functions among individuals at a fine spatial scale. In sexually polymorphic plants, the local sex ratio varies at a fine scale, and thus has the potential to generate this opportunity. We measured the spatial distribution of two wild populations of the gynodioecious plant *Silene vulgaris* and show that there is fine-scale heterogeneity in the local distribution of the sexes within these populations. We demonstrate that the largest variance in sex ratio is among nearest neighbors. This variance is greatly reduced as the spatial scale of social interactions increases. These patterns suggest the sex of neighbors has the potential to generate fine-scale differences in selection differentials among individuals. One of the most important determinants of social interactions in plants is the behavior of pollinators. These results suggest that the potential for selection arising from sex ratio will be greatest when pollen is shared among nearest neighbors. Future studies incorporating the movement of pollinators may reveal whether and how this fine-scale variance in sex ratio affects the fitness of individuals in these populations.

Keywords: *Silene vulgaris*, multilevel selection, population structure, context-dependence, local mate competition, gynodioecy

Introduction

The fitness consequences of interacting phenotypes—traits involved in social or sexual interactions—depend on the context in which these interactions occur (Moore et al. 1997). Social context is a ubiquitous feature of natural populations and can be created by demography, the spatial structuring of populations, and the patterns of interactions among individuals within populations. Social selection is a form of multilevel selection that acts on, and results from, the phenotypes of all interacting individuals in a population (Crook 1972; West-Eberhard 1979; Wolf et al. 1999). Thus, the phenotypes of individuals and their social partners act as both the targets and agents of selection, generating complex evolutionary dynamics across levels of biological organization (Moore et al. 1997). Social selection drives the evolution of combat traits in forked fungus beetles (Formica et al. 2011, 2012), intrasexual aggressive behavior in water striders (Eldakar et al. 2010), and reproductive conflicts in the body size of worker ants (Tsuji 1995). In each of these cases, the magnitude and direction of selection on the trait of interest is in part determined by the social context in which the trait is expressed.

We know little about the spatial scale on which social selection is likely to act in plants or animals. Social selection has largely been studied as effects on individuals arising from the average phenotype expressed in a population (e.g. the average body size of males; Formica et al. 2011), yet different social contexts can exist and vary across space and time at a fine scale within populations. Under these circumstances, individuals experience continuously varying, and potentially overlapping, social contexts across the population, and so understanding the scale on which organisms interact within populations is critical to understanding the potential fitness consequences of social interactions.

Wolf et al. (1999) presented a general model of social selection demonstrating how a covariance between the phenotypes of social partners affects selection on interacting phenotypes. The total selection differential on the trait of interest in this model is described by:

$$s_i = P_{ii}\beta_N + C^{ij'}\beta_S$$

where P_{ii} is the phenotypic variance of the trait, β_N is the non-social or natural selection gradient acting directly on the trait of the individual, $C^{ij'}$ is the covariance among individual trait values and the phenotypes of their social partners, and β_S is the social selection gradient representing the relationship between individual fitness and the trait values of social partners. The product $P_{ii}\beta_N$ is the non-social selection differential, representing selection directly on the trait of interest. The social selection differential $C^{ij'}\beta_S$ represents selection arising from the phenotypes of social partners. The critical metric that determines whether there is potential for social selection is this covariance between partners, or neighbors, $C^{ij'}$. When $C^{ij'}$ is non-zero, the total selection differential is the sum of the non-social and social selection differentials. However, when no consistent pattern of phenotypic covariance exists ($C^{ij'} = 0$), the traits of social partners (j') do not exert net selection on traits in the focal individuals, and only direct selection on the individual will affect phenotypic evolution.

The spatial scale of interaction is likely to be a primary determinant of $C^{ij'}$, especially in non-motile organism such as plants, where fitness impacting interactions are determined by physical proximity or the movement of animal vectors. Plants compete for resources in the soil and to avoid shading (Bengtsson et al. 1994; Dykstra et al. 2009; Van Etten & Chang 2009; Weiner & Freckleton 2010), and stand-level characteristics can result in both competition and facilitation for pollinators, in addition to affecting the apparency of the stand to herbivores (Stevens et al. 1995; Kelly 1996; Aspi et al. 2003; Donohue 2004; Bartkowska and Johnston 2014). Considerable attention has been paid to the degree to which the fitness effects of population- and community-level phenotypes depend on the spatial scale of interactions (Barrett and Thomson 1982; Aizen 1997; House 1992; Stehlik et al. 2008; Lin et al. 2015). If all individuals experience similar social contexts, as when stand-level patterns influence fitness, then we expect no opportunity for social selection. As the scale of interaction reduces, as expected under local competition with neighboring individuals or short range movements of pollinators, we expect greater opportunity to arise as individual plants experience variable contexts. The importance of spatial scale may be more severe in plants than in animals because the ability of plants to respond to selective interference through behavioral modification is likely limited to shifts in phenology (e.g. Rivkin et al. 2015; Weis et al. 2015).

In this study we focus on fitness effects arising from the sex of neighboring plants across various spatial scales using social selection analysis. Sex ratio is a social characteristic of populations or neighborhoods that is often linked with individual fitness because the frequency of available mates is defined by the demographic composition of the population (Fisher 1930; Hamilton 1967). Especially in plant populations, the spatial structure of the sexes generates a high degree of fine-scale variance in the sex ratio experienced by individuals (McCauley & Taylor 1997). Fine-scale variance in sex ratio is likely to result in strong fitness consequences because the fitness of individuals in a population will depend not only on their own sex allocation and behavior, but also on the availability of compatible mates and the strength of competition with neighboring individuals (Hamilton 1967).

In this paper we measure the spatial distribution of individuals within wild plant populations to examine the variation in local neighborhood sex ratios at varying scales. We address the following questions: 1) How do patterns of phenotypic covariance of sex vary among spatial scales and 2) What do these patterns suggest about the opportunity for social selection in these populations? We demonstrate that the greatest variance in sex ratio among individuals occurs at the smallest spatial scales and that the greatest covariance between the sex of focal individuals and the local sex ratio also occurs at the smallest spatial scales. These patterns suggest that the scale of mating will determine the degree to which selection differentials differ among individuals within the population. Selection differentials will be most strongly impacted by social interactions when pollen is shared among nearest neighbors, and dissipate if mating occurs over larger spatial scales. We discuss these results in terms of their implications for social selection arising from sex ratio, and highlight the generality and utility of the social selection framework for the study of plant mating systems and pollination biology.

Materials and Methods

Study system

Silene vulgaris (Caryophyllaceae) is a gynodioecious perennial plant that grows predominantly along roadsides in its introduced range in North America (Taylor & Keller 2007). There is tremendous variation in sex ratio among populations, ranging between 30% - 100% hermaphrodite (McCauley et al. 2000). As in many gynodioecious species, sex is determined through cytonuclear interactions: mutations in the mitochondrial genome cause the abortion of anthers, resulting in functionally female plants, while nuclear-encoded proteins can repress the action of the mitochondrial mutations, resulting in hermaphroditic plants (Frank 1989; Charlesworth & Laporte 1998). The species is pollinated both by diurnal (syrphid flies and bumble bees) and nocturnal insects (noctuid moths; Marsden-Jones & Turril 1957; Stone 2013). Seeds are passively dispersed when fruits dehisce, which may explain the high degree of genetic structuring of populations (Olson et al. 2006).

Mapping of populations

We sampled plants in a large, approximately 10 hectare pasture in Simmonsville, VA, wherein *Silene vulgaris* is patchily distributed. In October 2012 we ran 100 meter transects across the entire pasture, and selected two large patches of very high density for fine-scale mapping. Each plant was assigned a unique ID, and its sex was determined by the presence of mature anthers (hermaphrodite) versus aborted anthers (female). We mapped the spatial positions for all individuals in the two populations through trilateration: three tripods were spaced evenly around the perimeter of the population, and the GPS coordinates of these tripods were recorded using the Trimble TSC1 survey platform (Trimble Navigation, Sunnyvale, CA). The measurement of the GPS coordinates for each of these reference points was estimated as accurate to below a distance of one meter. The distance from each plant to each of these three tripods was recorded using the Sonin Multi-Measure Combo Pro sonic range finder (Sonin Inc., Charlotte, NC). We used a custom Python trilateration script to convert each of these ordered triplets of distances into GPS coordinates for each individual plant (script available at Dryad http://dx.doi.org/10.5061/dryad.c06n3). All of the following analyses were performed using R version 3.1.1 (R Core Team 2014).

Quantifying social context

We used the function dist to create matrices of the pairwise distance (in meters) between all plants for each subpopulation. We then quantified local hermaphrodite frequencies for each individual by tallying the number and sex of neighboring individuals at neighborhood sizes ranging from 0.5 – 6.0 m radii (in 0.5 m steps) from the focal plant. Throughout the following analyses the hermaphrodite frequency does not include the focal individual, which is the convention in social selection analysis (Wolf et al. 1999). We plotted these spatial relationships using the function ggplot in the package ggplot2 (Wickham 2009).

Variance in hermaphrodite frequency at different spatial scales

To determine whether the hermaphrodite frequency experienced by individuals significantly varied among spatial scales, we used the function leveneTest in the package car, which includes an implementation of the Brown-Forsythe test for heterogeneity of variances (Fox & Weisberg 2011). We tested whether mean hermaphrodite frequency experienced by individuals differed among spatial scales with a generalized linear mixed model with a binomial family error distribution and logit link using the function glmer in the package lme4 (Bates et al. 2014). In this model we regressed hermaphrodite frequency on sex, radius of spatial scale, the sex-by-spatial-scale interaction as fixed effects, and plant ID as a random effect. Although there was significant heterogeneity of variances in hermaphrodite frequency among spatial scales, this did not appear to have a strong effect on the fit of the mixed model (based on a visual inspection of the residuals and predicted values of the model) and so we did not adjust the model to account for heteroscedasticity. To determine the significance of these parameter estimates, we used the function Anova in the package car, specifying Type-III sums of squares (Fox & Weisberg 2011).

We calculated the phenotypic covariance among social partners ($C^{ij'}$) and the phenotypic variance of local hermaphrodite frequencies (P_{jj}) across spatial scales using the functions cov and var. We tested whether the $C^{ij'}$ terms were significantly different from zero using the function cor.test. Finally, we calculated the correlation of the local hermaphrodite frequencies experienced across the different spatial scales, and tested the significance of these correlations with the function corr.test in the package psych (Revelle 2015). Whenever a population is comprised of continuously overlapping social neighborhoods, the social phenotypes experienced by neighboring individuals will include some of the same individuals and thus violate assumptions of statistical independence. Some researchers, such as Bartkowska and Johnston (2014), have used permutation tests of non-overlapping social neighborhoods to estimate the degree to which violations of model assumptions are driving spurious results. This approach is less helpful when the patterns of phenotypic correlations are scale-dependent and the potential scales of interaction are large relative to the population as a whole, so caution should be used in interpreting the significance of results.

The spatial data, Python trilateration script, and the R scripts described in these methods are available at Dryad http://dx.doi.org/10.5061/dryad.c06n3

Results

We mapped 278 plants in the first population ("Burk1") and 390 plants in the second population ("Burk2"). The population-level hermaphrodite frequency of Burk1 was 0.658, and the population-level hermaphrodite frequency of Burk2 was 0.590. However, there was significant heterogeneity in the distribution of the sexes within each population (Table 1).

The mean hermaphrodite frequency of all individuals varied significantly among spatial scales (Table 2). There was a significant interaction of sex and spatial scale; hermaphrodites experienced a larger frequency of hermaphrodites among their neighbors, while females experienced a smaller frequency of hermaphrodites (Table 2). As the size of the spatial neighborhood increased, the hermaphrodite frequency experienced by both hermaphrodites and females converged on the population-level hermaphrodite frequency (Table 2; Figs 1 and 2).

The variance in hermaphrodite frequency greatly differed among spatial scales (Brown-Forsythe test, $P < 0.001$). Specifically, there was a dramatic contraction of variance in hermaphrodite frequency with increasing spatial scales (Fig. 3). The phenotypic covariance among social partners ($C^{ij'}$) also decreased markedly with increasing spatial scale (Fig. 4).

Finally, the correlations between the local hermaphrodite frequencies an individual experienced decreased as the size of the spatial scales diverged (Table 3). The strongest correlations were between the closest spatial scales, such that increasing off-diagonals of the correlation matrix show decreasing values. Thus, the hermaphrodite frequency that an individual experiences at one spatial scale becomes largely uncorrelated with hermaphrodite frequency experienced at spatial scales that differ by a meter or more. Taken together, these results all suggest that the hermaphrodite frequency that an individual experiences is strongly affected by the scale at which social interactions occur. This pattern may reflect differences in sampling variance of the sex ratio of the population, but would nonetheless be biologically relevant in the context of pollination or other inter-individual interactions.

Discussion

Social selection is generally studied as a population-level phenomenon. The questions most often asked with regard to social selection are questions of how population-level characteristics affect the fitness consequences of individual phenotypes. However, when a population exhibits fine-scale phenotypic structure there is potential for heterogeneity in local interactions across the population, which can result in different fitness consequences among individuals. Our results demonstrate that the distribution of the sexes in wild populations of *Silene vulgaris* creates the potential for dramatic differences in the social context experienced among individuals, depending on the scale of social interactions. We found that the patterns of phenotypic covariance of sex were greatest at the smallest spatial scales and disappeared at larger scales. These phenotypic covariances will have the greatest potential fitness effects on females, because a positive phenotypic covariance should exacerbate pollen limitation in female plants but not in hermaphrodite plants. This finding is particularly interesting in the context of vector-pollinated plant populations, because the scale of pollinator movement is often among nearest neighbors (Waser 1982). This heterogeneity in the social environment at a fine scale, and the resulting potential for heterogeneity in selection differentials among individuals in a population, demonstrates that an understanding of the evolution of interacting phenotypes requires understanding the scale on which those interactions occur.

If we make some assumptions about differences in fitness between hermaphrodites and females in these populations, we can use the model of social selection to determine how the patterns of phenotypic covariance we found might impact sex ratio evolution. Although we did not measure fitness in these populations, we can make reasonable estimates based on past work. Previous work suggests females experience a two-fold fitness advantage over hermaphrodites in fruit set or seed set (Olson et al. 2006; Dufay and Billard 2012). We can use this fitness measure, and consider sex as our trait of interest to calculate the natural selection differential for these populations (see supplemental material for details of this calculation). The resulting natural selection differential on sex is -0.35, representing the inherent advantage females have through higher seed production. In the absence of other influences from the social environment we would expect to see an increase in females if sex is at least partially heritable. However, when pollen moves among neighbors within a 1.5 m or smaller radius, the scales over which we found the largest values of $C^{ij'}$, a moderate to high strength of social selection will ameliorate these fitness differences between the sexes. Indeed, the social selection differential would completely counteract the natural selection differential against hermaphrodites when $\beta_S \geq 1.66$, such that there would be no difference in fitness between the sexes, despite the strong direct female advantage. Conversely, if pollen moves among plants at scales greater than 3.0 m the strength of social selection would need to be 10x greater to overcome female advantage, a magnitude that is unlikely to occur.

Previous studies of *S. vulgaris* suggest that the fitness effects of local sex ratios can be substantial. Olson et al. (2005) demonstrated that variance in the sex ratio among wild populations explained differences in relative fitness between the sexes. The relative fitness of females declined by 15% in wild populations because females suffered (and hermaphrodites benefited) from experiencing a locally high frequency of their own sex. Additionally, Olson et al. (2006) surveyed sex ratio at a fine scale in two wild populations of *S. vulgaris* and found a negative correlation between fruit set and local female frequency; the relative fitness of females was 19% lower than hermaphrodites due to the fine-scale spatial structure of the sexes within populations. Our results suggest that this type of social selection has the opportunity to occur simultaneously at several spatial scales within populations. We found that hermaphrodites and females will experience sex ratios biased toward their own sex, provided mating occurs over the scale of a few meters within populations. Thus, the population-level fitness effects that have previously been demonstrated also are likely to occur among individuals within the population.

In *S. vulgaris*, pollination is driven both by diurnal and nocturnal insects (Marsden-Jones & Turril 1957). Stone (2013) demonstrated that in *S. vulgaris* hermaphrodites were visited more often and for longer periods of time by diurnal insects, while nocturnal insects showed a less pronounced preference for hermaphrodites. Taken together, these results have several important implications. Moths have been shown to travel greater distances than bees or flies during pollination (Barthelmess et al. 2006). Whatever aspects of phenology (sexually dimorphic or otherwise) that affect which plants pollinators visit and what they do afterwards have the potential to greatly impact the fitness of individuals, given the difference in dispersal behavior among groups of pollinators. If pollinators have consistent preferences for plants based on the phenotype of individuals or the phenotypes of aggregations of individuals, those preferences may accelerate or constrain the effects of social selection.

Past experiments investigating the scale dependence of the fitness effects of social and ecological context in other plant species have revealed mixed results. Barrett and Thomson (1982) found no effect of sex ratio or density on fruit set in *Aralia nudicaulis* up to a distance of 40 meters, which they interpreted to reflect extremely long distance pollen dispersal. Aizen (1997) found that in *Alstroemeria aurea* neither density nor sex ratio explained large amount of variation in pollen receipt among individuals. However, there was a strong effect of sex ratio on seed output, suggesting that pollination effectiveness is impacted by social context. Conversely, there are many examples of species in which there is a direct relationship between fruit and/or seed set and the density of compatible mates in both wind- (Stehlik et al. 2008) and vector-pollinated plants (House 1992), and these patterns often vary across spatial scales (Lin et al. 2015). Bartkowska and Johnston (2014) recently demonstrated the scale dependence of sex ratio and density in *Lobelia cardinalis*. Plants experienced negative fitness effects based on the phenotypes of neighbors within 10 centimeters, which the authors interpret as competition for resources, while plants experienced positive fitness effects due to the phenotypes of neighbors at scales greater than 10 centimeters, which they interpret as evidence for facilitation of pollinator visits. These examples highlight how the fitness consequences of social phenotypes can depend on the scale over which interactions occur.

The environmental and genetic factors that drive the observed patterns of spatial structure may alter predictions of any short- or long-term responses to social selection. The environment—specifically light and moisture conditions—has been shown to affect sex expression and sex ratio in *S. vulgaris* (Dykstra et al. 2009) and *Geranium maculatum* (Van Etten & Chang 2009). Inbreeding also has been shown to significantly affect sex expression in *S. vulgaris*—the sex ratios of more inbred plants became increasingly female-biased in a greenhouse crossing study (Glaettli & Goudet 2006). Additionally, there is evidence of post-zygotic barriers to gene flow among plants that are spatially proximate and/or highly related (Glaettli et al. 2006). Taylor et al. (2001) demonstrated that the frequency of hermaphrodites of offspring is heritable, suggesting that the patterns of sex structuring may be maintained across generations. Any or all of these factors may determine the spatial distribution of plants in the wild or the timing of reproduction, which will determine the pool of potential mates that are available to individuals. Thus, any response to social selection will likely depend additionally on these environmental and genetic constraints.

The interactions that give rise to context-dependent processes are complex and likely vary among individuals within populations. The scale over which social interactions occur is critical to understanding how social context can affect phenotypic evolution. Whether and how social interactions demonstrate scale dependence is an open question in plants, and will vary with aspects of the mating system and ecology of the species. Social selection analysis presents a general empirical framework to investigate such questions, and provides standardized metrics that are directly comparable. Although studies of social selection have mostly been conducted in animal species, the approach is equally valid and useful when applied to plant species, especially with respect to competition for resources or sunlight, herbivory, and pollination.

Acknowledgements

We thank C. and L. Burk for permission to conduct this experiment in their pasture. A. Berardi, H. Edgington, V. Formica, N. Forrester, R. Hallock, T. Park, M. Pollack, D. Racke, M. Sebasky, E. Wice, and C. Wood assisted with fieldwork. We are grateful to J. Antonovics, L. Galloway, the members of the Brodie-Cox lab group, and two anonymous reviewers for comments on drafts of this manuscript. Funding was provided by National Science Foundation Doctoral Dissertation Improvement Grant number 1407155 to B.J.S. and E.D.B. III, National Science Foundation grant number 1027251 to E.D.B. III, and

Margaret Walton Scholarships from Mountain Lake Biological Station to B.J.S. and M.E.A.

Data Accessibility

Python and R scripts, GPS and phenotypic data are deposited in Dryad: http://dx.doi.org/10.5061/dryad.c06n3

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Figures and Tables

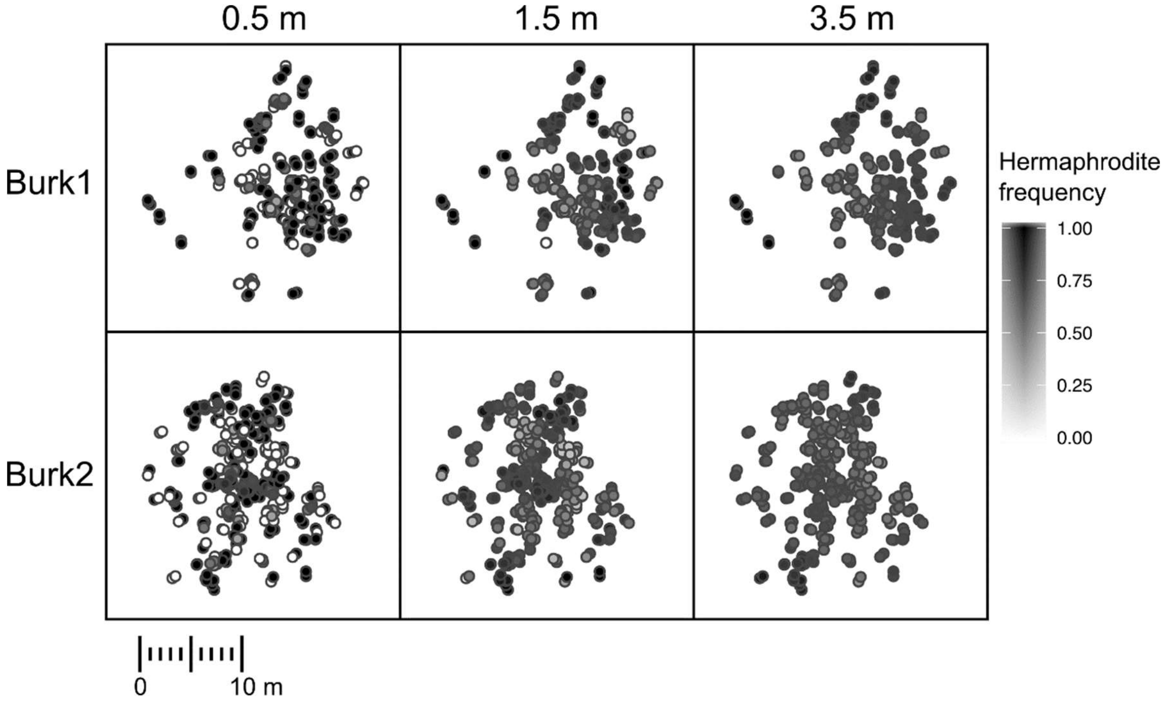

Figure 1. Locally-experienced hermaphrodite frequency for all individuals in two wild populations at different spatial scales. Each point represents an individual, and the shading of the point represents the subjective local frequency of hermaphrodites for that individual assessed at radii from 0.5 m, 1.5 m, and 3.5 m. Lighter shading represents individual plants that experienced a low frequency of hermaphrodites, darker shading represents individuals that experienced a high frequency of hermaphrodites. The X- and Y-axes are arbitrary Euclidian distance in m, with the scale as indicated by the scale bar.

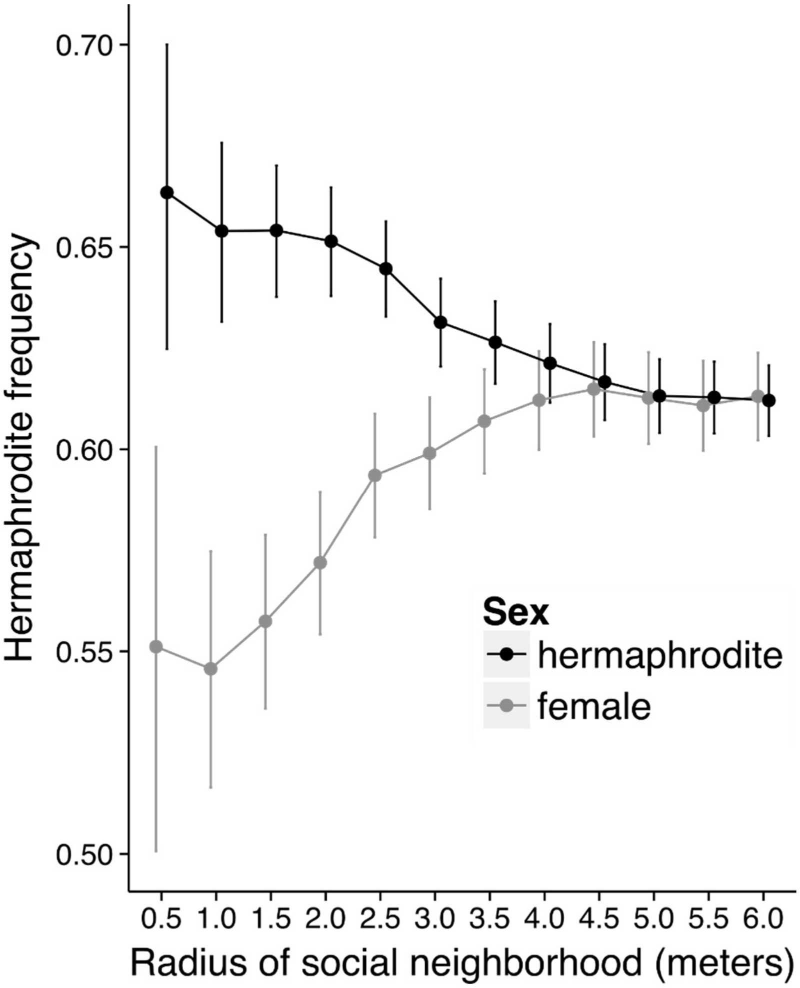

Figure 2. Hermaphrodite frequency experienced by hermaphrodites (black) and females (gray) across spatial scales measured in radii from 0.5 m – 6.0 m. The points are least square means of hermaphrodite frequency regressed on sex, radius of spatial scale, the sex-by-scale interaction as fixed effects, and plant ID as a random effect. Lines represent 95% confidence intervals of the LS means.

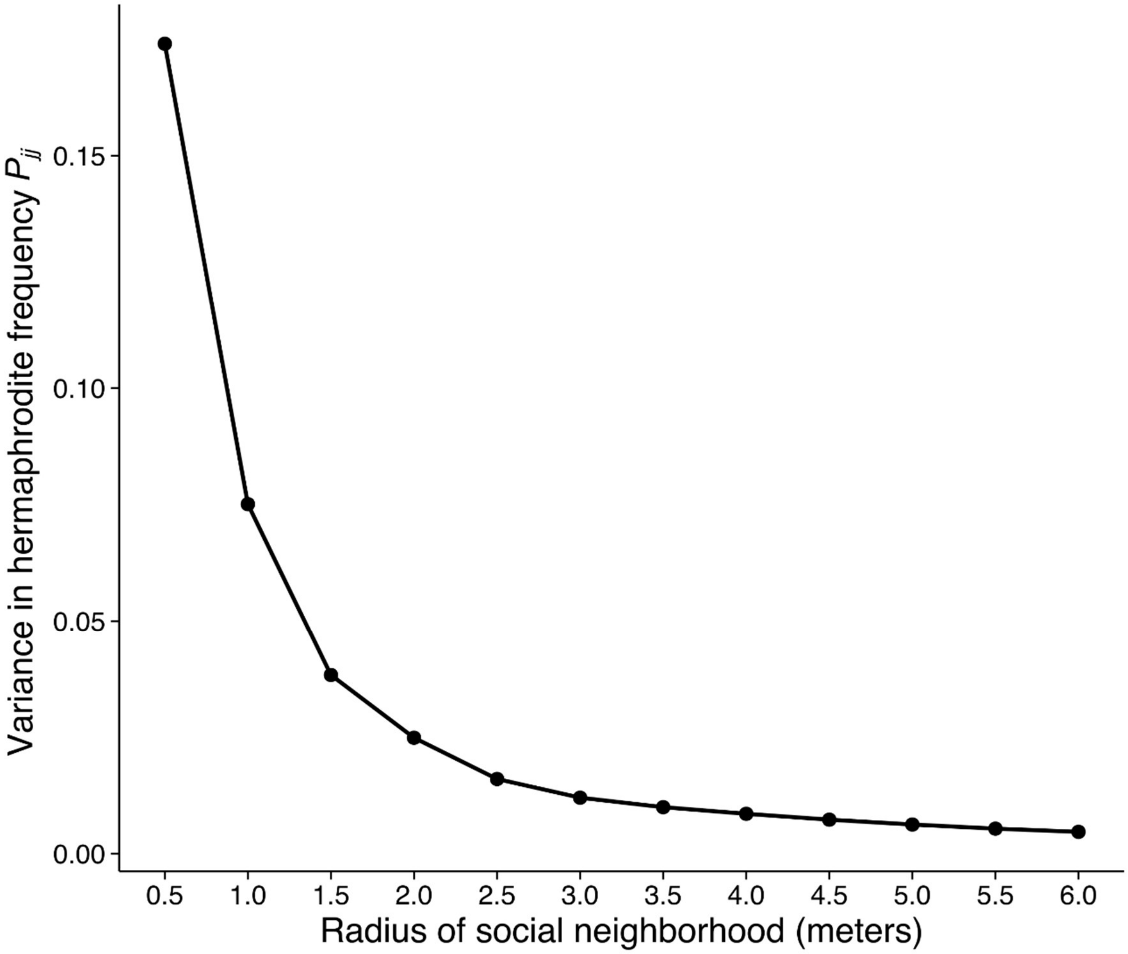

Figure 3. Variation in local hermaphrodite frequency decreases continuously with increasing spatial scale. Each point represents the variance in local hermaphrodite frequency assessed at spatial scales along the X-axis from 0.5 m – 6.0 m in 0.5 m steps.

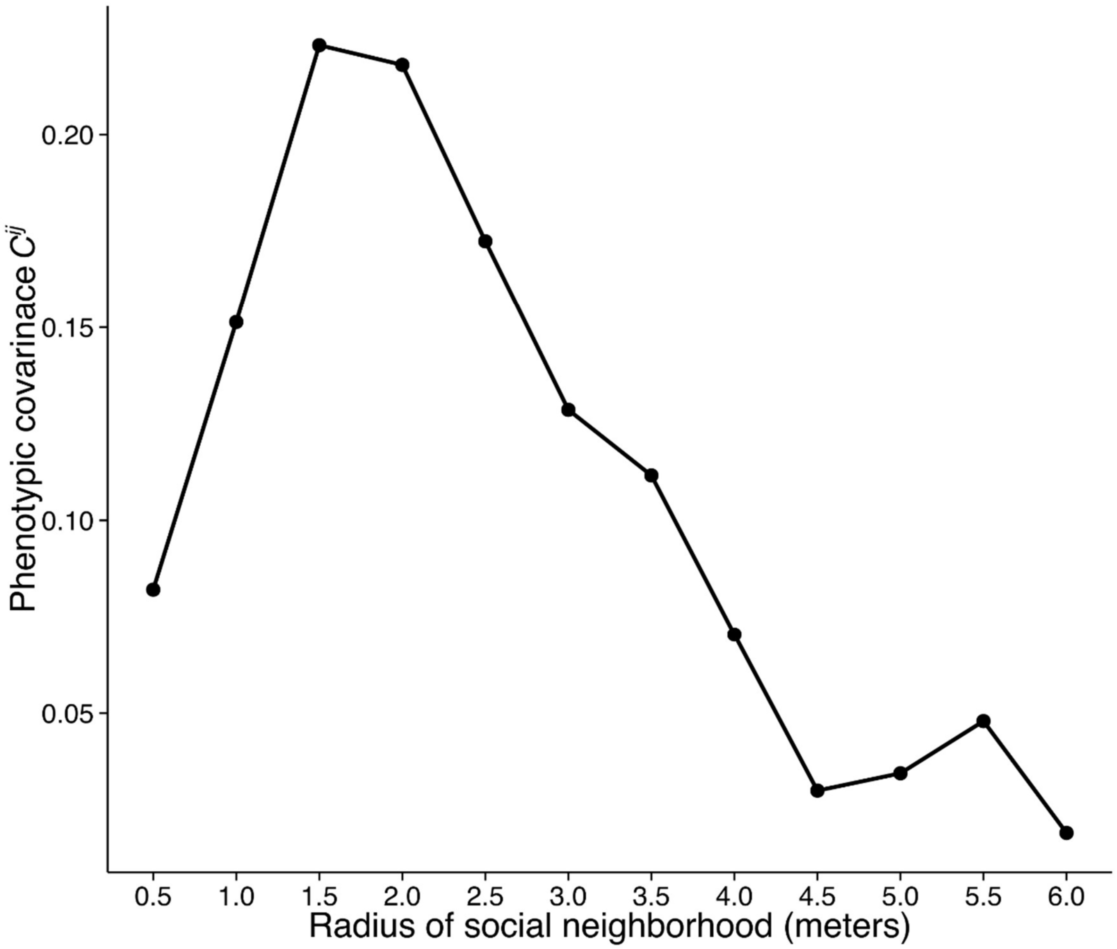

Figure 4. Phenotypic covariance decreases with increasing spatial scale. Each point represents the variance-standardized covariance between the sex of individuals and the local frequency of hermaphrodites assessed at spatial scales along the X-axis from 0.5 m – 6.0 m in 0.5 m steps.

Table 1

Mean and variance of sex ratio and number of social partners experienced by individuals across spatial scales measured as radii from 0.5 m to 6.0 m

Scale (m)	Mean hermaphrodite frequency	Variance in hermaphrodite frequency	Mean number of neighbors
0.5	0.630	0.174	2.0
1.0	0.611	0.080	5.8
1.5	0.622	0.043	11.9
2.0	0.621	0.027	20.0
2.5	0.625	0.018	30.0
3.0	0.621	0.014	41.5
3.5	0.621	0.011	54.2
4.0	0.618	0.009	68.5
4.5	0.615	0.007	83.8
5.0	0.613	0.006	99.3
5.5	0.612	0.006	116.1
6.0	0.611	0.005	133.1

Table 2

Type-III sum of squares test of significance for generalized linear mixed model of hermaphrodite frequency on sex, spatial scale, and the sex-by-scale interaction.

Parameter	χ^2	df	P-value
Sex	12.39	1	< 0.001
Spatial scale	95.70	11	< 0.001
Sex × scale	205.27	11	< 0.001

Table 3

Correlation matrix of local hermaphrodite frequencies across spatial scales. Rows and columns are the radius of the spatial scale, and values within are the correlation between the hermaphrodite frequencies individuals experience across scales indicated by the column and row pair. Cells are shaded to highlight the strength of the correlation; darker shading represents a higher correlation.

	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
0.5	1.00	0.70	0.56	0.49	0.39	0.31	0.23	0.18	0.12	0.12	0.12	0.08
1.0		1.00	0.78	0.65	0.55	0.45	0.38	0.32	0.24	0.22	0.21	0.17
1.5			1.00	0.83	0.69	0.58	0.48	0.42	0.33	0.29	0.26	0.23
2.0				1.00	0.86	0.75	0.64	0.55	0.43	0.38	0.33	0.29
2.5					1.00	0.88	0.78	0.68	0.56	0.50	0.46	0.40
3.0						1.00	0.90	0.81	0.70	0.63	0.56	0.49
3.5							1.00	0.91	0.82	0.73	0.67	0.58
4.0								1.00	0.93	0.85	0.77	0.69
4.5									1.00	0.94	0.87	0.80
5.0										1.00	0.94	0.86
5.5											1.00	0.94
6.0												1.00

Supplemental Methods

To estimate how the patterns of phenotypic covariance we measured might impact sex ratio evolution, we can use sex as the trait of interest (with hermaphrodites coded as 1 and females coded as 0) and estimate the natural and social selection differentials. We assigned values for fitness to each individual (1 for hermaphrodites, and 2 for females). In order to obtain standardized selection gradients we variance-standardized the index of sex, and obtained the values of relative fitness for each individual (0.73 for hermaphrodites, and 1.45 for females). We then regressed relative fitness on the standardized sex index, resulting in a value of $\beta_N = -0.35$. Because we are estimating a standardized selection gradient, the trait variance P_{ii} = 1, and so the resulting natural selection differential is $P_{ii}\beta_N = -0.35$.

To illustrate how the scale of social interactions affects the fitness consequences of sex, we can estimate the magnitude of social selection that would be necessary to ameliorate this female advantage. For this species it is advantageous for both hermaphrodites and females to be surrounded by hermaphrodites, and so we expect that β_S would be positive. At the 1.5 m scale we calculated a phenotypic correlation of $C^{ij'} = 0.21$, and so at that scale when the magnitude of social selection is $\beta_S = 1.66$ the natural and social selection differentials cancel out, and there is no difference in net selection on sex despite a two-fold fitness advantage in females. At the 6.0 m scale $C^{ij'} = 0.025$, and so when pollinators move across very large scales in the population the strength of social selection would need to be 10 times greater to overcome female advantage.

CHAPTER 2:

The effective pollinators of *Silene vulgaris* do not respond to variation in sex ratio[2]

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[2] Formatted as a co-authored manuscript: Sanderson, B.J., Augat, M.E., Enriquez, A.C., & Brodie III, E.D.

Abstract

Fitness in many plants depends on the transmission of pollen to receptive mates by pollinators. Sex ratio may determine the apparent reward status of a population because it determines the frequency of pollen-bearing flowers. If insects exhibit preferences for the sex of individual plants or for the sex ratio of aggregations of plants, those preferences might exaggerate or counteract the effects of pollen limitation or pollen competition on individual fitness. In this study we use experimental populations of the gynodioecious plant *Silene vulgaris* to demonstrate that while the recruitment of diurnal pollinators is affected by sex ratio, nocturnal pollinators do not exhibit preferences for either sex or sex ratio. We further demonstrate that only the nocturnal insects are effective pollinators. Taken together, these results suggest sex ratio is unlikely to generate selection on individuals through differences in pollinator behavior, and that whatever traits of individuals and groups of plants favor the visitation of nocturnal pollinators are the most likely to be linked with reproductive success.

Keywords: pollinator behavior, pollinator effectiveness, sex ratio, gynodioecy

Introduction

Fertilization in the majority of angiosperm species depends on the recruitment and behavior of pollinators, and the effective transmission of pollen to receptive flowers by those pollinators (Ollerton et al. 2011). Pollinator recruitment to individuals may depend on many factors, including the overall reward status of the population and the relative reward status of the individual compared to others within the patch (Kessler & Halitschke 2009; Knauer & Schiestl 2015). As a result, the fitness of individuals will likely be strongly affected by not only their own phenotypes, but also the phenotypes of their neighbors. In addition, flowers may be visited by a variety of potential pollinators that vary in their effectiveness, from insects that are pollen- or nectar-robbing to those that are completely effective at transferring pollen to receptive flowers (Schemske & Horvitz 1984). Understanding the fitness consequences of sexual traits in plants therefore requires an understanding of whether and how the recruitment and behavior of pollinators is affected by the frequency of those phenotypes in a population, and the extent to which this translates into differences in the effectiveness of pollen transfer.

For sexually polymorphic plants, the sex ratio of a patch is a critical context in which reproduction occurs (Ashman & Diefenderfer 2001). The ratio of different sexes in the patch can determine the reward status of the assemblage of plants, which can influence the frequency, abundance, and type of pollinators that visit (Burd 1995; Shykoff & Bucheli 1995; Eckhart 1999; Kessler & Halitschke 2009; Knauer & Schiestl 2015). For example, pollinators that are searching for pollen rewards may be more drawn to assemblages with a high frequency of pollen-bearing flowers (hermaphrodites or males), while nectar-seeking pollinators may be less responsive to the abundance of pollen. If pollinators exhibit this type of discrimination between sexual morphs, then the sex ratio of the patch may also determine the frequency with which individuals are visited by particular pollinators. When the preferred morph is rare, pollinators may forage on the less-preferred morph more frequently than if the preferred morph were common, because the optimal foraging strategy in a low-status patch may differ from that of a high-status patch (Goulson 1999). Alternatively, those pollinators may abandon a patch prematurely when the preferred morph is rare.

In addition to determining the assembly of pollinators available in a patch, the sex ratio will also potentially have large effects on the strength and form of selection on attractive traits (Ashman & Diefenderfer 2001). When seed set is pollen limited, there will be stronger selection on attractive traits through female fitness because the greatest variance in mating success among individuals is being driven by differences in the receipt of the limited amount of available pollen. The frequency of pollen-bearing flowers in the population is one factor that determines the degree to which plants are pollen limited. Conversely, when seed set is not pollen limited there will be stronger selection on traits through male fitness, because the greatest variance in mating success is no longer among which flowers convert to fruit, but rather which among the abundant pollen grains will successfully fertilize those flowers.

The fitness effects of sex ratio have been demonstrated in myriad studies in plant populations (Barrett & Thompson 1982; House 1992; Aizen 1997; McCauley & Brock 1998; Olson et al. 2005; Bartkowska et al. 2014). In addition to differences in sex ratio among populations, there can also be large within-population variation in sex ratio can lead to local and heterogeneous sex ratio bias among individuals (Sanderson et al. 2016). This type of fine-scale variation among individuals in a social phenotype, such as sex ratio, can result in social selection in which the phenotypes of social partners alters the strength and form of selection on an individual's phenotype (Wolf et al. 1999). How and whether local variation in sex ratio will affect individual fitness in plant populations depends on the behavior of pollinating insects. Specifically, if pollinators exhibit preferences for sex or sex ratio then the potential social selection arising from nearest neighbors is likely to be ameliorated. For example, a hermaphrodite surrounded by females may not enjoy elevated fitness through monopolization of those females if the pollinators exclusively visit hermaphrodites. Conversely, a female surrounded by other females might not experience pollen limitation if pollinators move great distances. Whether pollinators exhibit preferences for sexes, the distance that pollinators travel, and how or whether these individual-level preferences are affected by group composition, will determine whether and how selection arising from social context affects the fitness of individuals.

Visitation of flowers by insects is not the only factor influencing fertilization. When flowers are open for extended periods throughout the day they may be visited by numerous animals, only some of which may be effective pollinators. If a pollinator is both the most frequent and the most efficient we expect specialization to evolve (i.e. Stebbins 1970). However, when there are differences between the pollinators that are the most frequent and those that are the most efficient, the combined actions of those pollinators may result in opposing selection (Schemske & Horvitz 1984; Petterson 1991; Kwak 1993; Olsen 1996; Gómez & Zamora 1999). This type of mismatch between pollination syndrome and effective pollination may even occur when flowers seem particularly specialized: although the red, tubular flowers of *Ipomopsis aggregata* are most frequently visited by hummingbirds (which is expected from the pollinator syndrome), the fruits set by bumblebees set on average four times more seed (Mayfield 2001). Thus, understanding not just the behavior of pollinators, but also the fitness consequences of pollinator visitation, is critical for understanding how and whether the sex ratio of the patch can affect the fitness of individuals within the patch.

In this study we used experimental populations of the gynodioecious plant *Silene vulgaris* to assess 1) whether and how sex ratio affected the recruitment and behavior of pollinating insects and 2) whether there were differences in the effectiveness of pollinators. We demonstrate that diurnal pollinators were more abundant in populations with higher frequencies of hermaphrodites, while nocturnal pollinator abundances did not respond to sex ratio. Further, we found that although plants were visited much more frequently during the morning, the majority of fruit set occurred as a result of nocturnal pollination. Taken together, these results suggest that sex ratio variation, among and within populations of *Silene vulgaris,* is unlikely to affect the visitation of effective pollinators. Therefore, any fitness effects arising from sex ratio will be due to differences in the abundance of pollen, and not the behavior of pollinators. Furthermore, the traits that facilitate pollen dispersal by nocturnal insects are likely to be under the strongest selection.

Materials and Methods

Study system

Silene vulgaris (Caryophyllaceae) is a gynodioecious perennial herb that grows predominantly along roadsides in its introduced range in North America (Taylor & Keller 2007). As is common in gynodioecious species, sex is determined by cytonuclear interactions: mutations in the mitochondria or chloroplast result in the abortion of anthers, resulting in functionally male-sterile (i.e. female) plants, while nuclear-encoded proteins can counteract those cytoplasmic mutations, resulting in hermaphroditic plants (Frank 1989; Charlesworth & Laporte 1998; Taylor et al 2001). There is tremendous variation in sex ratio among populations, ranging between 30% - 100% hermaphrodite (McCauley et al. 2000). Additionally, there is significant variation in sex ratio at a fine scale within populations, such that there is the potential for continuously varying social environments among individuals (Sanderson et al. 2016). Flowers are open throughout the day and night, and are visited both by diurnal (syrphid flies and bumble bees) and nocturnal insects (noctuid and sphingid moths), although nocturnal pollinators have been hypothesized to be the 'evolutionarily important' pollinators (Marsden-Jones & Turril 1957; Pettersson 1991; Stone 2013). Seeds are passively dispersed when fruits dehisce, which may explain the high degree of genetic and phenotypic structuring of populations (Olson et al. 2006).

Pollinator recruitment and behavior

In July 2014 we established three experimental populations to assess whether pollinators exhibited a preference for the sex of individual plants or the sex ratio of aggregations of plants. These populations were located at the Mountain Lake Biological Station in Giles County, VA, and were surrounded by 5m × 5m deer netting to exclude herbivores. The plants used for this study were grown from wild-collected seed in a greenhouse at the University of Virginia a year prior to this study. Flowering plants were randomly permuted through these populations into treatment groups composed of low (30±4%), mid (70±4%) and high (92%) frequency of hermaphrodite plants. The variation in the low and mid treatment groups was due to the re-flowering rate of the limited number of female plants available for the study. Sex ratio treatments were randomly assigned to each population, and 24 plants were randomly permuted among treatment groups every three days. This process was repeated eleven times, and involved a total of 110 plants. Plants were arrayed in a hexagonal grid, with a distance between nearest neighbors of 0.83 meters, and an average inter-plant distance of 2.59±1.21 meters.

Audio narration of behavioral observations was recorded in each of the three populations simultaneously by three observers, each morning from 10:00 - 10:40 and every evening from 20:50 - 21:30, using Sony ICD-AX412 audio recorders (Sony Electronics Inc, San Diego, CA). When a new pollinator entered the population it was identified at a coarse level (e.g. bumble bee, syrphid fly, moth), and its behavior was recorded using a simple ethogram: searching the population, scanning individual plants, and landing on the flower of a plant. Pollinator behaviors were recorded daily from July

13th – July 29th and August 4th – August 16th, 2014, for a total of 174 observations. The audio files from these observation periods were transcribed to XML files using the program Transcriber (Barras et al. 2001). The XML files were collated into a long-form data table with a custom Python script. The resulting data table included the time stamp and duration in seconds for all observations of pollinators searching for plants in the population, scanning flowers of individual plants, landing on the flowers of individual plants. All of the following analyses were performed using R version 3.2.2 (R Core Team 2015).

Statistical analysis of pollinator behavior

To determine whether the hermaphrodite frequency of the population affected the number of pollinators that visited, or the amount of time pollinators spent in the population, we used linear mixed models implemented by the lmer function in the package lme4 (Bates et al. 2015). These analyses included the time of day (morning or evening), the hermaphrodite frequency of the population, the interaction between these two factors, and the total number of open flowers on all plants as fixed effects, and the date of observation as a random effect. Prior to analysis, the duration of pollinator visits was natural-log transformed to improve normality.

To determine whether and how pollinator recruitment and behavior was affected by the sex of individuals or aggregations of plants, we used generalized linear mixed models implemented by the lmer and glmer functions in the package lme4 (Bates et al. 2015). We ran four separate models to assess different components of pollinator behavior. The dependent variables in these models included whether an individual was visited by any pollinators during an observation period (0 or 1), the number of times an individual was visited, the duration in seconds of pollinator visits, and the straight-line distance in meters that the pollinator moved from the previous plant it had visited. These analyses included the sex of the plant, the hermaphrodite frequency, time of day (morning or evening), all factor interactions, and flower number as fixed effects, and the ID of the plant and the date of observations as random effects. Prior to analysis, the number of pollinator visits, and the duration of visits were natural-log transformed to improve normality.

Pollinator effectiveness

In June 2015 we established two experimental populations to determine whether there were differences in effectiveness between diurnal and nocturnal pollinators. These populations were located approximately 6.5 km from the previously described experimental populations, along a wildlife improvement strip on Salt Pond Mountain in Giles County, VA. Five hermaphrodite and five female plants were randomly assigned to each of these populations. All flowering stems on each plant were split into four treatment groups: 1) open from 7:00 AM - 7:00 PM, 2) open from 7:00 PM - 7:00 AM, 3) always open (positive control), and 4) never open (negative control). Pollinators were excluded from treatments during "closed" periods using tulle fabric bags that covered the entire cluster of stems. The plants were returned to the greenhouse after six days, and all flowers that were open during the trial were marked with cotton thread tied around the pedicle. Fruits developed on the plants in the greenhouse until mature, and then we counted the number of mature and aborted fruits. This experiment was replicated five times, for a total of 50 hermaphrodite and 50 female plants.

Statistical analysis of pollinator effectiveness

To determine whether diurnal and nocturnal pollinators differed in effectiveness we used a generalized linear mixed model implemented by the glmer function in the package lme4 (Bates et al. 2015). In this analysis we regressed fruit set (mature fruits / open flowers) on the sex of plants, the treatment group, and the interaction of sex and treatment as fixed effects, and the plant ID as a random effect. We specified a binomial error distribution with a logit link, weighted by the number of flowers.

For all of the above analyses we assessed whether model assumptions were violated by visual inspection of the residual values and a plot of predicted values as a function of the residual values. Hypothesis testing was performed using Wald's χ^2 implemented in the Anova function in the package car, specifying Type-III sum of squares (Fox & Weisberg 2011). To determine the significance of differences between combinations of categorical variables we performed a Tukey post-hoc analysis using the glht function in the package multcomp (Hothorn et al. 2008).

Results

The insects observed during the morning observation periods included a number of species of bumble bees and syrphid flies, while the predominant insects during the evening observation periods included species of noctuid and sphingid moths. The hermaphrodite frequency of the experimental populations affected the recruitment of morning, but not evening pollinators; there were nearly twice as many morning pollinators in the mid and high hermaphrodite frequency populations as in the low hermaphrodite frequency populations (Table 1; Figure 1A). Evening pollinators spent a larger amount of time visiting all of the populations, though this did not differ between the hermaphrodite frequency treatments (Table 1; Figure 1B).

At the plant level, pollinators did not show a consistent preference for either the sex of the plants or the frequency of hermaphrodites in the population, assessed through any of the components of behavior that we quantified (Table 2). Individuals were two to three times more likely to be visited in the morning than in the evening (Figure 2A-2B). Further, individuals were visited by a greater number of pollinators in the morning than in the evening (Figure 2C-2D). The average bout length of an evening pollinator visit was almost twice as long as those of morning pollinators (Figure 2E-2F). Finally, the average distance that pollinators traveled between plants did not differ between morning and evening pollinators (Table 2).

Despite the larger number of morning pollinators that aggregated in the populations, and the more frequent visitation of morning pollinators to individuals, evening insects account for the majority of fruit set. Flowers exposed only to evening insects had the same probability of setting fruit as flowers that were always open, while flowers exposed only to morning insects had a fruit set to proportional to that of flowers that were continuously bagged (Table 3; Figure 3). The fruit set of female plants was nearly double that of hermaphrodite plants in flowers exposed to evening insects.

Discussion

Reproduction in many plants depends on the recruitment of pollinators, and that the pollinator service transfers pollen to receptive mates. Because the frequency of pollen-bearing flowers might alter the apparent reward status of a population, we predicted that the hermaphrodite frequency of experimental populations of *Silene vulgaris* would affect the recruitment and behavior of pollinators. We found that hermaphrodite frequency did affect the recruitment of morning pollinators—there were nearly twice as many diurnal pollinators in experimental populations that contained 60% or more hermaphrodites. This effect of hermaphrodite frequency was not seen in nocturnal pollinators, however, which were equally abundant across all treatments. There were significant differences in effectiveness between diurnal and nocturnal pollinators—nearly all of the fruit set that we observed was due to the visitation of nocturnal insects. Therefore, although diurnal pollinators exhibit preferences for populations with a higher frequency of hermaphrodites, those preferences are unlikely to generate selection on individuals because diurnal insects are not effective pollinators in this species. Differences in fitness among individuals that result from variation in hermaphrodite frequency, then, will be driven by the severity of pollen limitation or pollen competition due to the frequency of pollen-bearing flowers in the population, and not the behavior of pollinating insects.

A number of recent studies have found similar effects of sex ratio on diurnal pollinator recruitment. In *Geranium maculatum*, bee and fly pollinators visited hermaphrodite plants more frequently than females, and this preference became more pronounced when females were rare (Van Etten & Chang 2014). Bee and fly pollinators of *Fragaria virginiana* showed similar preferences for pollen-bearing flowers: insects were present in greater abundances when hermaphrodites were more common (Ashman and Diefenderfer 2001). And in hummingbird-pollinated *Lobelia cardinalis*, both the sex ratio and density of local patches significantly affected visitation rate and rates of pollen transfer (Bartkowska & Johnston 2014).

Diurnal and nocturnal pollinators often differ in their preferences for floral rewards, and in their foraging behavior. These differences might explain the difference in responsiveness to sex ratio and the effectiveness of pollination that we observed between the two classes of insects. Diurnal pollinators, such as bumble bees and syrphid flies, often seek both nectar and pollen as rewards. Moths, on the other hand, only seek nectar rewards and lack the appropriate mouth parts to consume pollen (Barthelmess et al. 2006). Thus, the higher abundance of diurnal pollinators in populations with a higher frequency of hermaphrodites may be driven by the preference of those insects for pollen rewards. The greater effectiveness of nocturnal pollinators that we found here is similar to that seen in *Lonicera japonica*, in which bees consumed more pollen than they transferred, such that moths were much more effective at setting fruit (Miyake & Yahara 1998). If diurnal insects are similarly removing pollen and consuming it in *S. vulgaris*, the preference they exhibit for a high frequency of hermaphrodites may have negative fitness consequences, because hermaphrodites in those populations would be losing a greater proportion of their pollen to ineffective pollinators than in populations where hermaphrodites are rare and diurnal insects are less common.

Relatively little is known about pollination services provided by moths compared with other types of insect pollinators. A recent literature review found that although there are many studies that have demonstrated that moths are frequent visitors to plants, few have demonstrated that moths are effective pollinators (Hahn & Brühl 2016). Interestingly, one of the few studies to have robustly estimated moth pollination service was performed in *Silene vulgaris.* That study demonstrated that over the course of four seasons there was a fluctuating guild of diverse species of noctuid and sphingid moths that pollinated *S. vulgaris*, and that in general multiple visits by these moths were required to deliver sufficient pollen to achieve full seed set (Pettersson 1991). This type of temporal variation in the abundance and communities of moth pollinators is seen generally across ecosystems (Hahn & Brühl 2016). The lack of broadly available information about moth pollination service makes it difficult to extrapolate the results of this study. However, it is clear that nocturnal pollinators are the effective pollinators of this species, and so whatever traits are most attractive to those species are likely to be most closely linked with the fitness of individuals in *S. vulgaris*.

Some caution is warranted in interpreting the lack of an effect of sex ratio on pollinator behavior, due to the ephemeral nature of the sex ratio treatments. Plants were randomly permuted among populations every three days, such that both the sex ratio of the population as well as the identity of plants within the population changed. If the behavioral responses we predicted require learning and memory on behalf of pollinators, then we would not have been able to detect those responses. It is also possible that the variation in sex ratio that we used for our treatment groups (chosen to reflect natural variation in sex ratio among wild populations) was not extreme enough to elicit the predicted responses in pollinator behavior. Furthermore, it may be that pollinators are more sensitive to differences in density, or some interaction between density and sex ratio These results are qualitatively different from those previously reported in this species in Virginia, but they are similar to the results reported in a replicate study conducted in the Czech Republic (Stone 2013).

The results of this study have implications for the sexual selection and sex ratio evolution in gynodioecious species. Much theoretical and empirical work has been dedicated to understanding the role of female fitness advantages or hermaphrodite fitness costs due to pollen restoration in the evolution of gynodioecy (reviewed by Delph et al. 2007; Spigler & Ashman 2012). A relatively understudied factor, and one that is likely to affect most wild populations, is that of fine-scale spatial structure. Because populations of this species are highly structured there is fine-scale heterogeneity in the distribution of the sexes. This type of heterogeneity in a social phenotype generates the opportunity for social selection to drive differences in fitness among individuals at a fine spatial scale. In a previous study we demonstrated that even if females enjoyed a two-fold advantage through fruit or seed set, social selection arising from the phenotypic distribution of wild populations would be sufficient to ameliorate this fitness advantage (Sanderson et al. 2016). Because the effective pollinators do not exhibit differences in preference or behavior with respect to either the sex of plants or the sex ratio of aggregations of plants, local pollen limitation or pollen competition are likely to generate differences in fitness and selection among individuals within a population. Therefore, a greater understanding of how fine-scale sex ratio drives selection through both male and female components of fitness should provide novel insight into how social selection maintains this sexual polymorphism in wild populations.

Acknowledgements

We thank C. Alencar, A. Brumbaugh, R. Costello, K. Escoto, V. Formica, C. Thomason, and C. Wood for assistance with behavioral observations, and the George Washington and Jefferson National Forests for permission to conduct this research. Funding was provided by National Science Foundation Doctoral Dissertation Improvement Grant number 1407155 to B.J.S., National Science Foundation grant number IOS-1027251 to E.D.B. III, and Margaret Walton Scholarships from Mountain Lake Biological Station to B.J.S. and M.E.A.

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Figures and Tables

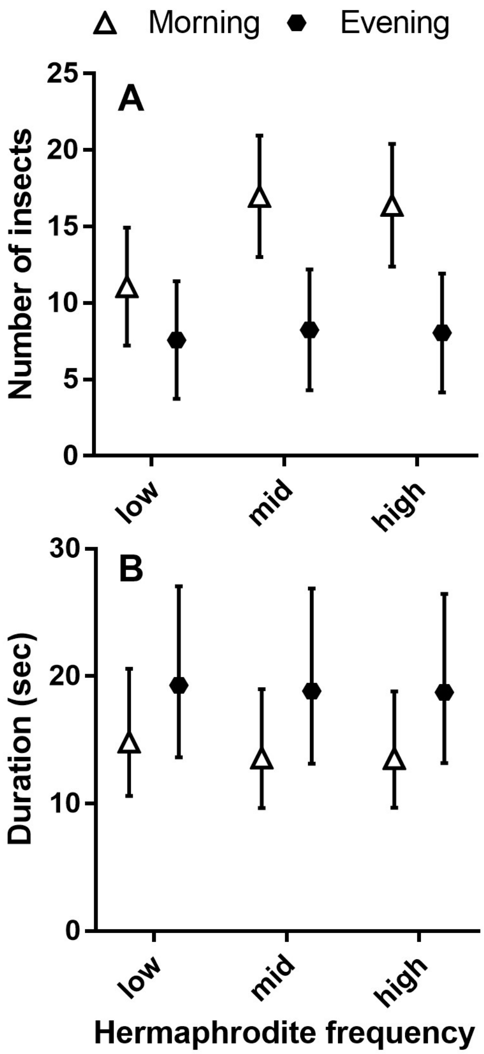

Figure 1. Population-level effects of sex ratio on pollinator behavior. Points are least square means of number of insects (A), or the duration of an insect's visit (B) regressed on the sex ratio of the population, the time of day, and the sex-ratio-by-time interaction, and flower number as fixed effects, and the date as a random effect. Lines represent 95% confidence intervals of the LS means.

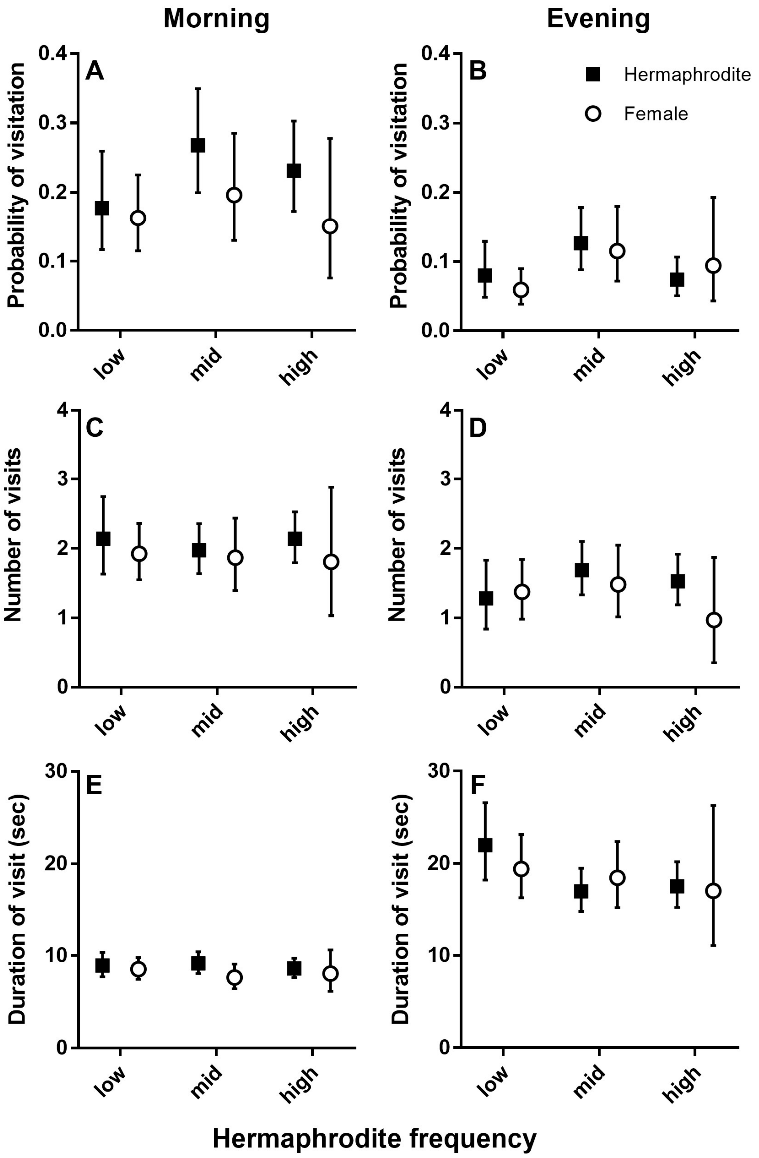

Figure 2. Plant-level effects of sex ratio on pollinator behavior. Points are least square means of the probability an individual is visited (A & B), the number of insects that visited an individual (C & D), or the duration of visits to individuals (E & F) regressed on the sex of the plant, the sex ratio of the population, the time of day, all pair-wise and three-way interactions, and flower number as fixed effects, and plant ID and date as random effects. The lines represent 95% confidence intervals of the LS means.

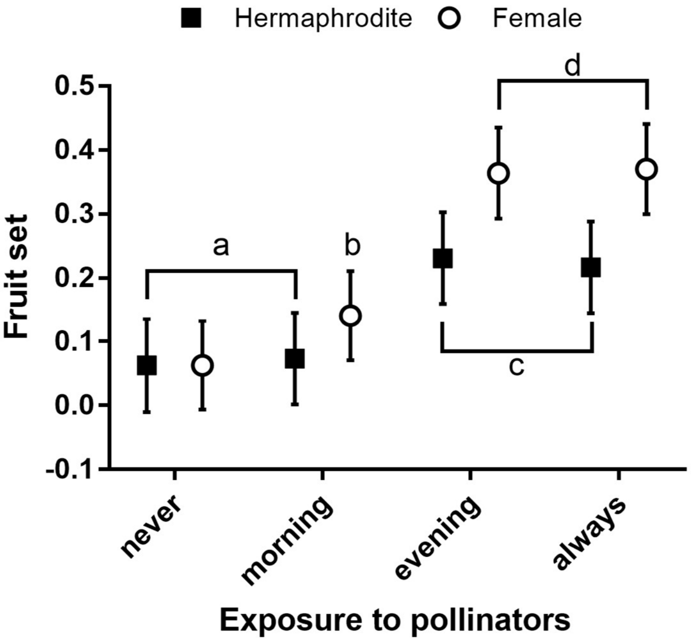

Figure 3. Differences in pollinator effectiveness throughout the day. Points are least squares means of fruit set (proportion of flowers that set fruit) regressed on the sex of the plant, the pollinator exposure treatment, the sex-by-treatment interaction as fixed effects, and plant ID as a random effect. The lines represent 95% confidence intervals of the LS means. Lower case letters represent a Tukey post-hoc analysis for differences between factor levels: groups a, c, and d significantly differ from each other, while b is not significantly different from either a or c.

Table 1

Summary of Wald χ^2 values of Type-III sum of squares hypothesis tests for general linear mixed models of number of insects recruited to populations and the amount of time insects spent in the populations regressed on the sex ratio of the population, the time of day, the sex-ratio-by-time interaction, and the total number of flowers in the population. The date of observation was included as a random effect. Emboldened values are significantly different from zero (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Source	df	Number of insects	Visit duration
Sex ratio	2	**7.25 ***	0.04
Time	1	**40.08 *****	**5.57 ***
Flower number	1	**4.05 ***	1.66
Sex ratio × time	2	4.86	0.13

Table 2

Summary of Wald χ^2 values of Type-III sum of squares hypothesis tests for generalized linear mixed models of the probability insects visit individuals, the number of insects that visit individuals, the duration of individual visits, and the distance traveled between mates, regressed on sex, the sex ratio, the time of day, all pair-wise and three way interactions, and flower number. The ID of the plant and the date of observation were included as random effects. Emboldened values are significantly different from zero (* P < 0.05, ** P < 0.01, *** P < 0.001).

Source	df	Prob. visit	Visit num.	Duration	Distance
Sex	1	0.17	0.49	0.35	0.53
Sex ratio	2	1.14	0.08	1.84	1.25
Time	1	**31.50** ***	**4.64** *	**101.95** ***	2.57
Flower number	1	**169.14** ***	**97.94** ***	0.78	3.12
Sex × sex ratio	2	1.32	0.17	1.78	0.18
Sex × time	1	0.42	0.50	0.42	0.02
Sex ratio × time	2	2.72	0.62	0.37	1.76
Sex × sex ratio × time	2	2.27	0.89	4.24	0.06

Table 3

Type-III sum of squares test of significance for general linear mixed model of fruit set regressed on sex, pollinator exposure treatment, and the sex-by-treatment interaction. The ID of the plant was included as a random effect.

Source	χ^2	df	P-value
Sex	2.33	1	0.127
Treatment	**90.62**	3	**< 0.001**
Sex × treatment	**11.18**	3	**0.012**

CHAPTER 3:

Sex ratio alters the strength and direction of selection on floral traits differently through male and female components of fitness in *Silene vulgaris*[3]

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[3] Formatted as a co-authored manuscript: Sanderson, B.J., Augat, M.E., & Brodie III, E.D.

Abstract

The sex ratio of a population defines the context in which sexual selection occurs because it determines the pool of available mates. Populations of gynodioecious plants typically exhibit extreme variation in sex ratio, which should result in variation in the fitness of individuals and also in the strength and direction of selection on floral traits. In this study we experimentally varied the sex ratio of *Silene vulgaris* and quantified selection through both the female and male components of fitness. We found that sex ratio strongly affected the fitness of individuals in the population through both male and female fitness components. Further, the strength and direction of selection on floral traits differed markedly across the sex ratios. Selection on flower size through fruit set was strongest when there was greater bias in sex ratio. Additionally, the direction of selection reversed across the sex ratio treatments—plants with the largest flowers had the highest fruit set when hermaphrodites were rare, while plants with the smallest flowers had the highest fruit set when hermaphrodites were common. However, there was no selection on flower size or shape through the male component of fitness. These findings present novel insight into how sex ratio creates a context for selection that contributes to the maintenance of sexual polymorphism in wild populations.

Keywords: Social selection, multilevel selection, gynodioecy, *Silene vulgaris*

Introduction

Social and sexual interactions are ubiquitous in nature, and yet studies of trait evolution rarely account for the fact that organisms are dynamically interacting with each other. When organisms interact socially or sexually, the phenotypes involved in those interactions are likely to experience selection arising from the phenotypes of social partners (Moore et al. 1997). Social selection is a form of multilevel selection that arises from social interactions within a population (Crook 1972; West-Eberhard 1979; Wolf et al. 1999). Social selection drives the evolution traits of involved in interactions such as aggression, courtship, and reproduction (Tsuji 1995; Eldakar et al. 2010; Formica et al. 2011). However, we still understand relatively little of the types of traits, and the conditions under which social selection is likely to act.

The study of social selection has largely focused on behavioral traits in animals, yet plants exhibit comparable social dynamics. The phenotypic qualities of neighbors have fitness effects on plants and drive selection arising from the height, stem number, and/or density of neighboring plants (Stevens et al. 1995; Kelly 1996; Aspi et al. 2003; Donohue 2004; Weinig et al. 2007). In these examples positive selection on an individual’s growth is countered by negative selection on overall stand size and/or apparency to herbivores (Agrawal et al. 2001; Weiner & Freckleton 2010). In animals, this type of selective interference can be ameliorated through phenotypic assortment: animals can behaviorally modify their social context by assorting with different individuals (Eldakar et al. 2010; Formica et al. 2011). Because plants are sessile it is likely that their ability to respond to selective interference through phenotypic assortment is limited, and thus the fitness consequences of social context may be more severe in plants than in animals.

The sex ratio of a population is a social context that is likely to affect fitness, and potentially also phenotypic selection, at multiple levels. First, the sex ratio of the population can affect the overall level of productivity of the population. The frequency of pollen-bearing flowers in the population can determine the degree to which fruit set is pollen limited, and so an increase in pollen-bearing flowers can increase the overall productivity of the population (McCauley & Taylor 1997; Olson et al. 2005). This relationship between variation in sex ratio and variation in fitness among populations creates the opportunity for inter-demic selection. Second, the sex ratio of a population can also affect the fitness of individuals within the population. The effects of pollen limitation or pollen competition that result from variation in sex ratio are likely to affect the sexes differently, and those effects may also vary among individuals depending on differences in sex allocation. (McCauley & Brock 1998; Bartkowska & Johnston 2014).

Finally, the sex ratio of the population may alter patterns of phenotypic selection on individuals within populations. The sex ratio of the patch will determine the extent of pollen limitation of fruit set and the degree of male-male competition for fertilization, and so the opportunity for sexual selection through both components of fitness is likely to vary with sex ratio (reviewed by Ashman & Diefenderfer 2001). For example, in a female-biased population fruit set is likely to be pollen limited, and so there will be greater variation among individuals in the female component of fitness than in the male component of fitness. This difference in the opportunity for selection between the sexes might to lead to differences in the form and magnitude of selection. Additionally, in hermaphroditic species, individuals experience different, and occasionally opposing, forms of selection through the male and female components of fitness, such that the strength and direction of phenotypic selection varies with differences in the functional gender expressed by individuals (Lloyd 1980; Campbell 1989; Delph & Ashman 2006). Selection on floral traits is likely to differ both among individuals that differ in their functional gender, but also as a result of variation in gender in the population.

Gynodioecious species, which maintain a sexual polymorphism between hermaphroditic and functionally female plants, exhibit large variation in sex ratio among populations, and as a result have a large opportunity for selection arising from sex ratio to drive different fitness consequences of sexual phenotypes. The theoretical and empirical consequences of sex ratio variation in gynodioecious species have been extensively explored, and have created strong predictions for the negative frequency-dependent fitness effects arising from sex ratio through the female components of fitness (Lewis 1941; Lloyd 1974; Charlesworth & Charlesworth 1978; McCauley & Brock 1998; Delph et al. 2007; Van Etten & Chang 2014). However, much less is known about the effects of sex ratio through the male component of fitness because of the difficulty with assigning paternity to plants in the wild (Spigler & Ashman 2012). Hermaphrodites have multiple, co-varying opportunities for fitness through pollen and seed, and so the context-dependent fitness consequences of sex ratio may also generate differences in sexual conflict through these two components of fitness (Ashman & Morgan 2004). An understanding of how and whether sex ratio results in differences in fitness and selection therefore requires the integration of both components of fitness.

In this study we used experimental populations of the flowering plant *Silene vulgaris* to determine whether and how the sex ratio of a population affects 1) the fitness of individual plants and hence the opportunity for sexual selection and 2) selection on floral characters through the male and female components of fitness. We quantified fruit set for all plants, and used microsatellite markers to assess realized paternity through pollen. We found that the sex ratio of the population has strong effects on fitness; both hermaphrodites and females enjoyed the highest fitness when they were the rare sex in the population. Further, the strength and direction of phenotypic selection on floral traits differed among the sex ratio treatments through the female, but not the male, component of fitness. Taken together, these results demonstrate how variation in sex ratio among populations of gynodioecious species serves as a context for selection on sexually polymorphic traits, and have implications for the evolution of those traits through the female component of fitness.

Materials and Methods

Study system

Silene vulgaris (Caryophyllaceae) is a gynodioecious, perennial herb that is broadly distributed through its introduced range in North America (Taylor & Keller 2007). Like most gynodioecious species, sex is determined by cytonuclear interactions: mutations in the mitochondria or chloroplast cause the abortion of anther development, resulting in functionally female plants, while genes in the nucleus counteract these mutations resulting in hermaphrodite plants (Frank 1989; Charlesworth & Laporte 1998; Taylor et al. 2001). Sex ratio varies greatly among populations, ranging from 30%-100% hermaphrodite (McCauley et al. 2000). There is also significant variation in sex ratio within populations, which creates the opportunity for social selection to drive differences in fitness among individuals at a fine scale (Sanderson et al. 2016). The species is visited by both diurnal (syrphid flies and bumble bees) and nocturnal insects (noctuid and sphingid moths), though only nocturnal insects are the effective pollinators (Chapter 2). Flowers in this species exhibit sexual size dimorphism; hermaphrodite flowers have larger calyces than female flowers, although female flowers have larger petals (Marsden-Jones & Turril, 1957; M. Augat, unpubl.).

Selection experiment

In June 2015, we established ten experimental populations along a wildlife improvement strip in the George Washington and Jefferson National Forests on Salt Pond Mountain near the Mountain Lake Biological Station in Giles County, VA. Each population was surrounded by 2 m × 2.5 m of deer netting to exclude herbivores. Populations were separated by 50 m to reduce the likelihood of gene flow between populations. The plants used in this experiment were grown from seed collected from a wild population at a greenhouse at the University of Virginia two years prior to this study. Plants were arranged in a rectangular array, with interplant distances of 0.5 m. Ten plants were assigned randomly to each population into treatment groups of 10%, 30%, 50%, 70%, and 90% hermaphrodite plants, with two replicates of each treatment. All open flowers were removed from the plants at the beginning of the experiment. Plants were then visited once a week for eight weeks. All open flowers were counted, and any developing fruits were marked with colored cotton thread tied around the pedicle to indicate on which week the developing fruit was first seen. We measured the calyx width, calyx length, petal width, and petal length from a single flower for each plant using calipers. After the experiment was completed, the plants were returned to a greenhouse at MLBS to allow the fruits to mature. Female fitness was assessed as the number of fruits that matured, and the proportion of flowers that set fruit. A leaf from each parental plant was collected for genetic paternity assignment.

Genetic analysis of relatedness

The majority of fruit set during this experiment occurred during the first four weeks, followed by a period of low flowering and fruiting for the subsequent four weeks. Therefore, we restricted our sampling of seeds to the first four weeks of the experiment, and all of our analyses through both the male and female components of fitness only included data from this period. Fruits were selected randomly, stratified by the frequency of hermaphrodites in the population of the parent plants (i.e., the sample contained a greater representation of fruits from populations with a high frequency of hermaphrodites, and a lower representation of fruits from populations with a low frequency of hermaphrodites). We chose this sampling scheme because hermaphrodites are the only plants capable of siring seed, and we wanted to ensure that all potential sires would be represented in our genotyped sample. In April 2016, 2,880 seeds were sown in Fafard 3B potting mix (Sun Gro Horticulture, Agawam, MA) in a greenhouse at the University of Virginia. Seeds were germinated on a mist bench, and when the seedlings reached the four leaf stage they were collected into 2mL deep 96-well plates (Greiner Bio-One, Monroe, NC), and stored at -20˚C.

DNA was extracted from dried and frozen tissue using a simple plate extraction method (Slotta et al. 2008). Briefly, a #BBB steel bead (Ballistic Products, Corcoran, MN) was added to each well of the 2mL 96-well plate and the tissue was homogenized using a Qiagen TissueLyser II (Qiagen, Valencia, CA). The homogenized tissue was incubated in 400 μL of an SDS extraction buffer (Edwards et al. 1991). DNA was precipitated from the supernatant using isopropanol, washed with 70% ethanol, and rehydrated with sterile water. All samples were genotyped at 10 microsatellite loci (Supplementary Table 1). PCR was performed using Qiagen's Multiplex PCR kit and multiplex protocol (Qiagen 2010), and capillary fragment analysis was performed at Yale's DNA Analysis Facility. Microsatellite genotypes were scored using the program GeneMarker (SoftGenetics, State College, PA). Paternity assignment was performed for all individuals with genotypes at 6 or more loci (1457 offspring, 98 parents) using full likelihood scores from COLONY version 2.0.6.1 (Jones & Wang 2010, Wang 2016). This included all seedlings that germinated (roughly 51% of the seeds sown) and all but two parents. In this analysis we specified that polygamy could occur in both sires and dams, that the mating system was monoecious to allow for selfing, provided the identity of the dam for all offspring, and excluded female plants from the list of potential sires. We included estimates of null allele frequencies calculated using the program Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004). COLONY assigned paternity to 718 offspring with a confidence of 80% or higher. The low assignment rate is likely due to a large degree of shared heterozygosity among parental and offspring plants, potentially due to the genetic structure of the source population of the parental plant.

We quantified male fitness by determining the projected number of seeds an individual hermaphrodite would have sired based on our genetic sample. We first divided the number of seeds assigned genetic paternity to each sire by the total number of seeds assigned paternity in the population, resulting in a proportion of seeds sired for each hermaphrodite. We multiplied this proportion by the number of fruits that we collected in the population, and the resulting value is the projected number of seeds sired that we use in the following analyses.

Statistical analysis

All of the following analyses were performed using R version 3.3.0 (R Core Team 2016). To determine whether sex ratio affected the overall productivity of the population, we regressed the total number of fruits that developed during the experiment on the sex ratio of the population using the lm function. To determine whether and how the sex ratio of the population affected the fitness of individuals, we used generalized linear mixed models implemented by the glmer function in the package lme4 (Bates et al. 2015). To quantify the effect of sex ratio through the female component of fitness, we regressed fruit set (mature fruits / open flowers) on the sex of the individual, the sex ratio of the population, and the sex-by-sex ratio interaction as fixed effects, and the population number and plant ID as random effects, specifying a binomial error distribution with a logit link, weighted by flower number. To quantify the effect of sex ratio through the male component of fitness, we regressed the projected number of seeds sired population on the sex ratio of the population as a fixed effect and the population ID as a random effect, specifying a Gaussian error distribution. To quantify the overall effect of sex ratio on individual fitness, we created an index of composite fitness through both the male and female components by summing the values of mean relativized fitness through fruit set and proportion seeds sired, following Morgan (1992). We then regressed this composite fitness index on the sex of the individual, the sex ratio of the population, and the sex-by-sex-ratio interaction as fixed effects, and the population ID as a random effect, specifying a Gaussian error distribution.

To quantify selection on floral traits, we first used a principal components analysis of petal and calyx length and width using the prcomp function in R (Table 1). All traits loaded positively and in roughly equal proportions to the first principal component, and so we interpret PC1 as representing variation in flower size. The loadings for calyx width and calyx length negatively co-varied for the second principal component, and so we interpret PC2 as representing variation in flower shape. Overall, these two principal components account for 83% of the variation in the floral phenotypes, and so we use PC1 and PC2 as our phenotypic measures for the following analyses. To estimate selection on floral phenotypes through the female component of fitness, we regressed fruit set on sex, the sex ratio of the population, PC1, PC2, and all two- and three-way interactions of the principal components, sex, and sex ratio as fixed effects, and the population number and plant ID as random effects, specifying a binomial error distribution with a logit link, weighted by flower number. To estimate selection on floral phenotypes through the male component of fitness, we regressed projected seed number on the sex ratio of the population, PC1, PC2, and the two-way interactions of sex ratio and PC1 and PC2 as fixed effects, and the population number as a random effect, specifying a Gaussian error distribution.

For all of these analyses we assessed whether model assumptions were violated by visual inspection of the residual values and a plot of predicted values as a function of the residual values. Hypothesis testing was performed using partial likelihood ratio tests implemented in the Anova function in the package car, specifying Type-III sum of squares (Fox & Weisberg 2011). We extracted least square means and confidence intervals from all of these models for visualization using the package lsmeans (Lenth 2016).

Results

The sex ratio of the population did not have a significant effect on the overall productivity of the patch, as estimated both by the total number of fruits that developed during the experiment and the total proportion of flowers that converted into fruits (Table 2; Figure 1). However, the sex ratio of the populations had a dramatic effect on the fitness of individuals within them. Both hermaphrodite and female plants experienced the highest fitness when they were the rarer sex (Table 3; Figure 2). These fitness effects differed between the sexes and between components of fitness. The fitness of female plants measured through fruit set increased two-fold with increasing hermaphrodite frequency, while the fitness of hermaphrodites through fruit set did not differ across the sex ratios (Figure 2A). However, the fitness of hermaphrodites through pollen was affected by sex ratio: the fitness of individual hermaphrodites decreased four-fold with an increasing frequency of hermaphrodites in the population (Figure 2B). The composite fitness of individual hermaphrodites and females was highest for each sex when they were rare (Figure 2C).

The sex ratio of the population also had a strong effect on the direction and magnitude of selection on floral traits in all individuals (Figure 3; Table 4). Selection through fruit set was strongest in the sex ratio treatments that were the most extreme, and weakest when hermaphrodites and females were represented in equal proportions. Further, the direction of selection reversed across the sex ratio treatments. When hermaphrodites were rare the plants with the largest flowers had the highest fitness through fruit set, while when hermaphrodites were common the plants with the smallest flower had the highest fitness through fruit set (Figure 3). There was no significant selection on flower size or flower shape through the male component of fitness (Figure 4; Table 4).

Discussion

Although sex ratio has the potential to alter the fitness consequences of sexual phenotypes at several levels, the results of this study suggest that the strongest effects of sex ratio will be through altering the context in which selection occurs within populations, rather than creating differences in reproductive output among populations. We found that sex ratio did not significantly affect the reproductive output of populations, estimated either as the total number of fruits that developed or the proportion of flowers that converted to fruits. This result suggests that at the interdemic level, populations with a greater proportion of hermaphrodites are not more productive than populations with fewer hermaphrodites. Within populations, however, sex ratio had effects both on the relative fitness of the sexes as well as on the direction and magnitude of selection on flower size. These results demonstrate that sex ratio acts a context for phenotypic selection on floral traits.

Both sexes experienced elevated fitness when they were rare, although the magnitude of this effect was greater in hermaphrodites than females. Hermaphrodite plants experienced a nearly two-fold advantage in composite relative fitness when they were the rare sex, while the female plants had much more modest advantage when they were rare. This difference between hermaphrodites and females suggests that the fitness effects of sex ratio are likely to be more severe for females than hermaphrodites. The fruit set of hermaphrodites was not affected by sex ratio, while female fitness through fruit set was strongly affected by sex ratio. However, because hermaphrodites also realize fitness through siring seeds, they experience greater composite fitness.

That the sex ratio of a population should result in frequency-dependent patterns of fitness is not a new concept—although commonly credited to Fisher, this concept is found in the writings of Darwin (Darwin 1871; Fisher 1930; Edwards 1998). This type of frequency-dependence is expected to result in the evolution of an equilibrium sex ratio that is roughly equal across populations over time. Indeed, in populations of dioecious species, especially animal species, there is a nearly ubiquitous 1:1 ratio of males to females. However, in sexually polymorphic plants dioecy is rare, and extreme variation in sex ratio among populations is the rule rather than the exception (Spigler & Ashman 2012). How this variation in sex ratio is maintained in populations, and whether it represents a key stepping stone in plant mating system evolution, and been the focus of intense study (Lewis 1941; Lloyd 1974; Charlesworth & Charlesworth 1978; McCauley & Brock 1998; Olson et al. 2005; Delph et al. 2007; Spigler & Ashman 2012). The general predictions that that emerge from these studies is that the frequency of females in the population will impact both the fitness of individuals within the population, as well as the overall productivity of the population. Our results suggest that the individual-level effects of sex ratio are greater than the population-level effects. However, it is worth noting that the fitness differences detected herein may not reflect effects through other components of female fitness, such as seed number or seed germination.

In addition to driving differences in fitness, sex ratio also created in differences in phenotypic selection on floral traits. Selection on flower size through the fruit set varied in both magnitude and direction across the sex ratio treatments. In populations in which hermaphrodites were rare, the individuals with the highest fruit set were those with the largest flowers, whereas in populations in which hermaphrodites were common the individuals with the smallest flowers had the highest fruit set. Flower size is sexually dimorphic in *Silene vulgaris*; hermaphrodites have larger flowers than females (Marsden-Jones & Turril 1957; M. Augat, unpubl.). This sexual dimorphism in flower size means that the negative frequency-dependence we see in the fitness consequences arising from sex ratio is also present in phenotypic selection through fruit set. The plants with the largest flowers are those that are more hermaphrodite-like in terms of sexual size dimorphism, and they have the highest fruit set when hermaphrodites are rare. If flower size is heritable, this would suggest that the degree of sexual dimorphism in a population may differ, depending on the history of sex ratio that population has experienced.

Although selection on flower size through fruit set was context-dependent, we did not quantify any significant selection on flower size or shape through the male component of fitness. This lack of a relationship between siring success and floral phenotypes is surprising, because the fitness of individual hermaphrodites through siring success negatively co-varied with their frequency in populations. Additionally, selection arising from sex ratio for greater "male-ness" of hermaphrodites is predicted to be an important component in the evolution of dioecy (reviewed by Spigler & Ashman 2012), and so we predicted we would see strong context-dependent selection through the male function in this study. These results suggest that flower size is a significant determinant of fitness through pollen receipt, but not pollen export. *S. vulgaris* is visited by many species of insects, including bumble bees and syrphid flies during the day, and noctuid and sphingid moths during the evening, although only the evening insects are effective pollinators (Chapter 2). The lack of a significant relationship between flower size and siring success may be the result of inefficient pollination by daytime insects, or suggest that the effective nocturnal pollinators respond to different, unmeasured floral traits that are more closely related to pollen export, such as nectar scent or volume, calyx or petal color, or anther exertion.

Although not statistically significant, the trends in the relationship between flower size and male fitness show a similar, but opposite, pattern of context-dependence as selection through fruit set. The trend when hermaphrodites were rare was for the smallest flowers to have the greatest number of seeds sired, while in the populations with the most hermaphrodites the plants with the largest flowers sired more seed. This opposition in selection between male and female components of fitness might reflect some difference in sex allocation among hermaphrodites—individuals with larger flowers may have invested more in the development of the gynoecium, and thus enjoy greater fruit set, while individuals with smaller flowers may have invested more in anther development. A similar phenotypic trade-off was found in *Fragaria virginica,* which reflected a negative genetic correlation between pollen and seed production (Ashman 2003). These results match the predictions of the "gender balance" hypothesis for the evolution of co-sexual flowers, in which phenotypic selection in hermaphrodites should be opposite in sign and roughly equal in magnitude through the male and female functions (Morgan 1992; Ashman & Morgan 2004).

The fact that we only quantified selection on flower size through fruit set and not pollen suggests that the effects of social selection in this species may contribute to cytonuclear linkage between the cytoplasmic genes that cause male sterility and the nuclear genes that contribute to flower size variation. Because selection on flower size is acting only through the female component of fitness, the genes passed on as a result of this selection are not only in the nucleus but also in the cytoplasm. Although it would be dampened by the random contribution of flower size genes from sires, this maternal inheritance could potentially create linkage between the genes contributing to flower size and the genes underlying sex determination in the population. The context-dependent nature of selection on flower size through fruit set could result in heterogeneity among populations with respect to sexual size dimorphism, and the extent to which size genes are linked sex determination.

Some caution is warranted in extrapolating these results beyond the current study. All of our estimates of selection were based on measurements from only a single flower. Phenology may shift throughout the breeding season as a result of shifts in the environment, and selection may differ over the course of the season as the community of pollinating insects changes. Additionally, we focused exclusively on fruit set as the female component of fitness, while many other studies of plant mating system evolution focus on seed set and the female advantage through seed rather than fruit set. Both of these shortcomings of the current study may have affected our estimates of the strength of selection on flower size. However, the significant patterns of selection through fruit set, and the general trends seen in the siring success results, match the predictions from the theoretical literature as well as past empirical studies. Thus, it is possible that our sample of seeds was not large enough to detect relatively weak selection through siring success in this experiment.

A growing body of literature has demonstrated the importance of understanding how social and ecological context shapes patterns of phenotypic selection in wild populations. Our results demonstrate that both the fitness consequences of sexual phenotypes, as well as the magnitude and direction of selection on floral traits, differ significantly among the sex ratio treatments. The reversal of the direction of selection on flower size across sex ratio treatments through fruit set in particular illustrates that the social context in which traits are expressed can have a dramatic impact on phenotypic selection. This suggests that the evolution of sexual phenotypes may strongly differ among populations with different sex ratios. How and whether this will affect mating system evolution depends on the heritability of sex and floral phenotypes, as well as the degree of gene flow between populations. What is clear, however, is that sex ratio is a critical context in which selection on sexual phenotypes occurs, and strongly affects both the fitness of individuals as well as the form of selection on floral traits.

Acknowledgements

We thank A. Brumbaugh, R. Costello, A. Enriquez, K. Escoto, S. Liechter, A. Yantes, C. Wood, J. Wood, and S. Wood for assistance with field work. The George Washington and Jefferson National Forests granted permission to conduct this research on Salt Pond Mountain in Giles County, VA. J. Wang provided assistance with the paternity analysis using COLONY. Funding was provided by National Science Foundation Doctoral Dissertation Improvement Grant number 1407155 to B.J.S., National Science Foundation grant number IOS-1027251 to E.D.B. III, and Margaret Walton Scholarships from Mountain Lake Biological Station to B.J.S. and M.E.A.

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Figures and Tables

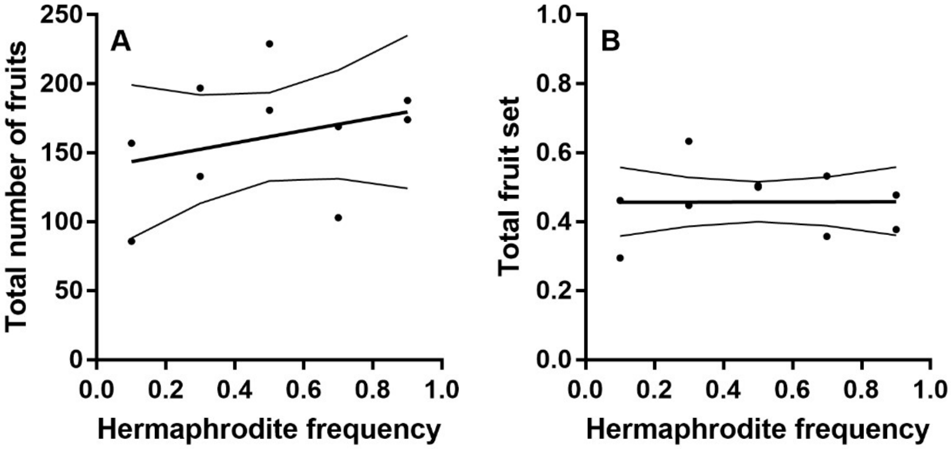

Figure 1. Effects of sex ratio on the overall reproductive output of populations. A: the total number of fruits that developed on all plants within the population. B: The proportion of all flowers in the population that developed into fruits. Center lines represent least square means from generalized linear models, and the exterior lines are 95% confidence intervals. Points are raw values of fruit number (A) and fruit set (B) for each of the 10 populations.

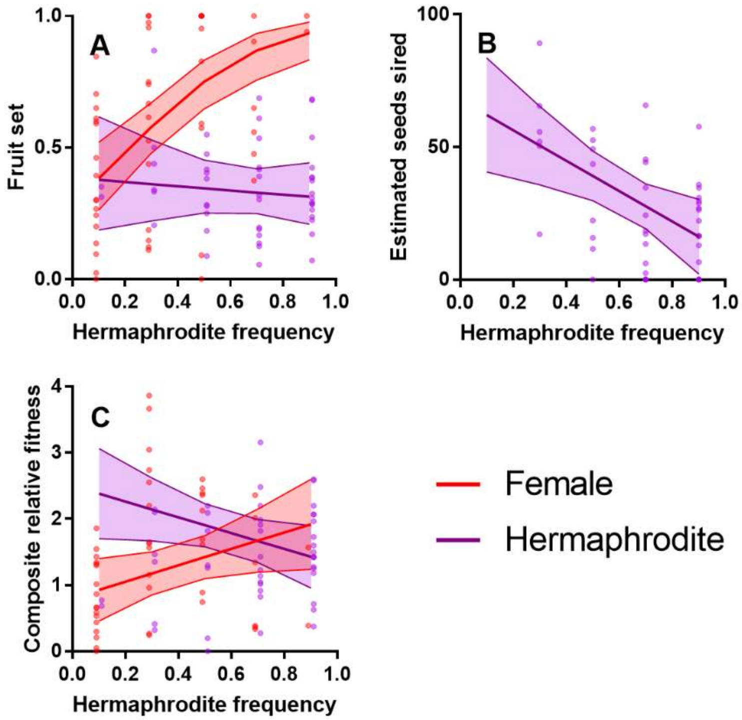

Figure 2. Individual-level fitness effects of sex ratio for hermaphrodites (purple) and females (red) estimated for both male and female components of fitness. A: the proportion of flowers that converted into fruits. B: the projected number of seeds sired by an individual hermaphrodite. C: the composite relative fitness through both fruit set and the projected seeds sired. Center lines represent least square means from generalized linear models, while the exterior lines are 95% confidence intervals. Points are the raw values of fruit set (A), projected number of seeds sired (B), and composite fitness (C), for hermaphrodites (purple) and females (red).

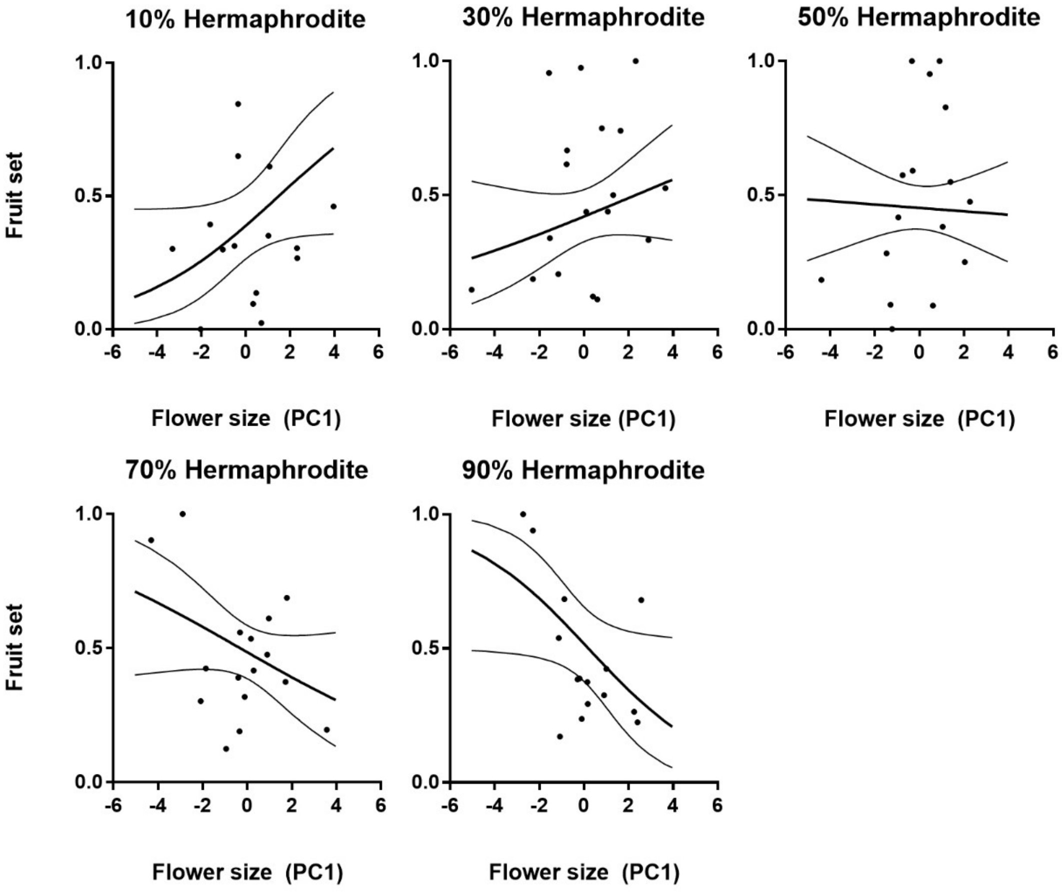

Figure 3. Phenotypic selection on flower size (PC1) through the female component of fitness. Center lines represent the partial regression of fruit set on PC1 from a generalized linear mixed model that included sex, sex ratio, PC1, PC2, and the two- and three-way interactions between sex, sex ratio, and PC1 and PC2 as fixed effects, and population ID and plant ID as random effects. The exterior lines are 95% confidence intervals. Points are raw values of PC1 and fruit set for all plants.

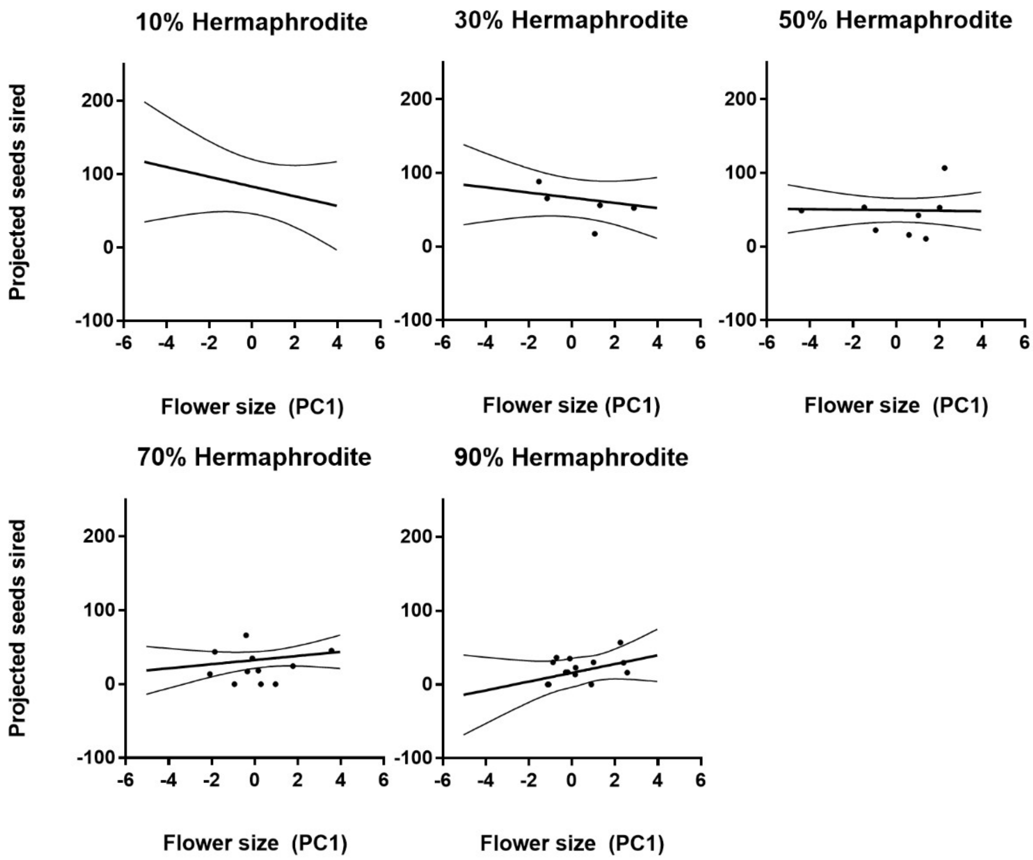

Figure 4. No significant phenotypic selection on flower size (PC1) through the male component of fitness. Center lines represent the partial regression of projected number of seeds sired on PC1 from a generalized linear mixed model that included sex, sex ratio, PC1, PC2, and the two- and three-way interactions between sex, sex ratio, and PC1 and PC2 as fixed effects, and population ID and plant ID as random effects. The exterior lines are 95% confidence intervals. Points are raw values of PC1 and projected seeds sired for all plants.

Tables

Table 1

Principle components analysis of floral morphologies. Calyx width and length, and petal width and length were measured in millimeters using digital calipers.

	PC1	PC2	PC1	PC2
Calyx width	0.601	0.279	0.749	-0.026
Calyx length	0.699	-0.637	-0.322	0.041
Petal width	0.364	0.651	-0.548	-0.378
Petal length	0.135	0.303	-0.188	0.925
Prop. Variance	0.597	0.231	0.143	0.029
Cum. variance	0.597	0.828	0.971	1.000

Table 2

Summary of likelihood ratio hypothesis tests for the effect of sex ratio on the total number of fruits that developed, and the total proportion of flowers that developed into fruits, over the four-week period of the experiment.

Source	df	N. fruit	Prop. fruit
Sex ratio	1	0.839 NS	0.001 NS

Table 3

Summary of partial likelihood ratio χ^2 values from hypothesis tests for the effects of sex, sex ratio, and their interaction on fitness quantified as the proportion of flowers that converted to fruits (fruit set), the projected number of seeds sired by individuals estimated by genetic paternity, and a composite fitness index of relative fitness through fruit set and proportion seeds sired. (* P < 0.05, ** P < 0.01, *** P < 0.001)

Source	df	Prop. fruit	N. sired	Composite
Sex	1	**23.558*** **		**16.654*** **
Sex ratio	1	**7.431** **	**14.058*** **	**4.708* **
Sex × sex ratio	1	**12.638*** **		**10.840*** **

Table 4

Summary of partial likelihood ratio χ^2 values from hypothesis tests of the relationship between petal size and fitness through both fruit set and the proportion of seeds sired by an individual. These models included also included the sex of the individual, the sex ratio of the population, and all interactions of these factors. (* P < 0.05, ** P < 0.01, *** P < 0.001)

Source	df	Fruit set	N. sired
Sex ratio	1	1.104	**14.420*** **
PC1 (flower size)	1	**3.901***	1.223
PC2 (flower shape)	1	0.150	1.362
Sex ratio × PC1	1	**5.210***	1.853
Sex ratio × PC2	1	0.394	0.869

Supplementary Tables

Supplementary Table 1

Microsatellite loci used for paternity assignment in this study. Effective number of alleles (eff. n. alleles), observed (H_O), and expected (H_E) heterozygosities, were estimated in GenoDive version 2.0b27 (Miermans and Van Tienderen 2004). Null allele frequencies were estimated using Micro-Checker version 2.2.3 (Van Oosterhout et al. 2004).

Locus	N. alleles	Eff. n. alleles	H_O	H_E	Null allele frequency
Sv123	7	2.535	0.437	0.606	0.132
Sv074	12	4.446	0.696	0.775	0.049
Sv028	7	3.622	0.655	0.724	0.058
Sv071	11	2.273	0.362	0.560	0.162
Sv040	14	5.987	0.726	0.833	0.053
Sv184	15	4.723	0.366	0.789	0.260
Sv033	10	3.189	0.626	0.687	0.061
Sv031	10	3.978	0.629	0.749	0.076
Sv063	10	4.327	0.689	0.769	0.064
Sv023	11	1.886	0.423	0.470	0.045

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