MATING SYSTEM EVOLUTION, PLANT-POLLINATOR INTERACTIONS, AND FLORAL ULTRAVIOLET PATTERNING IN *MIMULUS GUTTATUS*

by

Sarah Ann Bodbyl Roels

Submitted to the graduate degree program in Ecology and Evolutionary Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

_____
Chairperson: John Kelly

_____
Helen Alexander

_____
Bryan Foster

_____
Daniel Crawford

_____
Barbara Kerr

Date Defended: June 15, 2012

The Dissertation Committee for Sarah Bodbyl Roels
certifies that this is the approved version of the following dissertation:

MATING SYSTEM EVOLUTION, PLANT-POLLINATOR INTERACTIONS, AND FLORAL ULTRAVIOLET PATTERNING IN *MIMULUS GUTTATUS*

_____
Chairperson: John Kelly

Date Approved: June 15, 2012

ABSTRACT

The evolution and maintenance of plant mating systems has been a topic of great interest throughout the history of evolutionary biology. Despite a large body of literature describing mating system variation, critical knowledge is lacking regarding the mechanisms involved in mating system transitions, including the rate and magnitude of trait changes. I combine experimental evolution, pollinator behavioral studies, and classic phytomorphology to examine the mating system transition from outcrossing to self-fertilizing (selfing) in flowering plants. Using the wildflower *Mimulus guttatus* (yellow monkeyflower) as a model, I investigate which traits facilitate outcrossing via mutualistic relationships with pollinators and which traits evolve in response to pollination disruption. In Chapters 1 and 2, I demonstrate that rapid evolution of adaptive floral and genetic traits can occur in populations that lose pollinators and that trait evolution occurs sequentially. In Chapter 3, I find strong pollinator preference for specific floral traits, evidence that pollinator selection maintains attractive traits in wild populations. However, pollinators displayed limited ability to distinguish between subtle population variants and thus are unlikely drivers of ethological speciation. In Chapter 4, I document within and among population variation in a cryptic floral trait, ultraviolet (UV) patterning. UV patterning is a visual stimulant for pollinators, but I found it in a selfing *Mimulus* species, suggesting that UV patterning in flowers functions beyond pollinator attraction. My research has direct implications for forecasting plant adaptation as anthropogenic environmental disturbance increasingly decouples plant-pollinator relationships. Rapid adaptation is an option for some self-compatible, pollinator-reliant plants; however, a transition to selfing results in decreased genetic diversity which may expose populations to extinction with further environmental disturbances.

ACKNOWLEDGEMENTS

I dedicate this dissertation to my family. My parents, Tom and Sherri Bodbyl, have always encouraged me to follow my dreams and told me that I could succeed at whatever I put my mind to. My grandparents, Lois and Harry Bodbyl and Martha and Larry Yonkers, gave me undying love and support and taught me perseverance. My parents-in-law, John and Shirley Roels, have been amazing, tireless, academic role models. Thank you for supporting and encouraging me all these years.

Thanks to my past and present committee members, Helen Alexander, Bryan Foster, Town Peterson, Xingong Li, Dan Crawford, Steve Case, and Barbara Kerr, who have supported me through oral comprehensives, grant writing, and many experiment-related frustrations. Huge thanks to my brilliant advisor, John Kelly, who took me in and taught me so much about quantitative genetics, *Mimulus*, and the value of 'real science'. The Kelly lab, my academic siblings, are an eclectic group that I am proud to be a part of. Thank you graduate students Julius Mojica, Tara Marriage, Patrick Monnahan, Boryana Koseva, Katrina McClure, and Terra Lubin and postdocs Alison Scoville and Vanessa Koelling for advice, troubleshooting, companionship, and highly amusing conversations. Special thanks goes to the army of undergraduates and assistants that helped me with tens of thousands of measurements and seed counts, including Atiq Ahmadullah, Ronnette Chase Alone, Ider-Od Bat-Erdene, Rafferty Deeds, Peter Ebeling, Whitney Howard, Ian Kietzman, Sarah Mingle, Sierra Taul, and Alex Wayne.

The University of Kansas Department of Ecology and Evolutionary Biology has supported me in many ways. I am thankful for the hardworking greenhouse manager (Katie Nus) and her staff,

the always helpful office administration (Jaime Keeler, Dorothy Johanning, Linda Narcomey, and Kim Booth) and the support of the chair (Chris Haufler).

My surrogate family and friends at my home away from home, Fox Eye Ranch, have also contributed greatly to my time in Kansas. Thanks for all the memories and for doing your best to keep me sane.

Above all others, I want to thank my wonderful husband, Steve Roels. He has been my coach, cheerleader, editor, and house-husband throughout the dissertation process and I can not imagine a more fulfilling partnership.

Chapter specific acknowledgements:

Chapters 1 and 2—I thank Helen Alexander, John Willis, Tara Marriage, Casey McNeil, Steve Roels, Vanessa Koelling, Lena Hileman, and two anonymous referees for helpful comments on the manuscript. This work was supported by grants NIH GM073990, NSF DEB-054052, and NSF DEB-1010899, and by funding from the University of Kansas Botany Endowment Funds.

Chapter 3—I thank Sierra Taul and Elizabeth Lusher for help with bee experiments. Katie Nus kindly provided permission to use greenhouse space and tolerated frequent loose bees. Thanks to Chip Taylor for helpful comments on the manuscript. This study was supported by grants NSF DEB-1010899, the KU Botany Endowment, and the KU Office of Graduate Studies Doctoral Research Funds.

Chapter 4—I thank Aaron Paden and Klaus Schmitt for ultraviolet photography and equipment advice. Heather Shinogle and Prem Thapa of the University of Kansas Microscopy and Analytical Imaging Laboratory provided expert SEM assistance. Dan Crawford gave a

helpful review of the manuscript. This work was supported by the KU Office of Graduate Studies Doctoral Research Funds and KU EEB Department Summer Funding.

TABLE OF CONTENTS

INTRODUCTION

The evolution of plant mating systems has been a topic of great interest throughout the history of evolutionary biology. Numerous studies (e.g. Clements & Long 1923; Grant 1949; Stebbins 1957) describe natural mating system variation throughout the plant kingdom and convergence of characteristic suites of traits evolving in concert. Despite a large body of literature describing mating system variation, there is a critical gap in knowledge of the mechanisms involved in mating system transitions. For instance, the evolutionary transition from obligate cross-pollinating (outcrossing) to self-pollinating (selfing) is one of the most common transformations in the history of angiosperms (Stebbins 1974). This transition is associated with changes in floral biology, life history, and ecology and thus has manifold consequences for population genetics and evolution. However, the selective pressures that initiate mating system transitions and the rate and order in which floral traits evolve in response is unknown.

Pollinators play an essential role in the formation and maintenance of plant mating systems in many outcrossing and mixed mating (i.e. both outcrossing and selfing) angiosperm taxa. Plants interact with pollinators primarily through floral displays and pollinator preference for specific floral traits may result in strong directional selection for attractive floral morphology, enhancing plant reproductive success. Floral architecture, color, scent, texture, and nectar/pollen rewards are all traits known to facilitate pollinator attraction and fidelity. Many floral traits, such as size, shape, and color, have been studied extensively (see Patiny 2012). Others, such as the cryptic ultraviolet patterns found on petaloid flowers, have been overlooked. Despite the strong selective forces that pollinators impose upon plants, little is known about how pollinator preferences affect

plant fitness or facilitate and maintain divergence between populations and species. Changes in the abundance of pollinators may initiate mating system transitions in mixed mating plant populations. In particular, pollinator loss is thought to be one of the main selective forces driving the transition from outcrossing to selfing (Baker 1967; Kalisz et al. 2004). As plant-pollinator mutualisms erode, plants are predicted to evolve traits maximizing self-fertilization that are no longer intrinsically tied to pollinator preference. The convergent suite of self-fertilizing traits observed in nature is referred to as the 'selfing syndrome' (Orduff 1969).

Understanding what contributes to and maintains natural variation in plant mating systems becomes increasingly important as rapid, anthropogenic environmental changes modify plant-pollinator interactions (Eckert et al. 2009). Recent events, including habitat loss, climate change, invasive species, and novel disease, shift the abundance and composition of the available pollinator pool, disrupting plant reproduction (e.g. Kearns et al. 1998; Steffan-Dewenter et al. 2006). Accordingly, questions surrounding the evolution and ecology of mating system transitions have remained at the forefront of ecology and evolutionary biology and motivate my dissertation.

In the four chapters of this dissertation, I address the following objectives—

1: Investigate the immediate evolutionary consequences of pollinator limitation and assess the rate and nature of mating system adaptation.

2: Examine how the genetic architecture of populations is modified during mating system transformations.

3: Determine how pollinator preferences influence trait evolution and maintain divergence among populations and species.

4. Document population and species-level variation in ultraviolet patterning, an understudied floral trait, as a first step to understanding its evolutionary role in pollinator attraction and mating system divergence.

My four chapters provide a comprehensive quantification of the relationship between plant traits, plant-pollinator relationships, and mating system in *Mimulus guttatus*, the yellow monkeyflower. In the first two chapters, I investigate how *Mimulus* populations compensate for an abrupt loss of pollinators. I explore phenotypic and genetic changes in populations experiencing opposing pollinator regimes: abundant bumblebee (*Bombus*) pollinators vs. no pollinators. The mechanism, rate, and order of trait evolution during the mating system transition from outcrossing to selfing are poorly understood. This evolutionary transition has never been directly observed. In the third and fourth chapters, I investigate specific traits involved in the maintenance of pollinator mutualisms in outcrossing *Mimulus* populations. The third chapter explores the intimate relationship between bumblebees (*Bombus)* and *Mimulus*, using bee behavioral experiments to quantify pollinator preference for particular traits, constancy, and ability to discriminate among variably divergent populations. The fourth chapter focuses on a single pollinator attractive floral trait, ultraviolet patterning. In this chapter, I identify trait

variation within and among multiple *Mimulus* populations and investigate the contributions of pigment characters and cellular structures to that variation.

Mimulus (Phrymaceae) is an ideal plant genus for investigating mating system evolution and plant-pollinator interactions. *Mimulus* is a model system in ecology and evolution biology and has a rapidly expanding presence in functional genomics (Wu et al. 2008). The genus has numerous attributes that invite ecological study including tremendous phenotypic variation, high genetic diversity, occupation of numerous habitats, and mating systems ranging from completely outcrossing to obligately selfing to obligately asexual (Wu et al. 2008). *Mimulus* is easily propagated in the greenhouse. All species are self compatible, easily emasculated and hand pollinated, and most have only a 6–12 week generation time under experimental conditions (Wu et al. 2008). Numerous tools are also available for genetic and genomic studies, particularly in the *M. guttatus* and *M. lewisii* species complexes, including hundreds of thousands of raw expressed sequence tags (ESTs), over a thousand highly polymorphic gene-based markers, detailed linkage maps, BAC libraries, integrated genetic and physical maps, seed stocks, and a full genome sequence, all accessible to the scientific community via a centralized web site (Wu et al. 2008; http://www.mimulusevolution.org). I used *M. guttatus* (2n = 28), a mixed mating, hermaphroditic North American wildflower, as an experimental plant for my dissertation studies. *M. guttatus* populations show extensive variation in morphology and life history and exhibit adaptations for bee pollination (Campbell 1950; Vickery 1978). Previous studies have quantified heritable variation for a variety of mating system traits (detailed in Chapter 1), making *M. guttatus* an excellent species for studies of plant mating system evolution.

***Summary*—**I combine multiple methods, including experimental evolution, quantitative genetics, behavioral experiments, spectral analyses, and microscopy to understand both the forces that shape floral morphology, life history, and reproduction in *Mimulus* and the genetic architecture of mating system transitions. My work is a synthesis of both ecology and evolutionary biology. It contributes to the fields of plant mating systems biology, pollination ecology, and ecological genomics, the *Mimulus* research community, and groups interested in biotic response to anthropogenic environmental change.

LITERATURE CITED

Baker, H. G. 1967. Support for Baker's Law-as a rule. Evolution 21: 853–856.

Campbell, G. R. 1950. *Mimulus guttatus* and related species. El Aliso 2: 319–335.

Clements, F. E. and F. L. Long. 1923. Experimental Pollination: An outline of the ecology of flowers and insects. Carnegie Institute of Washington, Washington. 322 pages.

Eckert, C. G., Kalisz, S., Geber, M. A., Sargent, R., Elle, E., Cheptou, P-O., Goodwillie, C., Johnston, M. O., Kelly, J. K., Moeller, D. A., Porcher, E. Ree, R. H., Vallejo-Marín, M., and A. A. Winn. 2009. Plant mating systems in a changing world. Trends in Ecology and Evolution 25: 35–43.

Grant, V. 1949. Pollination systems as isolating mechanisms in angiosperms. Evolution 3: 82–97.

Kearns, C. A., Inouye, D. W. and N. M. Waser. 1998. Endangered mutualisms: The conservation of plant-pollinator interactions. Annual Review of Ecology and Systematics 29: 83–112.

Kalisz, S., Vogler, D. W. and K. M. Hanley. 2004. Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. Nature 430: 884–887.

Ornduff, R. 1969. Reproductive biology in relation to systematics. Taxon 18: 121–133.

Patiny, S. ed. 2012. Evolution of Plant-Pollinator Relationships. Cambridge University Press, Cambridge, UK. 504 pages.

Proctor, M., Yeo, P., and A. Lack. 1996. The Natural History of Pollination. Harper Collins Publishers, London. 479 pages.

Stebbins, G. L. 1957. Self-fertilization and population variability in the higher plants. American Naturalist 41: 337–354.

Stebbins, G. L. 1974. Flowering plants: evolution above the species level. Harvard University Press, Cambridge, MA. 399 pages.

Steffan-Dewenter, I., Klein, A-M., Gaebele, V., Alfert, T., and T. Tscharntke. 2006. Bee diversity and plant-pollinator interactions in fragmented landscapes. Pp. 387–407. *In* Plant-Pollinator Interactions: From Specialization to Generalization. N. M. Waser and J. Ollerton, eds. University of Chicago Press, Chicago.

Vickery, R. K. 1978. Case studies in the evolution of species complexes in *Mimulus.* Evolutionary Biology 11: 405–507.

Wilson, E. O. 1992. The diversity of life. Harvard University Press, Cambridge, MA. 424 pages.

Wu, C. A., Lowry, D. B., Cooley, A. M., Wright, K. M., Lee, Y. W. and J. H. Willis. 2008. *Mimulus* is an emerging model system for the integration of ecological and genomic studies. Heredity 100: 220−230.

Chapter 1. Rapid evolution caused by pollinator loss in *Mimulus guttatus*

ABSTRACT

Anthropogenic perturbations including habitat loss and emerging disease are changing pollinator communities and generating novel selection pressures on plant populations. Disruption of plant-pollinator relationships is predicted to cause plant mating system evolution, although this process has not been directly observed. This study demonstrates the immediate evolutionary effects of pollinator loss within experimental populations of a predominately outcrossing wildflower population. Initially equivalent populations evolved for five generations within two pollination treatments: abundant bumblebee pollinators vs. no pollinators. The populations without pollinators suffered greatly reduced fitness in early generations but rebounded as they evolved an improved ability to self-fertilize. All populations diverged in floral, developmental, and life history traits, but only a subset of characters showed clear association with pollination treatment. Pronounced treatment effects were noted for anther-stigma separation and autogamous seed set. Dramatic allele frequency changes at two chromosomal polymorphisms occurred in the no pollinator populations, explaining a large fraction of divergence in pollen viability. The pattern of phenotypic and genetic changes in this experiment favors a sequential model for the evolution of the multi-trait 'selfing syndrome' observed throughout angiosperms.

INTRODUCTION

Approximately 180,000 flowering plant species rely on pollinators, including at least 100 agricultural species (Committee on the Status of Pollinators in North America 2007). A variety of human disturbances, including climate change, habitat fragmentation, and the introduction of invasive species are disrupting plant-pollinator relationships (Memmott et al. 2007; Hegland et al. 2009). One consequence is that plants receive less outcross pollen in human disturbed habitats than in undisturbed areas (Eckert et al. 2009). While the ecological effects of pollinator limitation are starting to receive proper attention, the evolutionary consequences of pollinator declines have not been directly studied.

The reproductive assurance hypothesis posits that pollinator limitation will cause plant populations to evolve increased rates of self-fertilization (Baker 1967; Kalisz et al. 2004). Indeed, the transition from cross-pollination to self-pollination has occurred many times in the history of angiosperms and is associated with changes in floral biology, life history, and ecology (Grant 1949; Ornduff 1969). Highly selfing plant taxa routinely have reduced flowers, less vegetative mass, reduced anther-stigma separation (herkogamy), and develop more rapidly than closely related outcrossing taxa. This constellation of features is commonly called the "selfing syndrome" (Ornduff 1969). It remains unclear if the traits of the selfing syndrome evolve simultaneously or in a piece-meal fashion during the transition from outcrossing to selfing. Some features of the syndrome may evolve subsequent to mating system change. For example, smaller flowers may evolve within selfing populations, not because reduced corolla area is necessary for

self-fertilization, but simply because the investment in corolla tissue is unnecessary after a population has become predominantly selfing.

The *Mimulus guttatus* species complex exhibits the syndrome in a number of highly selfing lineages thought to be recently derived from outcrossing ancestors. *Mimulus guttatus* (2n = 28; Phrymaceae), the most common member of the complex is a self-compatible, hermaphroditic plant with extensive variation in morphology and life history (Wu et al. 2008). It exhibits adaptations for pollination by bees, including a wide, showy corolla with a landing platform and a touch-sensitive stigma. Previous studies of *M. guttatus* have demonstrated heritable variation for a variety of "mating system traits", i.e. rate of development, corolla width and length, style width and length, anther length, ovary size, ovary and pollen number, amount of red pigmentation on the corolla, and autogamous seed set (Carr & Fenster 1994; Robertson et al. 1994; Kelly & Arathi 2003; van Kleunen & Ritland 2004; Scoville et al. 2009).

There are numerous evolutionary lineages derived from *M. guttatus* (or a *guttatus*-like ancestor) that are thought to be highly selfing, including *M. nasutus, M. laciniatus, M. pardalis, M. longulus, M. clementinus, M. micranthus, M. platycalyx*, and *M. cupriphilus* (Grant 1924; Macnair et al. 1989; Vickery 1978; Ritland & Ritland 1989; Sweigart & Willis 2003). *M. nasutus* (2n = 28) is perhaps the most widespread of the selfing taxa and differs dramatically from *M. guttatus* in floral morphology and other mating system characters (Figure 1–1A). *M. nasutus* corollas are greatly reduced relative to *M. guttatus*, often cleistogamous. *M. nasutus* also exhibits reduced anther-stigma separation and produces less pollen per flower, but has larger leaves than *M. guttatus.* As expected from these morphological adaptations to selfing, autogamous seed set is

more than ten-fold greater in *M. nasutus* than *M. guttatus* (Fishman et al. 2002). Reduced flower size and reduced anther-stigma separation are common traits to the various selfing species in the complex – *M. platycalyx* (Ritland & Ritland 1989; Dole 1992), *M. micranthus* (Carr & Dudash 1996; Carr & Fenster 1994), *M. cupriphilus* (Macnair 1989; Macnair & Cumbes 1989).

While taxonomic data provide clear expectations regarding the traits that are likely to evolve with increased selfing, the expected pace of change is difficult to predict. How often do outcrossing populations harbor sufficient genetic variation in autogamy, the ability to set seed by selfing, to allow a rapid evolutionary response to environmental change? The rate that increased autogamy evolves is critical because pollinator decline will likely cause negative population growth and could ultimately lead to extinction. The likelihood of "evolutionary rescue"—wherein adaptation prevents extinction (Lynch & Lande 1993; Gomulkiewicz & Holt 1995)—depends on the nature of genetic variation in traits that are essential to survival and reproduction under the novel environmental condition.

Gomulkiewicz et al. (2010) recently analyzed a number of genetic models and conclude that genes with large phenotypic effects can often facilitate rescue. Studies of *M. guttatus* have demonstrated a contribution of major chromosomal polymorphisms to variation in pollination-related traits. The *Meiotic Drive* Locus on linkage group 11 (alternative alleles D/d) exhibits segregation distortion through female meiosis (Fishman & Saunders 2008), but also has phenotypic effects on pollen viability, floral development rate, and anther-stigma separation (Scoville et al. 2009). The drive-allele (D) is maintained at 30–40% in the Iron Mountain population (Fishman & Saunders 2008), which is the source of plants used in the present

experiement. The *LG6 Inversion* is a polymorphic inversion (C/c) with phenotypic effects on pollen and development traits (Lee 2009; Scoville et al. 2009). Floral traits are also affected by the inversion, but the estimated effects are heterogeneous among different mapping crosses (Scoville et al. 2009).

Here, we describe a novel experimental study documenting immediate evolutionary changes following pollinator loss. We measure changes in the means and genetic variances of a series of quantitative traits, and in allele frequencies at the two major chromosomal polymorphisms. These data are used to address the following questions: 1) What are the fitness consequences of pollinator loss? 2) Can a population of short lived plants evolve rapidly in response to pollinator loss? 3) If so, what traits evolve? 4) Are major polymorphisms critical to mating system evolution? 5) Do genetic variances change on the same time scale as trait means with a shift in mating system?

MATERIALS AND METHODS

***The pollinators*—**Bumblebees (genus *Bombus*) are perhaps the most important native pollinators for North American *Mimulus* species. *Bombus fervidus, B. griseocollis,* and *B. impatiens* are frequent visitors of *Mimulus ringens* (Mitchell et al. 2004), as is *B. vosnesenski* of *M. lewisii* (Schemske & Bradshaw 1999). *Mimulus guttatus*, the plant investigated here, is visited by many other *Bombus* species over its natural range. These include *B. balteatus, B. biarius, B. californicus*, *B. centralis,* and *B. flavifrons* in California (Thorp et al. 1983; Macnair et al. 1989) and *B. edwardsii, B. mixtus*, and *B. melanopygus* at Iron Mountain in Oregon, the source of

plants in this experiment (Arathi & Kelly 2004; *B. edwardsii* was recently recognized as a subspecies of *B. melanopygus*, occurring sympatrically in this region (Owen et al. 2010)). *M. guttatus* is not likely to be exclusively pollinated by *Bombus*. Frequent visitation by *Osmia bucephala* (Megachilidae) has been documented in the Oregon population at Dexter reservoir (Kelly & Willis 2002), while the introduced European honeybee, *Apis mellifera*, is now a frequent visitor to lower elevation populations of *M. guttatus* (Macnair et al. 1989; Martin 2004; Ivey and Carr 2005). Admittedly, studies documenting field visitation of flowers by potential pollinators do not prove effective transfer of outcross pollen. However, the diversity of visitors to *M. guttatus*, as well as the morphology of the flowers (Figure 1–1), strongly suggests that this species is not specifically adapted to any ***single*** pollinating species.

We used *B. impatiens* as the pollinator in our Bee treatment populations of this experiment. In part, this is a practical choice: colonies of *B. impatiens* are commercially available in the Midwest. *B. impatiens* occurs across eastern North America and thus does not pollinate Oregon populations of *M. guttatus*. However, it is closely related to western *Bombus* species (Cameron et al. 2007) and is intermediate in size to the documented visitors at Iron Mountain. *B. impatiens* worker bees are 8.5–16mm long, smaller on average than *B. melanopygus* but larger than *B. mixtus* (Franklin 1912). More importantly, both greenhouse and field studies have demonstrated that *Bombus* species are very effective pollinators of *M. guttatus*, regardless of whether a particular bumblebee species is from the location from which the plants are sampled (see Robertson et al. 1999; Arathi & Kelly 2004).

The plants—*Mimulus guttatus* (2n = 28, Phrymaceae) is a self-compatible wildflower ranging from Alaska to Mexico with natural selfing rates varying substantially among populations (Awadalla & Ritland 1997). As a starting point for experimental evolution, we synthesized a large outbred source population of genotypes derived from a single natural population. The source was created by randomly intercrossing plants from three distinct F2 populations. Each F2 was derived from crossing a single large flowered genotype to a small flowered genotype; these parentals were sampled from the high and low selected populations of an artificial selection experiment on corolla width (Kelly 2008; Lee 2009). Source genotypes are all derived from a single natural population located on Iron Mountain in Oregon (Willis 1993; Arathi & Kelly 2004). While the selfing rate is highly variable among *M. guttatus* populations (Ritland & Ganders 1987; Awadalla & Ritland 1997; Sweigart et al. 1999), Iron Mountain is primarily (76–91%) outcrossing (Willis 1993, 1996, 1999a,b). Source seed was distributed into two experimental treatment groups each containing two replicate populations each: **No Bee** treatment populations (**A1, A2**) and **Bee** treatment populations (**B1, B2**). Each replicate population consisted of four box flats with approximately 200 plants per box. Boxes did not create subdivision within populations. Gene flow occurred across boxes within each population and seed was randomly dispersed among boxes when sown for the next generation. In Generation 1, each box was sown with 20mg of source seed, but sowing density of subsequent generations was adjusted as necessary to maintain optimal adult density (~200/box) within flats.

The treatments—In each generation, for a total of five generations, seeds were grown to flowering plants following the same protocol. In all generations, No Bee populations (A1, A2) were maintained in a pollinator-free greenhouse. Plants could reproduce only by selfing, or perhaps by physical contact with flowers of neighboring plants. Bee treatment populations (B1,

B2) were grown in the same greenhouse except for two weeks during their flowering period. During this interval, Bee populations were rotated into a distinct greenhouse containing bumblebees (*Bombus impatiens*). We obtained colonies of approximately thirty bees from Koppert Biological Systems for each generation of the experiment. Replicates remained with the bees over two day rotations for the duration of two weeks. As in previous experiments using Koppert bumblebee hives (Arathi & Kelly 2004), we observed that bees typically visited >10 flowers per foraging run. Also as previously, we observed that visitation rates were high and that most flowers were visited multiple times during a rotation. Replicate populations were kept separate, and bees isolated within their hive between rotations to prevent gene flow between B1 and B2. Six weeks after setting seed to soil, all populations were transported to a growth room allowing the plants to senesce. Fruits were collected as they matured. We counted the number of reproductive plants per population and bulked seed to sow the next generation.

***Breeding design*—**In December of 2008, progeny of Generation 5 were grown simultaneously with source plants to maturity without selection. In this Generation 6, 245 seedlings were sampled from each population with half randomly assigned as dams and the other half as sires. Floral measurements were taken on the first two flowers produced at anthesis, including corolla width, throat width, tube length, and anther-stigma separation (Figure 1–1B). Floral measurements were averaged over both flowers, giving mean per-plant values. A pollen sample was collected from all individuals upon anthesis of a third flower and the number of viable and inviable pollen grains was estimated using a Coulter Counter Model Z1 dual (Kelly et al. 2002; Kelly & Arathi 2003). After measurements were completed, each sire was randomly paired with one dam within a population and hand-pollinated. This produced between 91 and 103 fully

outbred families per population (Family numbers: A1 = 102, A2 = 91, B1 = 103, B2 = 103, Source = 99). Finally, leaf tissue was collected from each sire/dam individual for subsequent DNA extraction and genotyping.

The progeny of these crosses, Generation 7, were grown up in two cohorts with three individuals of each outcross family (n = 1,494 total). Days to flower and the morphological measurements of Generation 6 were taken on each plant. In addition, the maximum width of the largest vegetative leaf was recorded upon anthesis of the second flower. The ability to set seed by self-fertilizing was evaluated by collecting seed produced from an unmanipulated fourth flower (no pollination). The inclusion of Generation 6 between the last generation of selection (Generation 5) and measurement of response (Generation 7) served two purposes. First, the random mating of Generation 6 adults allowed meaningful comparisons of trait mean values in Generation 7. Genotyping of Generation 6 plants (described below) confirmed that individuals within the No Bee and Bee populations were inbred to differing extents. Random mating of adults from Generation 6 produced outbred progeny within each population. Given that inbreeding directly changes means for these traits without selection (Holeski & Kelly 2006), outbred plants are necessary for meaningful comparisons of trait divergence across populations. Second, the controlled crosses generate a family structure for Generation 7 allowing us to estimate the genetic and environmental variance of each trait for each population.

***Genotyping*—**To quantify genetic changes associated with mating system evolution, each individual of Generation 6 was scored at four putatively neutral gene-based markers (MgSTS_461, MgSTS_755, MgSTS_523, and MgSTS_641) and two previously mapped

chromosomal polymorphisms. The length polymorphic marker HB5 was used as an indicator of the genotype at the meiotic drive polymorphism (D/d). The length of the HB5 allele is 277 base pairs (bp) at the derived locus classification, D, and all other allele lengths were scored as the ancestral allele, d. The derived inversion haplotype, C, was identified by a combination of alleles at two marker loci: allele length 240 at marker locus MgSTS_431 and allele length 201 at MgSTS_229. All other combinations were scored c (ancestral). DNA was extracted using our standard procedure (Marriage et al. 2009) followed by amplification using touchdown PCR (Hall & Willis 2005). All primer sequences can be accessed at www.mimulusevolution.org.

***Analysis*—**To determine if there was significant divergence among populations in the continuously distributed traits of Generation 7, we used one factor ANOVAs with Tukey-Kramer post-hoc tests. We also applied a two-level ANOVA to each of these traits with population (random) nested within pollination treatment. "Self seed"—the raw count of seeds produced via autogamy of the fourth flower produced by a plant—was highly right skewed with many zeros. For this response variable, we applied an overdispersed Poisson model with the log link function. Another autogamy variable, ln self seed, is the natural logarithm of [self seed +1], a variable considered in several analyses. Population and treatment effect analyses were performed in JMP® 8.0.2 (SAS Institute Inc., 2009) and Minitab® 16.1.1 (Minitab Inc., 2010).

The phenotypic effects of the two chromosomal polymorphisms on Generation 6 source plants were estimated using one-way ANOVA. We denote the effect on phenotype of having one or two derived alleles as e1 and e2, respectively. The contribution of each chromosomal polymorphism to the outbred mean of a population was estimated as $Z = 2*q*(1-q)*e1 + q^2*e2$,

where q is the population frequency of the derived allele (Table 1–1). The predicted divergence between trait means of the source and evolved populations (A1, A2, B1, B2) that can be attributed to allele frequency change at the chromosomal polymorphism is the difference in Z values between populations.

The mean inbreeding coefficient (F) of each population was estimated from genotypic data (Generation 6 plants) at the length polymorphic markers MgSTS_461, MgSTS_755, MgSTS_523, and MgSTS_641. We used the moment estimator for F of Lynch and Ritland (1999) calculated by the program COANCESTRY (Wang 2011); http://www.zsl.org/science/research/software). The F estimates were subsequently used in our calculations to estimate the additive genetic variance (V_A) and environmental variance (V_E) associated with measured traits in Generation 7. Assuming additive genetic inheritance, the predicted covariance of full-sibs is (1+F) V_A (Harris 1964). We used maximum likelihood to estimate variance components, first assuming a single V_A and V_E across populations (Model I). We then fit a more elaborate model allowing V_A to differ among populations (Model II). Finally, we allowed population specific values for both V_A and V_E (Model III). All models contained 6 fixed effects: trait means for each population (A1, A2, B1, B2, and Source) and a cohort effect (the difference in means between cohorts due to environmental effects). We compare Models (II to I and III to II) on a trait-to-trait basis using Likelihood Ratio Tests. There are four more parameters in Model II than Model I and four more in Model III than Model II, which suggests a critical value for each test of 9.49. These tests might be somewhat conservative given that variances are bounded to non-negative values (Self 1987). The computer programs to execute

these calculations were simplified from previous C programs (Kelly 2003; Kelly & Arathi 2003; Kelly 2008) and the code is available upon request.

RESULTS

***Phenotypic evolution*—**Mean fecundity within the Bee populations remained high over the five generations of pollination (Figure 1–2). Per capita seed production was greatly reduced in the No Bee populations in early generations, but improved substantially in generations 4–5. All measured traits differed significantly among populations in Generation 7 (Figure 1–3, Table 1–2; descriptive statistics are reported for all measured traits in Generations 6 and 7 in Tables 1–3 and 1–4). However, divergence was clearly explained by pollination treatment only for development rate (days to flower) and production of self-fertilized seed (Table 1–2). Despite large differences among populations, flower size was not clearly associated with treatment. The joint distribution of anther-stigma separation and self seed across populations (Figure 1–4) suggests that evolution in the former trait may be causally related to improvement of average plant fitness within the No Bee populations over time (Figure 1–2).

***Genetic divergence*—**All experimental populations maintained substantial allelic variation at our four putatively neutral markers (Table 1–5), although expected heterozygosity values were lowest in the No Bee populations. The mean inbreeding coefficient (F) was indistinguishable from zero in Generation 6 of the source population, which is expected given that these plants were generated by controlled outcrosses. F was moderate for Bee populations (F[B1] = 0.43, SE = 0.04; F[B2] = 0.33, SE = 0.04) and high for No Bee populations (F[A1] = 0.75, SE = 0.04;

F[A2] = 0.73, SE = 0.03). There were substantial changes in allele frequency at both chromosomal polymorphisms within experimental populations (Table 1–1; *LG6 Inversion*: X^2 = 137.8, DF = 8, $p < 0.0001$; *LG11 Drive locus:* $X^2 = 200.2$, DF = 8, $p < 0.0001$). The derived "alleles" for each (C, D) were at an intermediate frequency in the source population. C and D frequencies declined across experimental populations, but the reductions were substantially greater in the No Bee than Bee populations.

Hypothesis tests and estimates for quantitative genetic variance components (V_A and V_E) are summarized in Table 1–6. We cannot reject Model I (same V_A and V_E across all five populations) for average throat width (TW), average tube length (TL) or leaf width. The other corolla dimension (average CW) does exhibit marginally significant differences in V_A and V_E across populations. The most pronounced changes in variance components were observed for anther-stigma separation, days to flower, ln self seed, and the pollen traits. A consistent increase in V_A across experimental relative to the source population is evident for anther-stigma separation and ln self seed. Figure 1–5 illustrates the correspondence between mean values for ln self seed and the estimated heritability of this trait (obtained from Model II) across populations.

Predicting phenotypic from genetic divergence—In the source plants of Generation 6, the Inversion had significant effects on leaf width, anther-stigma separation, and pollen viability, while the Drive locus affected only pollen viability and ln total pollen (Table 1–7). Using these point estimates for effects on anther-stigma separation and pollen viability, our allele frequency estimates within populations A1, A2, B1, and B2 predict the amount of phenotypic change due to changes at the Inversion and Drive loci. Selection against C and D alleles appeared to explain a

large fraction of pollen viability evolution (Figure 1–3D**)**, as well as an incremental contribution to differences in anther-stigma separation **(**Figure 1–3C**)**.

DISCUSSION

Populations subjected to novel environmental stress are likely to have reduced reproductive success. If the stress is severe, populations may be driven extinct. However, theoretical (Lynch & Lande 1993; Gomulkiewcz & Holt 1995) and empirical (Bell & Gonzalez 2009) work has shown that populations with sufficient genetic variation may evolve rapidly enough to overcome extinction. The evolutionary rescue model predicts an initial population decline following environmental change, succeeded by an exponential increase of adapted genotypes. Our No Bee populations effectively experienced an immediate environmental change—a population adapted to outcrossing was denied pollinators. Mean fitness declined precipitously in the first two generations, but rebounded as selfing-efficient genotypes increased in frequency. The resulting population trajectories are thus consistent with evolutionary rescue (Figure 1–2).

Environmental conditions were not sufficiently harsh in the present experiment to provide a full test of the evolutionary rescue model. This is because pollinator loss did not cause negative population growth. Even in the first few generations, the No Bee populations were adequately fecund to seed the next generation at prescribed levels (absolute fitness > 1). In the field however, pollinator loss would likely cause negative population growth. Natural populations face harsher physical conditions and the potentially reinforcing effects of demographic stochasticity and inbreeding depression. A second issue is that while increased selfing may be advantageous

in the short term, this shift in mating system could prove detrimental in the long run. Elevated inbreeding has myriad consequences, including alteration of the distribution of genetic variation within populations, the balance between mutation and selection, the effective population size, gene flow, and metapopulation dynamics (Lande & Schemske 1985; Charlesworth 2003). Reduced genetic diversity may limit further adaptive evolution within a population and perhaps increase the likelihood of extinction (Stebbins 1957; Igic et al. 2008).

***Mating system evolution*—**The observed changes in the No Bee populations (A1 and A2) provide clear support for the reproductive assurance hypothesis—pollinator absence generated strong selection for traits that facilitate selfing. Across experimental populations, selfed seed set was strongly correlated with reduced anther-stigma separation (Figure 1–4); consistent with field results showing positive selection for reduced anther-stigma separation under pollen-limiting conditions (Fishman & Willis 2008). An unexpected outcome is that our Bee populations also evolved an increased capacity for selfing (Figures 1–3 through 1–5). Mean inbreeding coefficients at neutral markers are consistent with a selfing rate of approximately 0.5 in B1 and B2 (Table 1–5). In part, selection for selfing may be due to the fact that the 2-week pollination interval is nested within the flowering interval. Flowers that open before the arrival of bees, or after their departure, can only set seed by selfing or through physical contact between flowers of neighboring plants.

One hypothesis for frequent selfing in the Bee populations is that bumblebees were ineffective pollinators. Multiple lines of evidence speak against this explanation. Per capita seed set was uniformly high in the Bee populations, even in the first few generations when these populations

had limited capacity for autogamy (Figure 1–2). Second, previous experiments have shown that recurrent visitation of *M. guttatus* by *B. impatiens* yields seed set comparable to saturating hand-pollination (see Figure 2 of Arathi & Kelly 2004). In principle, visitation could induce selfing if a foraging bee carries pollen from anthers to stigma within a flower. However, *M. guttatus* has a touch sensitive stigma in which the pollen receiving surface quickly closes after first contact of the pollinator. This limits the opportunity for induced selfing. Direct evidence for infrequent induced selfing comes from a different set of experiments in which we measured seed set from the first 8 flowers (each on a different plant) visited by a bee on a foraging run (Julius P. Mojica, unpublished results). Bees were not likely carrying pollen at the beginning of a run and all flowers had not been previously visited. Thus, any seed from the first flowers visited should be due to selfing. An average 0.125 seeds were set by first flowers (SE = 0.125). In contrast, the average from flowers 3–8 of a foraging run was 29.8 (SE = 4.4). The striking difference in seed counts suggests that *Bombus impatiens* is an effective pollinator in this experimental system.

Other environmental features of our experiment may have favored intermediate selfing rates in our Bee populations. The automatic transmission advantage associated with selfing (Fisher 1941) implies that high inbreeding depression (ID) is necessary to maintain outcrossing even if pollination is not limiting (Lloyd 1979; Charlesworth & Charlesworth 1987). The Iron Mountain population does exhibit high ID (Willis 1993; Kelly 2003). However, the severity of ID is environmentally dependent (Carr & Dudash 1996; Armbruster & Reed 2005) and it is possible that ID is less severe under the growth conditions of this study. Perhaps more importantly, our populations were evolving with greatly reduced temporal variation in environmental conditions relative to the field. One of the major consequences and potential advantages of outcrossing is

the production of genetically variable progeny. Any advantage associated with variable progeny would likely be diminished in our experiment and might even have been a disadvantage.

The rapid changes in traits observed under our selection regime provide a useful contrast to the observed phenotypic divergence between *M. guttatus* and closely related but selfing species such as *M. nasutus* (Figure 1–1A), *M. micranthus*, and *M. cupriphilus*. Our No Bee populations evolved towards the "selfing phenotype" in some traits, but not others. As in the selfing species, anther-stigma separation decreased and self seed increased. However, corolla size was not substantially reduced in No Bee populations relative to Bee populations. Days to flower evolved opposite to the syndrome. Bee populations progress to flowering faster than No Bee populations (Table 1–3), on average within two days of when bumblebees first became available during each generation. These observations suggest that the evolution of selfing can occur in a sequential fashion, with change in some traits preceding others. The first steps may be the recruitment of standing genetic variation for traits key to reproductive assurance such as self seed production. Reduction in flower size may follow as secondary evolutionary response.

Fenster & Ritland (1994) suggested that pollinator limitation may have been responsible for the evolution of the selfing taxa *M. laciniatus* and *M. micranthus* and our results indicate the plausibility of this selective mechanism. However, Macnair & Cumbes (1989) proposed that selfing in *M. cupriphilus* evolved subsequent to colonization of copper mine tailings as a means to reduce maladaptive gene flow. Also, the selfing taxa in the complex tend to occupy relatively drier habitats than *M. guttatus* (e.g. Macnair et al. 1989; Kiang & Hamrick 1978) so edaphic adaptation might provide an alternative route for the evolution of selfing (see Macnair & Gardner

1999). Levin (2010) has recently argued that colonization of novel habitat may itself cause increased self-fertilization via plastic responses in traits like anther-stigma separation. In our experiment, physical conditions were constant and changes in mating system were genetically based and not due to plasticity. However, the experimental evolution methodology could be adapted to constant pollination with variable edaphic conditions and thus employed to investigate these alternative scenarios.

Major polymorphisms and the genetic basis of divergence—Large allele frequency changes occurred at both the *LG 6 Inversion* and the *LG 11 Meiotic Drive Locus* (Table 1–1). Previous studies had documented pronounced effects of both of these chromosomal polymorphisms on pollen viability; and strong but less consistent effects on floral pigmentation, rate of development and anther-stigma separation (Fishman & Saunders 2008; Scoville et al. 2009). In the current experiment, allele frequency changes at these two loci jointly explain most of the change in pollen viability (Figure 1–3D). Given that a major goal for Quantitative Trait Locus mapping is to understand morphological evolution at a genetic scale, these data provide an encouraging example. Unfortunately, we cannot determine whether these loci contributed to changes in ln self seed because this trait was not measured in Generation 6.

As noted in the Introduction, major polymorphisms may be critical to evolutionary rescue (Gomulkiewicz et al. 2010). The *Inversion* and *Drive Locus* are polymorphisms with major effects, but evolution at these loci may be more a consequence of mating system evolution than a cause. In particular, the observed increases in pollen viability are likely due to genetic purging. Inbreeding increases the frequency of homozygotes relative to heterozygotes and homozygosity

is apparently detrimental to the derived types (C and D) at each locus (Table 1–7; Fishman & Saunders 2008; Lee 2009; Scoville et al. 2009). The Driver (D) has a segregation advantage in female gamete formation of heterozygotes, but DD individuals have approximately 20% lower pollen viability. Less is known about the derived Inversion allele (C), but several experiments have found reduced pollen viability of CC plants. Homozygosity is highest in the No Bee populations, intermediate in the Bee populations, and lowest in the source population (Table 1–5).

An important contribution of major polymorphisms to standing variation is also suggested by changes in the additive genetic variance (V_A) of experimental populations (Figure 1–5; Table 1–6). If the genetic variance in a trait is due entirely to small contributions from many loci (the infinitesimal model), the variance should evolve much more slowly than the mean under directional selection. Changes in allele frequency at a major locus can cause V_A to increase or decrease, depending on allele frequencies (Agrawal et al. 2001). The apparent increase in V_A with increased ln self seed across populations (Figure 1–5) is worth considering from this perspective. The pattern suggests that alleles increasing autogamy may be uncommon and/or partly recessive in our source population. Selection favoring such alleles would have brought them to more intermediate frequencies, simultaneously increasing trait means and variances. Scalar epistasis is an alternative explanation for Figure 1–5 given that the source population is close to the lowest possible value for the trait (zero seed set). Interpretation is more straightforward for pollen viability given the direct measurements of causal loci (the Driver and the Inversion). Here, selection drove C and D from intermediate frequencies to relative rarity

(Table 1–1). This is likely the major cause for the striking reductions in V_A for pollen viability in A1, A2, and B1 relative to the source population.

Caveats—Our experimental populations were founded by genotypes from a single natural populations and the methods for population propagation were devised based on results from studies of that population. For example, microsattelite loci exhibit minimal spatial structure over the Iron Mountain site (Sweigart et al. 1999), and for this reason, we randomly mixed seed within populations between generations. However, plant populations routinely exhibit small scale structure owing to limited seed dispersal (Levin 1988), including other populations of *M. guttatus* (Ritland & Ganders 1987). In this situation, transfer of pollen between neighboring plants results in bi-parental inbreeding. Bi-parental inbreeding was excluded by design from this experiment, but could be an important factor in mating system evolution more generally (Uyenoyama et al. 1993).

Perhaps the most important issue, at least with regard to the generality of our results, is the amount and pattern of genetic variation for mating-system related traits. There are two reasons that the current experiment may ***underestimate*** the response to selection following pollinator loss. The first is that Iron Mountain contains only a fraction of the genetic variation present in *M. guttatus*, a species distributed broadly across the western United States. While the population is a natural unit for investigations of microevolution, response at the meta-population level might be greater if pollinator loss occurs on a broad geographic scale. Also, other populations of *M. guttatus* may actually have a greater initial capacity for response to selection for increased selfing. Iron Mountain is a highly outcrossing population (Willis 1993) and the genetic variance

in autogamy might be higher in populations with higher average selfing rates (e.g. Awadalla & Ritland 1997).

A second reason that we may be underestimating response is that our founding population contained only a subset of the genetic variation resident to Iron Mountain. All variation in the source population is due to segregation of heterozygosity from 3 F1 plants (see Methods). The sampling effect on our source population can be directly evaluated from the variance component estimates in Table 1–6. The estimated heritability of corolla width is 0.38 for the source population, exactly the value obtained from a large half sib / full sib breeding design (see Control population estimates from Table 3 of Kelly 2008). Source heritability estimates for anther-stigma separation, days to flower, and pollen number are lower than in the background population. The notable exception is pollen viability where the source population heritability (0.45) is about twice the estimate for the entire population (see Table 3 of Scoville et al. 2009). This can be largely explained by the fact that the Inversion and Drive polymorphisms, each with large effects on pollen viability, have more intermediate allele frequencies in our source than in the background population. Here, we note that the variance component estimates of Table 1–3 were obtained from outbred plants (Generation 7) created through controlled crosses (Generation 6). As a consequence, differences in genetic variances can be attributed to differences in allele frequencies. However, the estimates of Table 1–3 do not capture the effects of inbreeding on genetic variation owing to deviations from Hardy-Weinberg equilibrium or inter-locus associations. These associations likely affected response to selection, but were eliminated or at least diminished by the breeding design of Generations 6–7.

***Summary*—**The extent of phenotypic changes evident after only five generations indicates that rapid adaptation is possible if a population loses pollinators. Self-compatible, pollinator-reliant plants experiencing mounting human-induced environmental stress might adapt rapidly in the short term. However, the resulting decrease in genetic diversity may leave them vulnerable to extinction with further environmental alterations. We found that a substantial fraction of phenotypic change could be explained by large changes in allele frequency at two chromosomal polymorphisms; an intriguing observation in light of recent theoretical work on evolutionary rescue. The increase of genetic variance along with mean trait expression of self fecundity is consistent with a scenario in which rare alleles in the source population increased in frequency as populations evolved increased selfing. Finally, our results favor a sequential model for the evolution of the selfing syndrome. Traits essential to increasing selfing efficiency (e.g. anther-stigma separation) evolve first, followed by evolution of other characters (e.g. flower size) subsequent to mating system change.

LITERATURE CITED

Agrawal, A. F., Brodie, E. D. and L. H. Rieseberg. 2001. Possible consequences of genes of major effect: transient changes in the G-matrix. Genetica 112: 33–43.

Arathi, H. S. and J. K. Kelly. 2004. Corolla morphology facilitates both autogamy and bumblebee pollination in *Mimulus guttatus*. International Journal of Plant Science. 165: 1039–1045.

Armbruster, P. and D. H. Reed. 2005. Inbreeding depression in benign and stressful environments. Heredity 95: 235–242.

Awadalla, P. and K. Ritland. 1997. Microsatellite variation and evolution in the *Mimulus guttatus* species complex with contrasting mating systems. Molecular Biology and Evolution 14:1023–1034.

Baker, H. G. 1967. Support for Baker's Law-as a rule. Evolution 21: 853–856.

Bell, G. and A. Gonzalez. 2009. Evolutionary rescue can prevent extinction following environmental change. Ecology Letters 12: 942–948.

Cameron, S. A., Hines, H. M. and P. H. Williams. 2007. A comprehensive phylogeny of the bumble bees (*Bombus*). Biological Journal of the Linnean Society 91: 161–188.

Carr, D. E. and M. R. Dudash. 1996. Inbreeding depression in two species of *Mimulus* (Scrophulariaceae) with contrasting mating systems. American Journal of Botany 83: 586–593.

Carr, D. E. and C. B. Fenster. 1994. Levels of genetic variation and covariation for *Mimulus* (Scrophulariaceae) floral traits. Heredity 72: 606–618.

Charlesworth, D. and B. Charlesworth. 1987. Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics 18: 237–268.

Charlesworth, D. 2003. Effect of inbreeding on genetic diversity of populations. Philosophical Transactions of the Royal Society B: Biological Sciences 358: 1051–1070.

Committee on the Status of Pollinators in North America. 2007. Fruits of their labour: Status of pollinators in North America. National Academies Press, Washington D.C. 307 pages.

Dole. J. A. 1992. Reproductive assurance mechanisms in three taxa of the *Mimulus guttatus* complex (Scrophulariaceae). American Journal of Botany 79: 650–659.

Eckert, C. G., Kalisz, S., Geber, M. A., Sargent, R., Elle, E., Cheptou, P-O., Goodwillie, C., Johnston, M. O., Kelly, J. K., Moeller, D. A., Porcher, E. Ree, R. H., Vallejo-Marín, M., and A. A. Winn. 2009. Plant mating systems in a changing world. Trends in Ecology and Evolution 25: 35–43.

Fenster, C. B. and K. Ritland. 1994. Evidence for natural selection on mating system in *Mimulus* (Scrophulariaceae). International Journal of Plant Science 155: 588–596.

Fisher, R. A. 1941. Average excess and average effect of a gene substitution. Annals of Eugenics 11: 53–63.

Fishman, L., Kelly, A. J. and J. H. Willis. 2002. Minor quantitiative trait loci underlie floral traits associated with mating system divergence in *Mimulus*. Evolution 56: 2138–2155.

Fishman, L. and A. Saunders. 2008. Centromere-associated female meiotic drive entails male fitness costs in monkeyflowers. Science 322: 1559–1562.

Fishman, L. and J. H. Willis. 2008. Pollen limitation and natural selection on floral characters in the yellow monkeyflower, *Mimulus guttatus*. New Phytologist 177: 802–810.

Franklin, H. J. 1912. The Bombidae of the New World. Transactions of the American Entomological Society 38: 177–486.

Gomulkiewicz, R. and R. D. Holt. 1995. When does evolution by natural selection prevent extinction? Evolution 49: 201–207.

Gomulkiewicz, R., Holt, R. D., Barfield, M. and S. L. Nuismer. 2010. Genetics, adaptation and invasion in harsh environments. Evolutionary Applications 3: 97–108.

Grant, A. L. 1924. A monograph of the genus *Mimulus*. Annals of the Missouri Botanical Gardens 11: 99–398.

Grant, V. 1949. Pollination systems as isolating mechanisms in angiosperms. Evolution 3: 82–97.

Hall, M. C., and J. H. Willis. 2005. Transmission ratio distortion in intraspecific hybrids of *Mimulus guttatus*: Implications for genomic divergence. Genetics 170: 375–386.

Harris, D. L. 1964. Genotypic covariances between inbred relatives. Genetics 50: 1319–1348.

Hegland, S. J., Nielsen, A., Lázaro, A., Bjerknes, A.-L. and Ø. Totland. 2009. How does climate warming affect plant-pollinator interactions? Ecology Letteters 12: 184–195.

Holeski, L. M. and J. K. Kelly. 2006. Mating system and the evolution of quantitative traits: An experimental study of *Mimulus guttatus*. Evolution 60: 711–723.

Igic, B., Lande, R. and J. R. Kohn. 2008. Loss of self-incompatibility and its evolutionary consequences. International Journal of Plant Science 169: 93–104.

Ivey, C. T., and D. E. Carr. 2005. Effects of herbivory and inbreeding on the pollinators and mating system of *Mimulus guttatus* (Phrymaceae). American Journal of Botany 92: 1641–1649.

Kalisz, S., Vogler, D. W. and K. M. Hanley. 2004. Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. Nature 430: 884–887.

Kelly, J. K. and J. H. Willis. 2002. A manipulative experiment to estimate bi-parental inbreeding in Monkeyflowers. International Journal of Plant Science 163: 575–579.

Kelly, J. K. 2003. Deleterious mutations and the genetic variance of male fitness components in *Mimulus guttatus*. Genetics 164: 1071–1085.

Kelly, J. K. 2008. Testing the rare alleles model of quantitative variation by artificial selection. Genetica 132: 187–198.

Kelly, J. K. and H. S. Arathi. 2003. Inbreeding and the genetic variance of floral traits in *Mimulus guttatus*. Heredity 90: 77–83.

Kelly, J. K., Rasch, A. and S. Kalisz. 2002. A method to estimate pollen viability from pollen size variation. American Journal of Botany 89: 1021–1023.

Kiang, Y. T., and J. L. Hamrick. 1978. Reproductive isolation in the *Mimulus guttatus*-*M. nasutus* complex. American Midland Naturalist 100: 269–276.

Lande, R. and D. W. Schemske. 1985. The evolution of self-fertilization and inbreeding depression in plants. I. Genetic models. Evolution 39: 24–40.

Lee, Y. W. 2009. Genetic Analysis of Standing Variation for Floral Morphology and Fitness Components in a Natural Population of *Mimulus guttatus* (common monkeyflower). Biology, Ph.D. thesis. Duke University, Durham, N.C.

Levin, D. A. 1988. Local differentiation and the breeding structure of plant populations. Pp. 305–329. *In* L. D. Gottleib, and S. K. Jain, eds. Plant Evolutionary Biology. Chapman and Hall, New York.

Levin, D. A. 2010. Environment-enhanced self-fertilization: implications for niche shifts in adjacent populations. Journal of Ecology 98: 1276–1283.

Lloyd, D. G. 1979. Some reproductive factors affecting the selection of self-fertilization in plants. American Naturalist 113: 67–79.

Lynch, M. and R. Lande. 1993. Evolution and extinction in response to environmental change Pp. 234–250. *In* P. M. Kareiva, J. G. Kingsolver, and R. B. Huey, eds. Biotic Interactions and Global Climate Change. Sinauer, Sunderland, Mass.

Lynch, M. and K. Ritland. 1999. Estimation of pairwise relatedness with molecular markers. Genetics 152: 1753–1766.

Macnair, M. R. 1989. A new species of *Mimulus* endemic to copper mines in California. Botanical Journal of the Linnean Society 100: 1–14.

Macnair, M. R., and Q. J. Cumbes. 1989. The genetic architecture of interspecific variation in *Mimulus*. Genetics 122: 211–222.

Macnair, M. R., Macnair, V. E. and B. E. Martin. 1989. Adaptive speciation in *Mimulus*: an ecological comparison of *Mimulus cupriphilus* with its presumed progenitor *Mimulus guttatus*. New Phytologist 112: 269–279.

Macnair, M. R. and M. Gardner. 1999. The Evolution of Edaphic Endemics Pp. 157–171. *In* Howard, D.J., and Berlocher, S.H, eds. Endless Forms: Species and Speciation. Oxford University Press, Oxford.

Marriage, T. N., Hudman, S., Mort, M. E., Orive, M. E., Shaw, R. G. and J. K. Kelly. 2009. Direct estimation of the mutation rate at dinucleotide microsatellite loci in *Arabidopsis thaliana* (Brassicaceae). Heredity 103: 310–317.

Martin, N. 2004. Flower size preferences of the honeybee (*Apis mellifera*) foraging on *Mimulus guttatus* (Scrophulariaceae). Evolutionary Ecology 6: 777–782.

Memmott, J., Craze, P. G., Waser, N. M. and M. V. Price. 2007. Global warming and the disruption of plant-pollinator interactions. Ecology Letters 10: 710–717.

Mitchell, R. J., Karron, J. D., Holmquist, K. G. and J. M. Bell. 2004. The influence of *Mimulus ringens* floral display size on pollinator visitation patterns. Functional Ecology 18: 116–124.

Ornduff, R. 1969. Reproductive biology in relation to systematics. Taxon 18: 121–133.

Owen, R. E., Whidden, T. L. and R. C. Plowright. 2010. Genetic and morphometric evidence for the conspecific status of the bumbe bees, *Bombus melanopygus* and *Bombus edwardsii*. Journal of Insect Science. 10: 1–18.

Ritland, C. and K. Ritland. 1989. Variation of sex allocation among 8 taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). American Journal of Botany 76: 1731–1739.

Ritland, K. and F. R. Ganders. 1987. Covariation of selfing rates with parental gene fixation indexes within populations of *Mimulus guttatus.* Evolution 41: 760–771.

Robertson, A. W., Diaz, A. and M. R. MacNair. 1994. The quantitative genetics of floral characters in *Mimulus guttatus*. Heredity 72: 300–311.

Robertson, A. W., Mountjoy, C., Faulkner, B. E., Roberts, M. V. and M. R. Macnair. 1999. Flower choice by bumblebees collecting pollen from *Mimulus guttatus* (Scrophulariaceae): the effects of pollen quality and reward depletion. Ecology 80: 2594–2606.

Schemske, D. W. and H. D. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). Proceedings of the National Academy of Science 96: 11910–11915.

Scoville, A., Lee, Y. W., Willis, J. H. and J. K. Kelly. 2009. Contribution of chromosomal polymorphisms to the G-matrix of *Mimulus guttatus*. New Phytologist 183: 803–815.

Self, S. G. and K. Y. Liang 1987. Asymptotic properties of maximum likelihood estimators and likelihood ratio tests under nonstandard conditions. Journal of the American Statistical Association 82: 605–610.

Stebbins, G. L. 1957. Self-fertilization and population variability in higher plants. American Naturalist 91: 337–354.

Sweigart, A., Karoly, K., Jones, A. and J. H. Willis. 1999. The distribution of individual inbreeding coefficients and pairwise relatedness in a population of *Mimulus guttatus.* Heredity 83: 625–632.

Sweigart, A. L. and J. H. Willis. 2003. Patterns of nucleotide diversity are affected by mating system and asymmetric introgression in two species of *Mimulus*. Evolution 57: 2490–2506.

Thorp, R. W., Horning D. S. Jr., and L. L. Dunning. 1983. Bumble bees and cuckoo bumble bees of California. Bulletin of the California Insect Survey 23: 1–79.

Uyenoyama, M. K., Holsinger, K. E. and D. M. Waller. 1993. Ecological and genetic factors directing the evolution of self-fertilization. Oxford Surveys in Evolutionary Biology 9: 327–381.

van Kleunen, M. and K. Ritland. 2004. Predicting the evolution of floral traits associated with mating system in a natural plant population. Journal of Evolutionary Biology 17: 1389–1399.

Vickery, R. K. 1978. Case studies in the evolution of species complexes in *Mimulus.* Evolutionary Biology 11: 405–507.

Wang, J. 2011. COANCESTRY: a program for simulating, estimating and analysing relatedness and inbreeding coefficients. Molecular Ecology Resources 11: 141–145. doi: 10.1111/j.1755–0998.2010.02885.x

Willis, J. H. 1993. Partial self fertilization and inbreeding depression in two populations of *Mimulus guttatus*. Heredity 71: 145–154.

Willis, J. H. 1996. Measures of phenotypic selection are biased by partial inbreeding. Evolution 50: 1501–1511.

Willis, J. H. 1999a. Inbreeding load, average dominance, and the mutation rate for mildly deleterious alleles in *Mimulus guttatus.* Genetics 153: 1885–1898.

Willis, J. H. 1999b. The role of genes of large effect on inbreeding depression in *Mimulus guttatus.* Evolution 53: 1678–1691.

Wu, C. A., Lowry, D. B., Cooley, A. M., Wright, K. M., Lee, Y. W. and J. H. Willis. 2008. *Mimulus* is an emerging model system for the integration of ecological and genomic studies. Heredity 100: 220–230.

Table 1–1. Genotype counts for Generation 6 plants of each population at the Inversion and Drive chromosomal polymorphisms.

Inversion	cc	Cc	CC	Total	C-type frequency
Source	71	78	26	175	0.371
A1	145	2	1	148	0.014
A2	95	19	10	124	0.157
B1	106	45	11	162	0.207
B2	105	35	3	143	0.143
Drive	**dd**	**Dd**	**DD**	**Total**	**D-type frequency**
Source	41	96	40	177	0.497
A1	107	34	31	172	0.279
A2	138	11	17	166	0.136
B1	78	77	32	187	0.377
B2	36	67	55	158	0.560

Table 1–2. Tests for trait mean differences in Generation 7 for each measured trait. (**A)** Tests for differences among populations (population as factor)**.** (**B)** Tests with population nested within treatment.

(A)			**(B)**			
Population			**Treatment**		**Population (Treatment)**	
Trait	**F ratio**	**P value**	**F ratio**	**P value**	**F ratio**	**P value**
Days to flower	23.70	*<.0001*	45.91	*0.0228*	1.00	*0.3677*
Avg. CW (mm)	5.62	*0.0002*	1.91	*0.3456*	3.71	*0.0221*
Avg. TW (mm)	13.01	*<.0001*	1.82	*0.3541*	8.65	*0.0002*
Avg. TL (mm)	10.53	*<.0001*	2.10	*0.3230*	6.69	*0.0013*
Avg. A/S (mm)	36.92	*<.0001*	2.46	*0.2891*	19.19	*<.0001*
Leaf Width (mm)	11.86	*<.0001*	14.13	*0.0679*	1.48	*0.2273*
ln Total Pollen	13.67	*<.0001*	1.57	*0.4337*	10.68	*<.0001*
PV	14.02	*<.0001*	4.62	*0.1787*	4.81	*0.0083*
	LRT	**P value**	**LRT**	**P value**	**LRT**	**P value**
Self Seed	133.4	*<.0001*	109.8	*<.0001*	12.64	*0.0018*

Table 1–3: Least squares means and standard deviations are reported for each measured trait of Generation 6. Abbreviations are CW (Corolla Width), TW (Throat Width), TL (Tube Length), A/S (Anther-stigma Separation), and PV (Pollen Viability).

Generation 6		Population				
		No Bee		Bee		Source
Trait		A1	A2	B1	B2	F3M
Avg. CW (mm)	mean	17.59	18.00	18.49	17.14	18.63
	st. dev.	3.79	2.97	3.01	2.46	2.61
Avg. TW (mm)	mean	8.82	8.39	9.31	8.83	9.15
	st. dev.	1.46	1.27	1.45	1.16	1.28
Avg. TL (mm)	mean	11.45	10.86	11.92	11.27	11.58
	st. dev.	1.78	1.31	1.42	1.23	1.39
Avg. A/S (mm)	mean	1.66	1.96	1.50	1.91	2.32
	st. dev.	1.30	1.05	0.96	1.07	1.19
Leaf Width (mm)	mean	17.34	17.43	17.92	17.56	18.67
	st. dev.	4.59	4.51	4.64	4.79	4.35
ln Total Pollen	mean	4.82	4.66	5.16	5.08	4.98
	st. dev.	0.71	0.68	0.66	0.69	0.77
PV	mean	0.52	0.50	0.56	0.39	0.51
	st. dev.	0.23	0.24	0.22	0.21	0.25

Table 1–4: Least squares means and standard deviations for each measured trait of Generation 7. Abbreviations are as in Table 1–3, although additional traits are reported here.

Generation 7		Population				
		No Bee		Bee		Source
Trait		A1	A2	B1	B2	F4M
Days to Flower	mean	29.04	28.61	27.60	27.30	30.34
	st. dev.	4.28	4.09	3.30	3.65	4.47
Avg. CW (mm)	mean	18.39	17.96	18.54	17.94	17.40
	st. dev.	2.94	2.96	2.78	2.80	3.29
Avg. TW (mm)	mean	9.37	9.03	9.18	8.90	8.69
	st. dev.	1.02	1.14	1.11	1.14	1.17
Avg. TL (mm)	mean	11.54	11.09	11.54	11.34	10.87
	st. dev.	1.44	1.36	1.30	1.32	1.56
Avg. A/S (mm)	mean	0.86	1.45	1.67	1.92	1.86
	st. dev.	0.76	1.18	1.11	0.99	1.22
Leaf Width (mm)	mean	17.06	16.45	15.86	15.73	14.86
	st. dev.	3.30	3.68	3.57	3.81	3.87
Self Seed (raw)	mean	37.98	27.43	16.35	9.74	5.81
	st. dev.	51.30	42.54	36.00	20.59	14.34
ln Total Pollen	mean	9.10	9.12	9.28	9.07	8.94
	st. dev.	0.45	0.44	0.45	0.52	0.58
PV	mean	0.68	0.65	0.65	0.60	0.55
	st. dev.	0.18	0.19	0.18	0.20	0.24

Table 1–5: Neutral marker data for genotyped individuals of Generation 6. Unique alleles, defined by length in bp, are listed for each of four markers along with their respective frequency in each population. N is the total number of individuals genotyped from each population. H_E is the expected heterozygosity for each locus. H_O is the observed heterozygosity.

MARKER DATA		POPULATION				
	Marker **e461**	**A1**	**A2**	**B1**	**B2**	**Source**
ALLELE	*181*	0.00	0.05	0.11	0.08	0.11
	188	0.49	0.06	0.39	0.55	0.44
	192	0.50	0.90	0.50	0.37	0.45
	N	***112***	***180***	***161***	***145***	***183***
	H_E	0.50	0.19	0.59	0.56	0.59
	H_O	0.07	0.08	0.28	0.32	0.61
	Marker **e523**	**A1**	**A2**	**B1**	**B2**	**Source**
ALLELE	*211*	0.21	0.09	0.25	0.25	0.32
	213	0.78	0.90	0.72	0.73	0.67
	217	0.01	0.01	0.03	0.01	0.01
	N	***117***	***182***	***128***	***118***	***145***
	H_E	0.35	0.18	0.42	0.40	0.45
	H_O	0.06	0.09	0.28	0.34	0.37

Table 1–5. (Continued)

MARKER DATA		POPULATION				
	Marker **e755**	**A1**	**A2**	**B1**	**B2**	**Source**
ALLELE	*481*	1.00	0.96	0.87	0.90	0.83
	485	0.00	0.04	0.13	0.10	0.17
	N	***78***	***88***	***89***	***89***	***146***
	H_E	0.00	0.08	0.23	0.17	0.28
	H_O	0.00	0.04	0.16	0.12	0.24
	Marker **e641**	**A1**	**A2**	**B1**	**B2**	**Source**
ALLELE	*316*	0.38	0.50	0.40	0.34	0.27
	393	0.44	0.24	0.39	0.45	0.47
	410	0.18	0.25	0.21	0.21	0.26
	N	***94***	***134***	***163***	***129***	***185***
	H_E	0.63	0.62	0.65	0.64	0.64
	H_O	0.26	0.21	0.50	0.52	0.71

Table 1–6. Model-specific estimates of the variance components reported for each trait in each population. Variance estimates are standardized such that the trait variance of the Source population is 1. The LRT value next model 2 is the test of model 2 vs model 1, while the value next to model 3 is for model 3 vs model 2. For each trait, **bold** indicates the model favored by the LRT.

			V_E					V_A				
Trait	**Model**	**LRT**	Source	A1	A2	B1	B2	Source	A1	A2	B1	B2
Avg. CW	1		0.74					0.26				
	2	7.0	0.61					0.39	0.20	0.20	0.21	0.12
	3	**10.4**	**0.62**	**0.74**	**0.78**	**0.53**	**0.99**	**0.38**	**0.26**	**0.22**	**0.47**	**0.01**
Avg. TW	**1**		**0.78**					**0.22**				
	2	4.7	0.70					0.30	0.09	0.20	0.23	0.21
	3	6.2	0.64	0.83	0.81	0.66	0.94	0.36	0.17	0.19	0.34	0.06
Avg. TL	**1**		**0.68**					**0.32**				
	2	5.9	0.55					0.45	0.27	0.23	0.22	0.22
	3	9.3	0.71	0.81	0.68	0.55	0.67	0.29	0.19	0.32	0.45	0.33
Avg. A/S	1		0.80					0.20				
	2	28.3	0.56					0.44	0.00	0.26	0.28	0.11
	3	**38.3**	**0.98**	**0.71**	**0.86**	**0.72**	**0.82**	**0.02**	**0.29**	**0.14**	**0.28**	**0.18**
Days to Flower	1		0.92					0.08				
	2	16.5	0.71					0.29	0.14	0.21	0.00	0.03
	3	**16.5**	**1.00**	**0.97**	**0.72**	**1.00**	**0.92**	**0.00**	**0.03**	**0.28**	**0.00**	**0.08**

Table 1–6. (Continued)

			V_E					V_A				
Trait	**Model**	**LRT**	Source	A1	A2	B1	B2	Source	A1	A2	B1	B2
Leaf Width	**1**		**0.68**					**0.32**				
	2	6.4	0.57					0.43	0.16	0.26	0.29	0.25
	3	5.2	0.69	0.76	0.65	0.55	0.84	0.31	0.24	0.35	0.45	0.16
ln Total Pollen	1		0.83					0.17				
	2	23.4	0.54					0.46	0.10	0.00	0.10	0.23
	3	**13.4**	**0.93**	**0.76**	**1.00**	**0.66**	**0.86**	**0.07**	**0.24**	**0.00**	**0.34**	**0.14**
ln Self Seed	1		0.69					0.31				
	2	**17.5**	**0.98**					**0.02**	**0.66**	**0.63**	**0.44**	**0.25**
	3	1.5	1.00	0.57	0.69	0.66	0.76	0.00	0.43	0.31	0.34	0.24
PV	1		0.82					0.18				
	2	**25.0**	**0.55**					**0.45**	**0.05**	**0.06**	**0.05**	**0.19**
	3	2.2	0.70	0.91	0.90	0.86	0.78	0.30	0.09	0.10	0.14	0.22

Table 1–7. The effect of chromosomal polymorphisms on traits in Source plants of Generation 6. **(A)** F-test summary with chromosomal polymorphism states scored 0 (ancestral homozygote), 1 (heterozygotes), and 2 (derived homozygote). * denotes significance when α = 0.05, ** significance when α = 0.001. **(B)** Effect summary of chromosomal polymorphism on source trait means for all significant F-tests in panel A.

(A) One-way ANOVAs with genotype as factor

Polymorphism:	**Inversion [C]**				**Drive [D]**			
	df num	**df dem**	**F ratio**	**P value**	**df num**	**df dem**	**F ratio**	**P value**
Avg. CW	2	172	2.31	0.103	2	176	0.46	0.635
Avg. TW	2	172	2.64	0.074	2	176	0.26	0.775
Avg. TL	2	172	1.35	0.263	2	176	0.31	0.734
Avg. A/S	2	172	6.06*	0.003	2	176	1.53	0.218
Leaf Width	2	172	4.17*	0.017	2	176	1.45	0.237
ln Total Pollen	2	170	2.61	0.077	2	174	4.65*	0.011
PV	2	170	6.45*	0.002	2	174	18.88**	<.0001

(B) Polymorphism effect on Source		**Derived allele count**	
Inversion [C]	**Ancestral mean**	1	2
Avg. A/S (mm)	1.97	0.63	0.64
Leaf Width (mm)	18.81	0.49	-2.35
PV	0.59	-0.13	-0.17
Drive [D]			
ln Total Pollen	8.24	-0.26	-0.53
PV	0.65	-0.11	-0.32

Figure 1–1. (A) Trait differences between outcrossing and selfing *Mimulus* congeners—anterior perspective of flowers from *Mimulus guttatus* (left) and *M. nasutus* (right). **(B)** Diagram of floral measurements. *M. guttatus* flower, dorsal view, with upper corolla removed to show reproductive structures. Floral measures reported in study depicted with brackets.

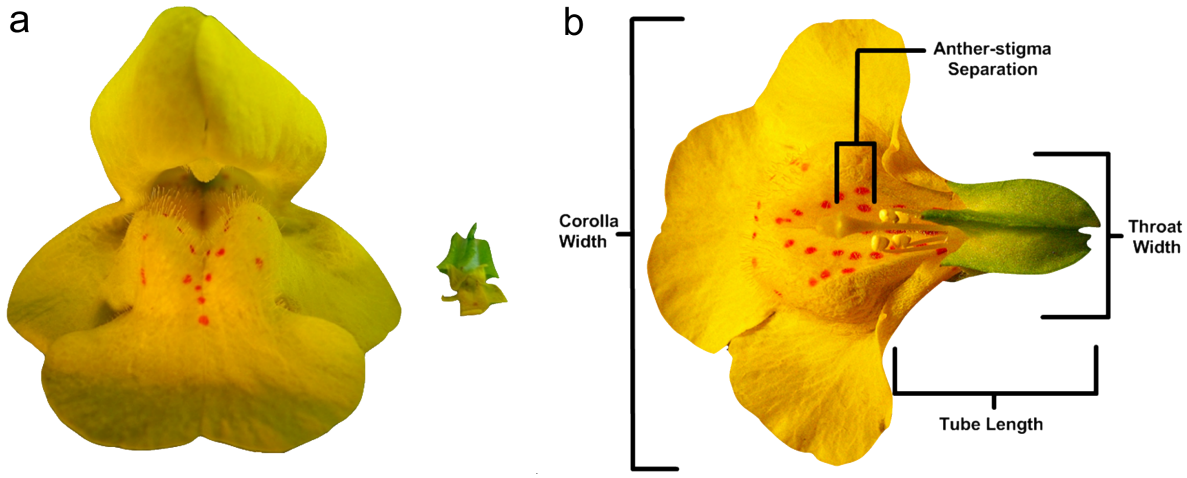


Figure 1–2. Population fitness over five generations of evolution. Per capita seed production (mg) for each population and generation calculated by dividing bulked seed collected from each population by the number of adult plants. As a consequence, standard errors could not be estimated.

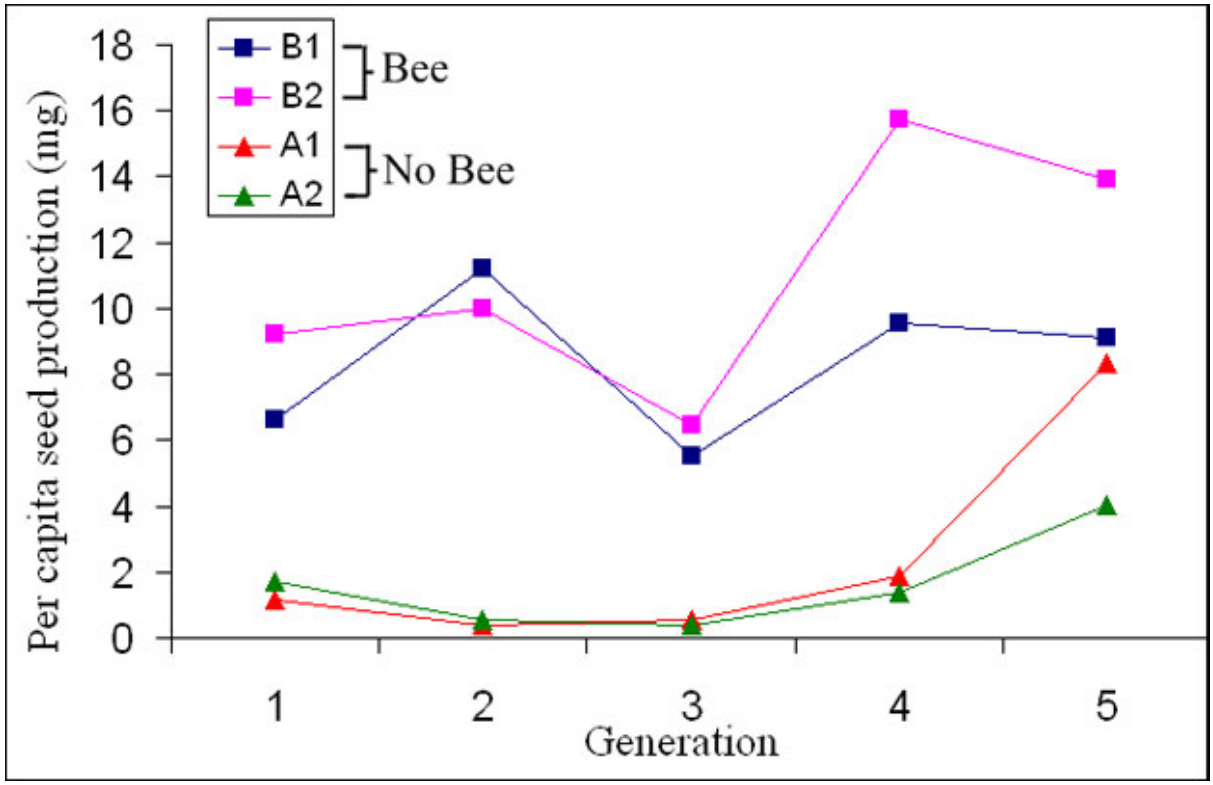


Figure 1–3. Character divergence and chromosomal polymorphism effects. Population trait means are given for (**A**) corolla width, (**B**) self seed, (**C**) anther-stigma separation, and (**D**) pollen viability after five generations of selection for the No Bee (A1, A2), Bee (B1, B2), and source populations. Means sharing letters above bars are not significantly different from one another (Tukey-Kramer post-hoc). Error bars are +/- 1 SEM. In panels (**C, D)**, the predicted effects of the two chromosomal polymorphisms are depicted with arrows for each population (direction and magnitude of trait change expected given the allele frequency difference from the source population). Values at arrow origins are predicted units of trait change. Panel (**C**) includes only the Inversion while (**D**) is combined effects of Inversion and Drive on pollen viability.

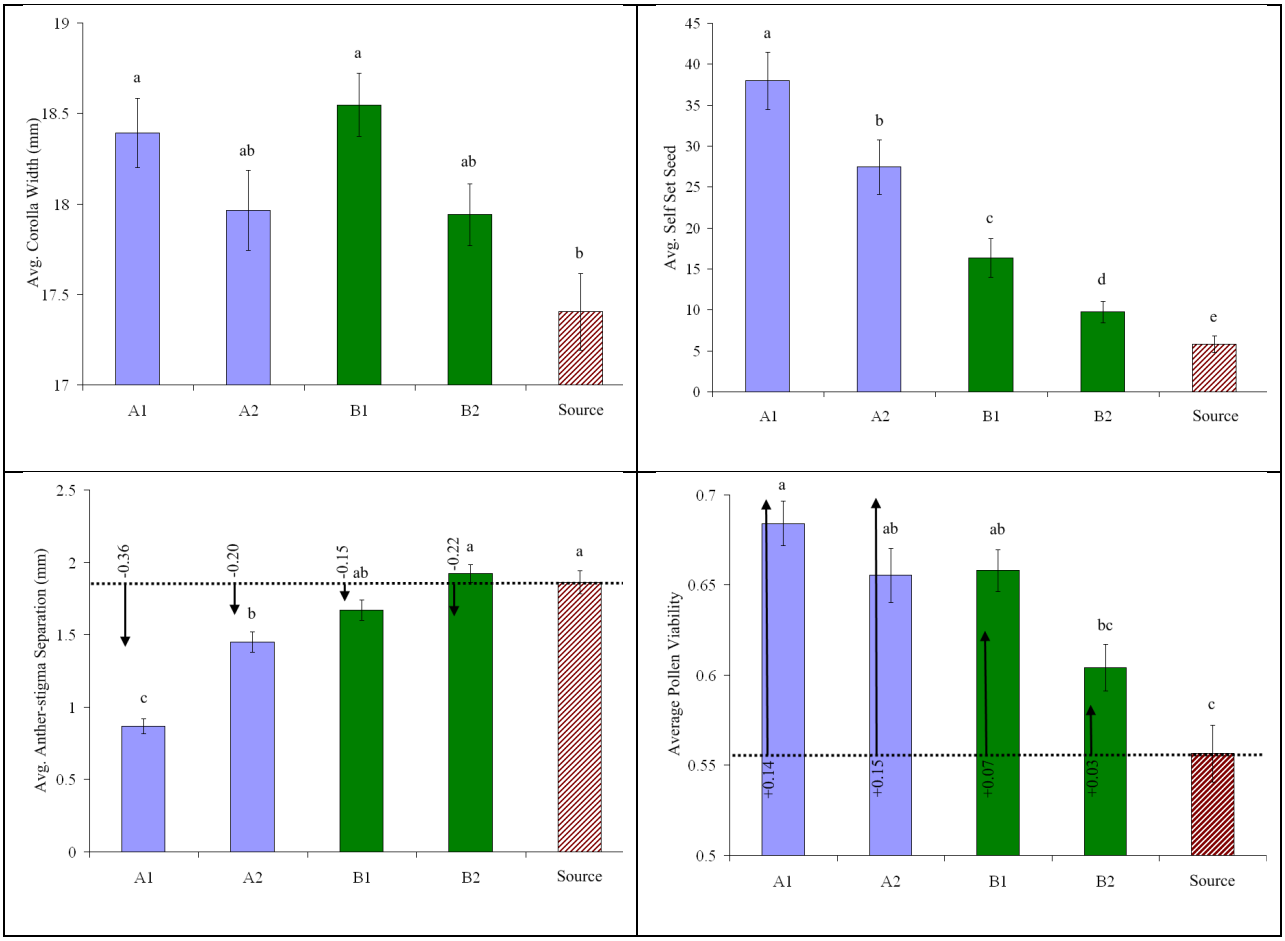


Figure 1–4. Bivariate plot of the negative relationship between mean anther-stigma separation and mean self seed by experimental population for Generation 7. Error bars are +/- 1 SEM.

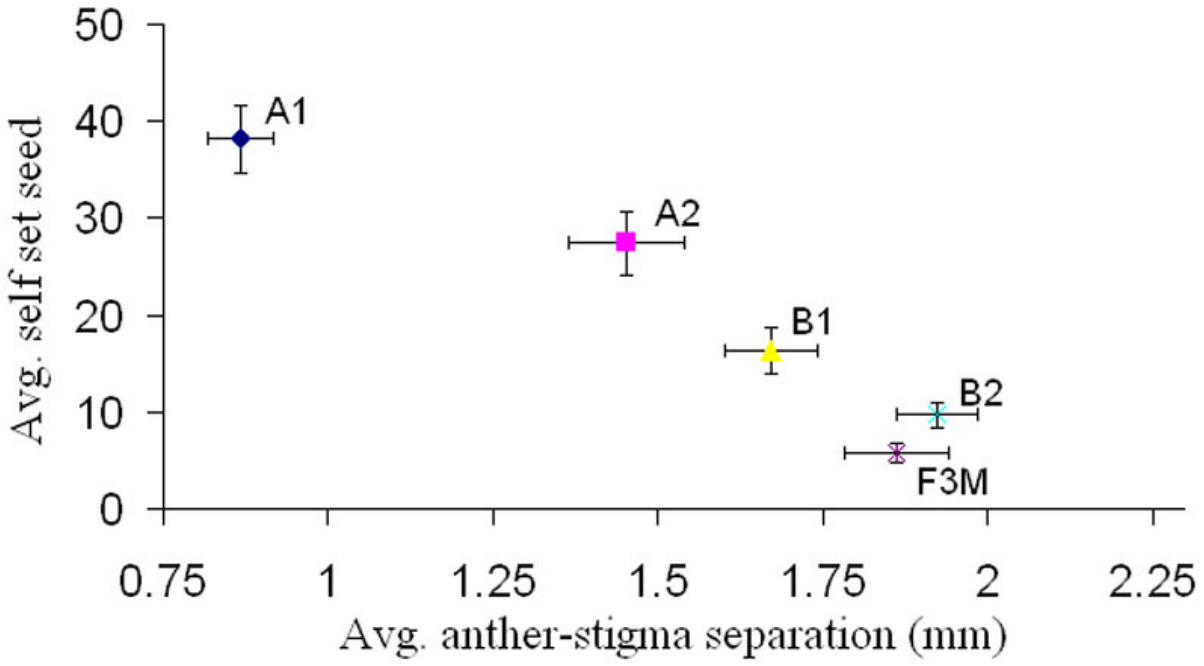
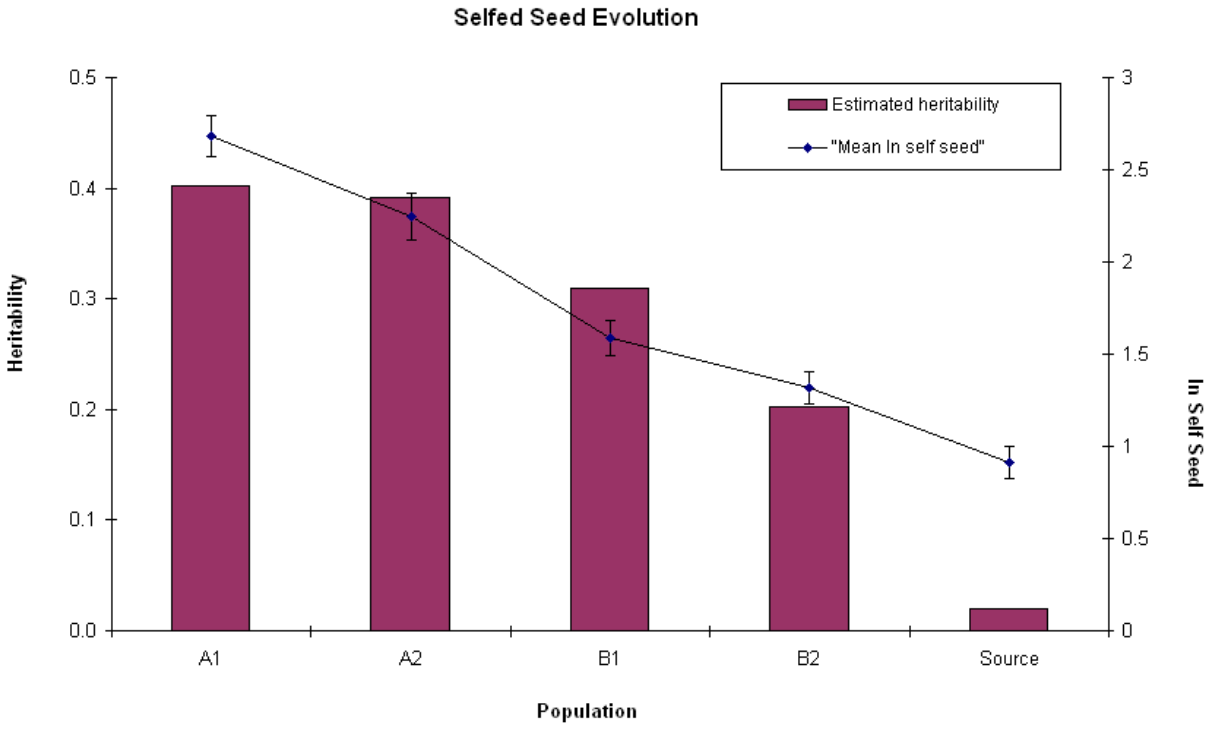


Figure 1–5. Mean autogamy positively covaries with heritability in Generation 7 plants. Error bars are +/- 1 SEM.



Chapter 2. 'Dropping the *Bombus'*: continued adaptation to pollinator loss in *Mimulus guttatus.*

ABSTRACT

Despite extensive theory regarding mating system transitions observed throughout angiosperm history, empirical studies demonstrating the rate and nature of plant adaptation are lacking. This study is the continuation of an experiment examining the immediate evolutionary consequences of pollinator loss within a primarily outcrossing *Mimulus* (monkeyflower) population. Experimental populations experienced four additional generations of pollination treatments: abundant bumblebee pollinators vs. no pollinators. Populations continued to diverge in some floral, developmental, and life history traits, but not others. One floral character, tube length, diverged significantly by treatment type, paralleling the extended floral morphology observed in a naturally occurring *Mimulus* selfing lineage. Elevated levels of self-fertilization continued in populations without pollinators (No Bee), but no additional evolution was observed. Rapid allele frequency changes continued at two chromosomal polymorphisms, evidence of selection and purging in the No Bee populations. The results provide additional support for the hypothesis that mating system transitions from outcrossing to selfing occur sequentially.

INTRODUCTION

Plants employ a diverse array of reproductive strategies from asexual vegetative cloning to complete dependence on a single pollinator species, including a variety of mixed mating (both

outcrossing and self-fertilizing) strategies in between (Darwin 1876, 1877; Barrett 1995; Charlesworth 2006). Mirroring the diversity of reproductive strategies, floral phenotypes in angiosperms range from large, showy, outcrossing flowers to greatly reduced, self-fertilizing flowers (Ritland & Ritland 1989; Sicard & Lenhard 2011). Despite the variety and complexity of plant mating system strategies, most studies of mating system evolution have focused on the importance of a single comparison: the relative amounts of fertilization through outcrossing and selfing (Lloyd & Schoen 1992). Traditional theory predicts that plant populations should evolve to be either fully outcrossing or fully selfing (Fisher 1941; Lloyd 1979; Lande & Schemske 1985), with mixed mating strategies only functioning as temporary transitional states. However, the frequency of mixed mating observed in natural populations exceeds expectations of populations currently undergoing a mating system transition (Goodwillie et al. 2005). The evolutionary processes maintaining mixed mating have yet to be definitively determined in any plant species and the proposed underlying theory remains controversial (Flaxman 2000; Plaistow et al. 2004; Goodwille et al. 2005). The factors contributing to selection for mixed mating systems are numerous and fall into two main categories: environmental and genetic. Environmental factors include frequency dependent selfing, pollen discounting, reproductive assurance, and variable rates of pollination. Some genetic factors are overdominance, biparental inbreeding, and selective interference (Lande et al. 1994, Winn et al. 2011, review in Goodwillie et al. 2005).

The evolutionary transition from outcrossing to selfing has never been directly observed in nature but it has occurred repeatedly in angiosperms and is associated with changes in floral biology, life history, and ecology (Grant 1949; Ornduff 1969; Barrett et al. 1996). The most

widely cited ecological forces prompting the evolution of selfing are limited mate availability and pollinator loss, whereby selfing ability provides reproductive assurance (Darwin 1876; Stebbins 1957; Lloyd 1992). Alternative hypotheses include rapid adaptation to novel, challenging environments, such as adaptation to toxic copper mine tailings (Macnair et al. 1989), and anti-herbivory selection for smaller flowers that incidentally increase self-fertilization via allometric decreases in the physical distance between male and female reproductive parts, called herkogamy (Eckert et al. 2006). Highly selfing plant taxa routinely have reduced flowers, less vegetative mass, reduced herkogamy, and develop more rapidly than closely related outcrossing taxa— a set of features commonly called the 'selfing syndrome' (Ornduff 1969, Sicard & Lenhard 2011). The order in which selfing syndrome traits evolve is a long-standing question in plant biology, but our recent work suggests a pattern of sequential evolution (Bodbyl Roels & Kelly 2011).

Concerns about anthropogenic environmental change have fueled research investigating potential impacts on plant mating systems, such as the effects of pollinator loss (Eckert et al. 2009). The likelihood of plant mating systems adapting to a rapidly changing planet depends not only on the selection pressures initiating mating system transitions but also the extent to which populations are capable of evolving (i.e. extent of heritable variation). Theoretical work suggests populations may avoid extinction by rapidly increasing the frequency of adaptive genotypes, if sufficient genetic variation is present in traits essential to survival and reproduction under the novel conditions (Lynch & Lande 1993; Gomulkiewicz & Holt 1995). This 'evolutionary rescue' has been demonstrated via experimental evolution in yeast (Bell & Gonzalez 2011) and perhaps in monkeyflowers (Bodbyl Roels & Kelly 2011).

Inbreeding depression (ID) is thought to be fundamental to the evolution of mating systems (e.g. Nagylaki 1976; Lloyd 1979; Charlesworth & Charlesworth 1987; Charlesworth et al. 1990; Winn et al. 2011). The rate of mating system transitions in plants depends, in part, on the strength of ID in a population and whether or not it can be reduced or eliminated by inbreeding. ID is a reduction in fitness of inbred relative to outbred individuals (Darwin 1876; Wright 1977; Charlesworth & Charlesworth 1987). Most ID is due to deleterious, partially recessive alleles (e.g. Charlesworth & Charlesworth 1987; Dudash & Carr 1998, Charlesworth & Willis 2009) that are exposed to selection as inbreeding increases homozygosity. The purging hypothesis (Charlesworth & Charlesworth 1987) posits that accumulated genetic load, or lowered mean population fitness, should be reduced as inbreeding occurs over time. Thus, highly selfing populations are expected to have lower expression of ID while outcrossing populations are expected to accumulate genetic load. The range of ID expressed in mixed-mating populations is predicted to fall between that of exclusive outcrossing and selfing, and be dependent on the history of selfing in the population (Lloyd 1979). However, recent work has demonstrated an overabundance of mixed mating taxa with high ID, potentially maintained in an evolutionary stable state by selective interference (Winn et al. 2011).

The number of deleterious alleles present in a genome and their effects on fitness influence the rate at which they can be purged. Theoretical models indicate few deleterious alleles of large effect can be rapidly purged from a population while many deleterious alleles of small effect will persist in the short term and perhaps even fix with genetic drift in small populations (Lande & Schemske 1985; Charlesworth et al. 1990; Shultz & Willis 1995; Lynch 1996). Recent

Kondrashov model simulations estimate purging rates of moderately deleterious alleles to be rapid (200 generations) and mildly deleterious alleles to be much slower (12,000 generations—Winn et al. 2011). The rate at which populations are capable of reducing ID through purging is essential for predicting a response to mating system selection pressures. Populations capable of rapid ID reduction may quickly evolve self-fertilization following various environmental changes, such as a loss of pollinators (Lande & Schemske 1985). In contrast, strong ID may slow or even prevent transitions to selfing; extensive purging may result in too few selfing adaptive genotypes persisting in a population for continued existence (e.g. Lynch et al. 1995; Lynch 1996). However, purging is not a guaranteed outcome of inbreeding and can be prevented by strong selection or extreme recessivity (Lande et al. 1994; Kelly 2007).

This chapter describes the continuation of an experimental evolution study examining the immediate consequences of pollinator loss in a primarily outcrossing *Mimulus guttatus* (yellow monkeyflower) population (Bodbyl Roels & Kelly 2011). The initial study documented rapid phenotypic and genetic changes that increased self-fertilization capacity in populations with no access to pollinators. Our results suggested a sequential model for the evolution of selfing, wherein traits essential to self-fertilization evolve first and other traits typically observed in natural selfing populations (e.g. small flower size) may evolve in further generations. By extending the study through additional generations of selection, we addressed the following questions:

1) What are the continuing fitness effects of a transition to selfing?

2) If mating system traits evolve sequentially, what is the order and timescale of trait evolution?

3) Does genetic load present in a mixed-mating population hinder the mating system transition to selfing?

METHODS

We grew plants from bulk seed produced by each experimental population (A1, A2, B1, B2) from Generation 5 of the previous study following the protocol in Bodbyl Roels & Kelly 2011. Populations experienced four additional generations (6–9) of bumbleebee vs. no pollinator treatments; we exposed bee treatment population replicates (Bee = B1, B2) to a hive of *Bombus impatiens* for two weeks during their flowering period and withheld pollinators from no bee treatment population replicates (No Bee = A1, A2), as in the previous study. After bulk seed was collected from Generation 9, we grew 250 individuals per population in individual 2" X 2" pots, along with the Source population (F5M) from the previous study. We recorded a number of phenotypic measurements on these individuals, named Generation (Gen) 10. We first recorded the number of experiment days from seed sowing to flowering (Days to Flower) for the first flower produced by each plant. We next measured corolla width (CW) and anther-stigma separation (A/S, also herkogamy) for both the first and second flowers. We then marked the third and fourth flowers produced by each plant and allowed them to self-fertilize naturally. Finally, we averaged seed collected from the two matured pods per plant, resulting in the variable Self Seed.

After phenotypic measures were complete, we randomly arranged individuals within each population in family groups of three. Within each group, one sire pollinated two dams, creating

fully outcrossed half-sibling progeny, hereafter referred to as Generation 11. We planted individuals from each cross in four cohorts. Cohorts 1 and 2 grew in the greenhouse from April to June. Cohort 1 contained a single representative from 109, 118, 109, and 116 families of populations A1, B1, B2, and the Source, respectively, and two representatives from 60 A2 families. Cohort 2, planted two weeks after Cohort 1, contained a single representative from each family. Cohorts 3 and 4 grew from January to April of the following year. Cohort 3 sampled 100 individuals per population and Cohort 4 sampled 250 individuals per population, maximizing family diversity. As for Gen 10, we recorded multiple phenotypic traits in Gen 11. We recorded Days to Flower for the first flower of individuals in all cohorts of Gen 11. Next we averaged floral measures CW, throat width (TW), tube length (TL), and A/S for the first two flowers of Cohorts 1, 2, and 3 (See Figure 1B of Bodbyl Roels & Kelly 2011). At anthesis of the second flower, we measured the width of the largest leaf (Leaf Width). A trait not considered in the previous study, the distance on the plant stem between the first (above the cotyledons) and second internode, we measured at anthesis of the first flower (Internode). We assayed all four cohorts for self-fertilized seed production. The third and fourth flowers produced by Cohorts 1 and 2 naturally self fertilized; we averaged seed counts by individual (Self Seed). We harvested self-fertilized seed for Cohorts 3 and 4 from the fourth flower produced by each plant. For Cohorts 1 and 2, we recorded the mass of the self-fertilized seeds from each plant (Seed Mass) and averaged Seed Mass by the count of seeds produced by each plant (Mass/Seed). Trait values referred to hereafter are population means unless noted otherwise.

We sampled apical meristem and young leaf tissue from 96 individuals of each population of Gen 10 for genotyping. We assessed population allele frequencies at the same four gene-based

markers [MgSTS_461, MgSTS_641, MgSTS_755, MgSTS_523] as in the previous study. We also genotyped populations at the same two chromosomal polymorphisms as in the previous study, the chromosomal inversion [markers e229, e431] and the female meiotic drive locus [marker HB5]. The length of the HB5 allele is 277 base pairs (bp) at the derived locus classification, D, and we scored all other allele lengths as ancestral, d. We identified the derived inversion haplotype, C, by a combination of alleles at two marker loci: allele length 240 at marker locus MgSTS_431 and allele length 201 at MgSTS_229. We scored all other genotypes as ancestral, c.

Analyses—To assess phenotypic trait divergence among populations in Gen 11, we used one-way ANOVA with Tukey-Kramer post hoc tests. To determine if trait divergence was significant by treatment type, we also applied two-level ANOVA to each trait with population (random effect) nested within pollination treatment (fixed effect). One trait, Self Seed, was highly right skewed with many zero values. For this variable, we applied an overdispersed Poisson model (GLM) with the log link function. We additionally characterized self-fertilization with a binomial variable, Autogamy, coding plants as either producing self seed (1) or not (0). Reported as a population mean, Autogamy is the proportion of plants producing at least one selfed seed.

To compare trait evolution among generations, we standardized trait divergence by subtracting experimental population trait means (A1, A2, B1, and B2) from the Source population mean for each generation. This method compared the fully outbred Gen 11 to the fully outbred Gen 7 of the previous study and compared Gen 10 to Gen 11. We performed all statistical analyses using

JMP® 8.0.2 (SAS Institute Inc., 2009) and Minitab® 16.1.1 (Minitab Inc., 2010).

RESULTS

PHENOTYPE EVOLUTION

We measured phenotypic traits on a total of 1,156 and 2,578 individuals in Gens 10 and 11, respectively (Tables 2–1, 2–2). Comparing populations of the fully outbred Gen 11, all measured traits significantly diverged by population (One-way ANOVA, all $p < 0.001$, Table 2–3). Traits significant by *treatment* in Gen 11 were TL, Autogamy, and Self Seed (Table 2–3). No Bee populations demonstrated a *ten-fold* increase in Self Seed with respect to the Source population. Bee populations doubled Self Seed with respect to the Source (Table 2–2, Figure 2–1). No Bee populations also had elevated Autogamy relative to Bee and Source populations (Table 2–2). TL increased in the No Bee populations relative to Bee and Source populations (Table 2–2, Figure 2–1). Days to Flower was nearly significant (Table 2–3), with Bee populations flowering more quickly than No Bee and Source populations (Table 2–2).

Other informative traits divergent among populations but not treatments include Leaf Width, Mass/Seed, A/S and Internode. Leaf Width increased in all experimental populations, with the greatest gains occurring in the No Bee populations (Table 2–2, Figure 2–1). Self Seed and Mass/Seed revealed differences in seed allocation when considered by treatment type; No Bee populations produced more total seed than Bee and Source populations, but individual seeds weighed more in Bee populations (Table 2–2). A/S separation was not significant by treatment type (Table 2–3) but No Bee populations had reduced herkogamy when compared with the other

populations (Table 2–2, Table 2–4). Internode distances increased for populations B1, B2, and A2 over the 9 generations of selection when compared to the Source population (Table 2–2, Table 2–4).

Among generation comparisons—We compared six traits (Days to Flower, CW, TL, A/S, Leaf Width, and Self Seed) from the fully outbred Gen 11 to those of the fully outbred Gen 7 of the previous study (Figure 2–1). We observed no substantial divergence (difference of population mean from source mean) from the previous study in CW and A/S, with the exception of an increase in CW by population A1 in Gen 11. Days to Flower, TL, Leaf Width, and Self Seed all diverged from Gen 7 in mean trait expression. Gen 11 experimental populations flowered from 1.5 to 3 days earlier than the Gen 7 Source population (Figure 2–1). Leaf width increased in all Gen 11 populations (Figure 2–1). TL increased in No Bee populations, but not in Bee populations (Figure 2–1). The trait Self Seed decreased in Gen 11 Bee populations, i.e. the difference from Source was reduced relative to same contrast for Gen 7 plants. Two-sample t-tests for mean Self Seed among Gen 7 and Gen 11 by population found significant differences for only populations B1 and Source (Table 2–5).

We compared patterns of divergence in four traits (Days to Flower, CW, A/S, and Self Seed) across the transition from Gen 10, with populations variably inbred, to Gen 11, with all populations equivalently outbred. We observed no significant divergence between generations for A/S and Self Seed (Figure 2–2). In Days to Flower, population B1 flowered later in Gen 10 and earlier in Gen 11, relative to the Source. For CW, both Bee populations had larger flowers relative to the Source in Gen 10 and smaller flowers in Gen 11.

GENETIC DIVERGENCE

Neutral markers—Changes in allele frequency were more pronounced for No Bee populations than Bee and Source populations in the four putatively neutral markers (Table 2–6). Additionally, No Bee populations displayed substantially less heterozygosity than expected (Table 2–6). Considering the allele frequencies reported in Gen 6 of the previous study (Supplemental Table 3, Bodbyl Roels & Kelly 2011, Chapter 1), Gen 10 No Bee populations continued divergence of allele frequencies at each marker; however, we observed very little change in Bee and Source populations. Specifically, markers e461, e755, and e641 had nearly identical frequencies of each allele as those in Gen 6. The only exception was marker e523, in which the frequency of allele size 217 increased in both Bee and Source populations.

Polymorphisms—Gen 10 chromosomal polymorphism allele frequencies are reported in Table 2–7. The derived allele of the meiotic drive locus (D) maintained an intermediate frequency in the Bee and Source populations and reduced frequency in the No Bee populations. Comparison with the previous study (Gen 6 frequencies of 0.50, 0.56, 0.38, 0.13, 0.28, for Source, B2, B1, A2, and A1, respectively) revealed the derived allele continued to be selected against only in No Bee populations. The derived allele for the *chromosomal inversion* (C) was completely lacking in No Bee populations (Table 2–7) compared with the previous study (Gen 6 frequencies of 0.37, 0.14, 0.21, 0.16, 0.01, for Source, B2, B1, A2, and A1, respectively). Bee and Source population allele frequencies for the inversion remained equivalent to those in Gen 6.

DISCUSSION

Summary of previous results—Our No Bee experimental populations experienced one of the key environmental stressors hypothesized to select for selfing in nature: a rapid, novel loss of pollinators. Thus, we initially predicted phenotypic trait evolution in a direction consistent with the selfing syndrome. Our expectation was fulfilled in some traits, but not others. In the first five generations of the study, No Bee populations rapidly responded to pollinator loss by evolving heightened self-fertilization ability. Self seed production was negatively correlated with herkogamy, the distance between the reproductive parts within the flower. Evolution towards reduced herkogamy was likely a mechanistic driver for increased seed production. Flowering phenology also evolved, but observed differences in phenology were attributed more to experimental design (e.g. timing of bumblebee pollination) and to trait plasticity than to the mating system shift. A substantial proportion of phenotypic divergence among populations was explained by allele frequency changes in two chromosomal polymorphisms. No other traits showed significant among treatment divergence.

After an additional four generations of experimental evolution, traits immediately essential to selfing in previous generations, A/S and Self Seed, remained significantly divergent by treatment type but did not show continued evolution from the previous study (Figure 2−1). Instead, flower length (TL), increased in No Bee populations relative to Bee and Source populations, in agreement with flower shape divergence predicted by comparisons between naturally selfing and outcrossing taxa (Grant 1949; Ornduff 1969; Corbet 2000). In *Mimulus,* the flowers of the selfing *M. nasutus* are nearly twice as long (TL) as they are wide (CW), while *M. guttatus*

flowers are twice as wide as they are long (Fishman et al. 2002). Flower length has also been demonstrated to correlate with ovule number and was favored in a previous pollen-limitation study of *M. guttatus* (Fishman & Willis 2008). Reduced floral size is a common feature of selfing lineages throughout angiosperms (Grant 1949; Ornduff 1969; Barrett et al. 1996), including *Mimulus* selfing species *M. nasutus, M. platycalyx, M. micranthus,* and *M. cupriphilus* (Fishman et al. 2002; Ritland & Ritland 1989; Dole 1992; Carr & Fenster 1994; Macnair 1989). Large flowers typically function in pollinator attraction (review in Kingsolver et al. 2001; Elle & Carney 2003), thus reduced resource allocation to showy corollas is expected as populations transition to selfing. The lack of CW reduction observed in the No Bee populations did not follow the predicted course of selfing trait evolution, but benevolent greenhouse conditions, where individuals were not resource limited, may have contributed to this trend.

The observed lack of reduced floral morphology in No Bee populations may also be influenced by the timing of self-fertilization. Mixed mating populations of *M. guttatus,* including the Iron Mountain population in this study, primarily display delayed selfing (Dole 1990, 1992; Arathi et al. 2002, but see Dudash & Ritland 1991; Leclerc-Potvin & Ritland 1994). Delayed selfing is self-fertilization occurring after the opportunity for outcrossing has passed (Lloyd 1979, 1992; Sakai 1995), satisfying the reproductive assurance hypothesis (Darwin 1876; Baker 1955, 1967). Delayed selfing is selectively advantageous when mates are abundant but pollinators are unreliable (e.g. Kalisz et al. 1999) and delayed selfing incurs no cost (e.g. pollen discounting) if pollinators are abundant (Holsinger 1991). Delayed selfers are not predicted to experience selection for reduced flower size because maintenance of pollinator-attracting traits facilitates outcrossing when pollinators are available (Armbruster et al. 2002; Kalisz et al. 2012). However,

prior selfing, occuring before opportunity to outcross via pollinators (Lloyd 1979), is promoted by reduced A/S and expected to produce reduced allocation to floral size (Charlesworth & Charlesworth 1987; Lloyd 1987; Takebayashi & Morrell 2001).

In contrast to *M. guttatus,* selfing lineages such as *M. nasutus* produce flowers, often cleistogamous, that prior self (Diaz & MacNair 1998). Although reduced A/S separation was correlated with increased Self Seed in our experimental populations (Figure 4, Bodbyl Roels & Kelly 2011), No Bee populations had not evolved significantly reduced A/S with respect to other populations after nine generations of selection. Thus, there may be a fundamental shift from delayed to prior selfing that occurs in the mating system transition from outcrossing to selfing, preceding the evolution of reduced floral traits. Previous work with crosses between *M. nasutus* and *M. guttatus* provide some support for this hypothesis; F_2 hybrids all possessed *M. guttatus*-like non-cleistogamous flowers (Fishman et al. 2002). Fishman et al. suggest cleistogamy (and thus prior selfing) in *M. nasutus* may be a threshold trait of sorts, responding to both the external environment, internal resources and genetic environment (Lila Fishman, pers. comm.). Further research is warranted to clarify the relationship between selfing mode and floral trait evolution in *Mimulus.*

Inbred vs. Outbred Comparisons—Comparing the fully outbred individuals of Gen 11 to individuals with varying inbreeding coefficients in the previous Gen 10 provided an opportunity to observe trait specific effects of inbreeding. Traits unaffected by the genotypic rearrangement caused by inbreeding (e.g. deviations from Hardy-Weinberg equilibrium or inter-locus associations) would not be expected to change from Gen 10 to Gen 11. Four traits (Days to

Flower, CW, A/S, and Self Seed) showed no inbreeding effects in No Bee populations (Figure 2–2). However, two traits (CW and Days to Flower) reversed sign, from Gen 10 to 11. Bee populations had larger CW than the Source in Gen 10, but smaller CW in Gen 11. Similarly, population B1 flowered more quickly than the Source in Gen 10, but more slowly in Gen 11. The sign reversals, potentially signatures of outbreeding depression or heterosis in the Bee populations, would only be expected with some self-fertilization. In our previous study, we estimated inbreeding coefficients for Bee populations at 0.30–0.40. Moderate levels of inbreeding in Gen 10 Bee populations, either due to selfing or pollinator-assisted geitonogamy, could result in the observed trait mean differences from the fully outbred Gen 11. However, I interpret between-generation comparisons cautiously because Gen 7 and Gen 11 were not grown simultaneously, thus large environmental variance among generations is probable. A more rigorous test of inbreeding or outbreeding depression could be performed by comparing trait means among simultaneously grown individuals of Gen 6, 7, 10 and 11.

Phenotypic plasticity—Identical genotypes may express dramatically different phenotypes due to interaction between the environment and developmental processes; this variation is known as phenotypic plasticity (see Scheiner 1993). The inconsistent patterns observed in among generation comparisons of flowering phenology are likely due to the highly plastic nature of the trait. Our previous study estimated the proportion of environmental variance in flowering date found in our populations to range from 0.72–1.00 (Bodbyl Roels & Kelly 2011, Table 3), high values for a quantitative trait. The four Gen 11 cohorts likely experienced different environmental conditions, such as variation in daily mean temperatures, because they were grown at different times of the year. A comparison across cohorts shows significant G x E

interaction for Days to Flower. The reaction norms for each population were lower for the late spring planted cohorts (1 and 2) than for the early spring cohorts (3 and 4, Figure 2–3A), but no significant crossovers by treatment type were observed. Of all of our measured traits, flowering phenology is the most likely to be selected upon in nature to be plastic. The Source population for this experiment was originally derived from Iron Mountain, Oregon. This *M. guttatus* population inhabits a montane slope seep and experiences highly variable yearly growing conditions where maintaining a plastic flowering phenology may be an adaptive advantage. Despite the apparent plasticity in Days to Flower, it is still a heritable trait with demonstrated evolution in *Mimulus* (e.g. Kiang & Hamrick 1978; Willis 1999a; Hall & Willis 2006; Kelly & Mojica 2011). Through all nine generations of the experiment, No Bee populations were observed to flower at a slower pace than the initial Source population; the trend toward rapid flowering may be due to greenhouse adaptation and a weak selection pressure created by discarding individuals not flowering within ~60 days.

The phenotypic plasticity observed in Days to Flower implied seasonality in the environmental conditions of the greenhouse. Thus, an important consideration for our long-term study is whether or not selfing ability also contained a large G x E component. A comparison across cohorts for Self Seed also displayed differences in reaction norms for each population, but the trait did not appear to be correlated with the observed plasticity in flowering phenology (Figure 2–3B). In other words, the rapid flowering time present in Cohorts 1 and 2 did not correlate with either an increase or decrease in self-fertilized seed production.

GENETIC BASIS OF DIVERGENCE

Genes with large phenotypic effects may play a role in the immediate response to mating system selection (Gomulkiewicz et al. 2010). The *Meiotic Drive* locus on linkage group 11 (alternative alleles D/d) and the *LG6 inversion* (C/c) are major chromosomal polymorphisms in *M. guttatus* that contribute to variation in pollination traits. The Drive locus has phenotypic effects on pollen viability, herkogamy, and floral development rate (Scoville et al. 2009). Similarly, the Inversion affects pollen and development traits (Lee 2009; Scoville et al. 2009) although estimated floral trait effects are ambiguous, differing among mapping crosses (Scoville et al. 2009). Derived alleles (D/C) of both the Drive and Inversion polymorphisms initially segregated at 0.50 and 0.37, respectively, in the Source population. We expected the derived alleles would decline in No Bee populations because homozygosity, increased by inbreeding, is detrimental to both derived types due to reductions in pollen viability (Fishman & Saunders 2008; Lee 2009; Scoville et al. 2009). Our expectation was met in all experimental populations for the full 9 generations of the experiment, with No Bee treatments most affected (Table 2–7, also see Table 3 of Bodbyl Roels & Kelly 2011). By Gen 10, the drive allele (D) was at a low frequency and there was a complete loss of the derived inversion allele (C) in the No Bee populations. However, Bee population allele frequencies for the Drive and Inversion loci (0.40 and 0.13, respectively) did not change since Gen 6, perhaps reaching new equilibria (Table 2–7, Table 3 of Bodbyl Roels & Kelly 2011). A similar pattern was observed in the neutral markers, where continued allele frequency changes and increased homozygosity from the previous study only occurred in No Bee populations (Table 2–6). Thus, the rapid response phase (Gens 1–5) of our No Bee populations to pollinator loss was dominated by allele frequency changes in standing variation of genes with large fitness effects while a second phase (Gens 6–9) began to accumulate polygenic, minor

effects (as proposed in Orr 1998). A detailed study of QTL underlying floral divergence between *M. guttatus* and its selfing descendant *M. nasutus*, supports the hypothesis that floral traits frequently observed in established selfing populations accumulate slowly (Fishman et al. 2002). Floral traits mapped to many polygenic QTL with small effects, suggesting that the evolution of selfing in *M. nasutus* was a process of gradual adaptation rather than a single mutational step.

As populations transition from outcrossing to selfing, theory predicts a rapid loss of inbreeding depression (ID) as genetic load is purged, if load is due to a few genes of large effect (Lande & Schemske 1985; Charlesworth & Charlesworth 1987; Charlesworth et al. 1990). Inbreeding depression is high in *M. guttatus* (Dole & Ritland 1993; Willis 1993; Latta & Ritland 1994; Dudash et al. 1997) and primarily due to mildly deleterious, partially recessive alleles (Dudash and Carr 1998; Willis 1999a,b). In our study, the derived alleles of the two chromosomal polymorphisms reduced fitness in homozygotes and, accordingly, their frequencies in No Bee populations rapidly declined over the duration of the nine generation study. The polymorphisms represent a few deleterious genes of large effect, contributing to ID in mixed-mating populations of *Mimulus,* which are rapidly purged during a mating system transition to selfing. Willis (1999a) quantified rapid purging of similar genes of large effect when inbreeding *M. guttatus*. Additionally, no outbreeding depression or heterosis was observed when the No Bee populations were outcrossed in Gen 11, despite large amounts of inbreeding depression observed in the fitness of No Bee populations in Gens 1–5 (Figure 2, Bodbyl Roels & Kelly 2011). The lack of heterosis when outbred, combined with a ten-fold increase in self-fertilized seed production from origin of the experiment, suggests No Bee populations experienced substantial purging of genetic load as well as a rapid increase in alleles favorable for selfing.

Future directions—Naturally self-fertilizing populations have adapted to an entire series of environmental factors shaping the evolutionary trajectory of plant populations over time. Our experiment investigates the most immediate consequences of pollination loss but we recognize that our experimental protocols do not completely reflect the time scale or complexity of environmental forces that shape wild selfing lineages. We propose three additional tests, using our experimental evolution framework, to further elucidate the rate and nature of the mating system shift from outcrossing to selfing. First, repeat the entirety of this experiment in highly controlled environmental conditions (e.g. growth chamber). The benefits of a controlled environment are twofold, eliminating much of the confounding environmental variation and allowing for much more precise estimates of phenotypic response. Second, duplicate the entire study in the field, substantially increasing the effects of environmental variance and the likelihood of population extinction, but providing a more realistic scenario. Third, study selfing evolution from a genomic perspective (as highlighted in Sicard & Lenhard 2011) by locating novel, highly divergent genetic regions between mixed-mating and selfing populations, and determining the effects of those regions on phenotype. The third test is currently being developed in our lab and is expected to provide new insight into the genetic architecture of mating system divergence.

CONCLUSION

Our two-part study is the first to quantify the evolutionary response of plant populations experiencing pollinator loss. The initial phase of adaptation, with five generations of pollinator

treatments, is reported in Chapter 1. Here, results from four additional generations of pollinator treatments indicate continuing phenotypic and genetic evolution in some, but not all, floral, life history, and reproductive traits. Floral length increased in No Bee populations, as expected in comparison with other selfing lineages. Production of self fertilized seed remained high in No Bee populations, but trait means were unchanged from those in Chapter 1. Elevated homozygosity and evidence of purging were also observed in the No Bee populations. The lack of an evolutionary trend towards reduced floral morphology in No Bee populations is contrary to expectations based on naturally selfing *Mimulus* lineages; possible explanations include benevolent greenhouse conditions and the mode of self-fertilization. Greenhouse adaptation, experimental design, and specific selection pressures imposed by the *Bombus* pollinators were likely causes of the divergence in Bee populations from the original Source population. Data from this extension of our previous study support our prior conclusion: mating system transitions from outcrossing to selfing occur sequentially, with traits essential to continued survival and reproduction evolving first. Our two-part study, spanning 9 selective generations, demonstrates that rapid mating system evolution is possible for plant populations experiencing sudden pollinator loss.

LITERATURE CITED

Arathi, H. S., Rasch, A., Cox, C., and J. K. Kelly. 2002. Autogamy and floral longevity in *Mimulus guttatus*. International Journal of Plant Science 163: 567–753.

Armbruster, W. S., Mulder, C. P. H., Baldwin, B. G., Kalisz, S., Wessa, B., and H. Nute. 2002. Comparative analysis of late floral development and mating-system evolution in tribe Collinsieae (Scrophulariaceae, s.l.). American Journal of Botany 89: 37–49.

Baker H. G. 1955. Self-compatibility and establishment after "long distance" dispersal. Evolution 9: 347–348.

Baker H. G. 1967. Support for Baker's Law as a rule. Evolution 21: 853–856.

Barrett, S. C. H. 1995. Mating-system evolution in flowering plants: micro- and macroevolutionary approaches. Acta Botanica Neerlandica. 44: 385–402.

Barrett, S. C. H., Harder, L. D., and A. C. Worley. 1996. The comparative biology of pollination and mating in flowering plants. Philosophical Transactions Royal Society B 351: 1271–1280.

Bell, G. and A. Gonzalez. 2011. Adaptation and evolutionary rescue in metapopulations experiencing environmental deterioration. Science 332: 1327–1330. doi: 10.1126/science.1203105

Bodbyl Roels, S. A. and J. K. Kelly. 2011. Rapid evolution caused by pollinator loss in *Mimulus guttatus*. Evolution 65: 2541–2552.

Carr, D. E. and C. B. Fenster. 1994. Levels of genetic variation and covariation for *Mimulus* (Scrophulariaceae) floral traits. Heredity 72: 606–618.

Charlesworth, D., and B. Charlesworth. 1987. Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics 18: 237–268.

Charlesworth, D., Morgan, M. T., and B. Charlesworth. 1990. Inbreeding depression, genetic load, and the evolution of outcrossing in a multilocus system with no linkage. Evolution 44: 1469–1498.

Charlesworth, D. 2006. Evolution of plant breeding systems. Current Biology 16: R726–R735. doi:10.1016/j.cub.2006.07.068

Charlesworth, D. and J. H. Willis. 2009. The genetics of inbreeding depression. Nature Reviews Genetics 10: 783–796.

Corbet, Sarah A. 2000. Butterfly nectaring flowers: butterfly morphology and flower form. Entomologia Experimentalis et Applicata 96: 289–298.

Darwin, C. 1876. The effects of cross- and self-fertilization in the vegetable kingdom. John Murray, London, UK.

Darwin, C. 1877. The different forms of flowers on plants of the same species. John Murray, London, UK.

Diaz, A., and M. R. MacNair. 1998. The effect of plant size on the expression of cleistogamy in *Mimulus nasutus.* Functional Ecology 12: 92–98.

Dole, J. A. 1990. Role of corolla abscission in delayed self-pollination of *Mimulus guttatus* (Scrophulariaceae). American Journal of Botany 77: 1505–1507.

Dole, J. A. 1992. Reproductive assurance mechanisms in three taxa of the *Mimulus guttatus* complex (Scrophulariaceae). American Journal of Botany 79: 650–659.

Dole, J. and K. Ritland. 1993. Inbreeding in two *Mimulus* taxa measured by multigenerational changes in the breeding coefficient. Evolution 47: 361–373.

Dudash, M. R., and K. Ritland. 1991. Multiple paternity and self-fertilization in relation to floral age in *Mimulus guttatus* (Scrophulariaceae). American Journal of Botany 78: 1746–1753.

Dudash, M. R., Carr, D. E., and C. B. Fenster. 1997. Five generations of enforced selfing and outcrossing in *Mimulus guttatus:* Inbreeding depression variation at the population and family level. Evolution 51: 54–65.

Dudash, M. R. and D. E. Carr. 1998. Genetics underlying inbreeding depression in *Mimulus* with contrasting mating systems. Nature 393: 682–684.

Eckert, C. G., Samis, K. E, and S. Dart. 2006. Reproductive assurance and the evolution of uniparental reproduction in flowering plants. Pp. 183−203. *In* L. D. Harder and S. C. H. Barrett, eds. The Ecology and Evolution of flowers. Population English Edition. Oxford University Press, UK.

Eckert, C. G., Kalisz, S., Geber, M. A., Sargent, R., Elle, E., Cheptou, P-O., Goodwillie, C., Johnston, M. O., Kelly, J. K., Moeller, D. A., Porcher, E. Ree, R. H., Vallejo-Marín, M., and A. A. Winn. 2009. Plant mating systems in a changing world. Trends in Ecology and Evolution 25: 35–43.

Elle, E. and R. Carney. 2003. Reproductive assurance varies with flower size in *Collinsia parviflora* (Scrophulariaceae). American Journal of Botany 90: 888−896.

Fisher, R. A. 1941. Average excess and average effect of a gene substitution. Annals of Eugenics 11: 53–63.

Fishman, L., Kelly, A. J., and J. H. Willis. 2002. Minor quantitative trait loci underlie floral traits associated with mating system divergence in *Mimulus.* Evolution 56: 2138–2155.

Fishman, L., and A. Saunders. 2008. Centromere-associated female meiotic drive entails male fitness costs in monkeyflowers. Science 322: 1559–1562.

Fishman, L., and J. H. Willis. 2008. Pollen limitation and natural selection on floral characters in the yellow monkeyflower, *Mimulus guttatus.* New Phytologist 177: 802–810.

Flaxman, S. M. 2000. The evolutionary stability of mixed strategies. Trends in Ecology and Evolution 15: 482–84.

Goodwillie, C., Kalisz, S., and C. G. Eckert. 2005. The evolutionary enigma of mixed mating systems in plants: occurrence, theoretical explanations, and empirical evidence. Annual Review of Ecology, Evolution, and Systematics 36: 47–79.

Grant, V. 1949. Pollination systems as isolating mechanisms in angiosperms. Evolution 3: 82–97.

Gomulkiewicz, R. and R. D. Holt. 1995. When does evolution by natural selection prevent extinction? Evolution 49: 201–207.

Gomulkiewicz, R., Holt, R. D., Barfield, M. and S. L. Nuismer. 2010. Genetics, adaptation and invasion in harsh environments. Evolutionary Applications 3: 97–108.

Hall, M. C. and J. H. Willis. 2006. Divergent selection on flowering time contributes to local adaptation in *Mimulus guttatus* populations. Evolution 60: 2466–2477.

Holsinger, K. E. 1991. Mass-action models of plant mating systems: the evolutionary stability of mixed mating systems. American Naturalist 138: 606–622.

Kalisz, S., Vogler, D., Fails, B., Finer, M., Shepard, E., Herman, T., and R. Gonzales. 1999. The mechanism of delayed selfing in *Collinsia verna* (Scrophulariaceae). American Journal of Botany 86: 1239–1247.

Kalisz, S., Randle, A., Chaiffetz, D., Faigeles, M., Butera, A., and C. Beight. 2012. Dichogamy correlates with outcrossing rate and defines the selfing syndrome in the mixed mating genus *Collinsia.* Annals of Botany 109: 571–582. doi:10.1093/aob/mcr237

Kelly, J. K. 2007. Mutation-selection balance in mixed mating populations. Journal of Theoretical Biology 246: 355–365.

Kelly, J. K. and J. P. Mojica. 2011. Interactions among flower size QTLs of *Mimulus guttatus* are abundant but highly variable in nature. Genetics 189: 146–171.

Kiang, Y. T. and J. L. Hamrick. 1978. Reproductive isolation in the *Mimulus guttatus – M. nasutus* complex. American Midland Naturalist 100: 269–276.

Kingsolver, J. G., Hoekstra, H. E., Hoekstra, J. M., Berrigan, D., and S. N. Vigneri. 2001. The strength of phenotypic selection in natural populations. American Naturalist 157: 245–261.

Lande, R. and D. W. Schemske. 1985. The evolution of self-fertilization and inbreeding depression in plants. 1. Genetic models. Evolution 39: 24–40.

Lande, R., Schemske, D.W., and S. T. Schultz. 1994. High inbreeding depression, selective interferences among loci, and the threshold selfing rate for purging recessive lethal mutations. Evolution 48: 965–978.

Latta, R. and K. Ritland. 1994. The relationship between inbreeding depression and prior inbreeding among populations of four *Mimulus* taxa. Evolution 48: 806-817.

Leclerc-Potvin, C. and K. Ritland. 1994. Modes of self-fertilization in *M. guttatus* (Scrophulariaceae): a field experiment. American Journal of Botany 81: 199–205.

Lee, Y. W. 2009. Genetic Analysis of Standing Variation for Floral Morphology and Fitness Components in a Natural Population of *Mimulus guttatus* (common monkeyflower). Biology, Ph.D. thesis. Duke University, Durham, N.C.

Lloyd, D. G. 1979. Some reproductive factors affecting the evolution of self-fertilization in plants. American Naturalist 113: 67–79.

Lloyd, D. G. 1987. Allocation to pollen, seeds, and pollination mechanisms in self-fertilizing plants. Functional Ecology 1: 83–89.

Lloyd, D. G. 1992. Self- and cross-fertilization in plants. II. The selection of self-fertilization. International Journal of Plant Sciences 153: 370–380.

Lloyd, D. G. and D. J. Schoen. 1992. Self- and cross- fertilization in plants. I. Functional dimensions. International Journal of Plant Science 153: 358–369.

Lynch, M. 1996. A quantitative-genetic perspective on conservation issues. Pp. 471–501. *In* J. Avise and J. Hamrick, eds. Conservation genetics: case histories from nature. Chapman and Hall, New York.

Lynch, M. and R. Lande. 1993. Evolution and extinction in response to environmental change Pp. 234–250. *In* P. M. Kareiva, J. G. Kingsolver, and R. B. Huey, eds. Biotic Interactions and Global Climate Change. Sinauer, Sunderland, Mass.

Lynch, M., Conery, J. and R. Burger. 1995. Mutation accumulation and the extinction of small populations. American Naturalist 146: 489–518.

Macnair, M. R. 1989. A new species of *Mimulus* endemic to copper mines in California. Botanical Journal of the Linnean Society 100: 1–14.

Macnair, M. R., Macnair, V. E., and B. E. Martin. 1989. Adaptive speciation in *Mimulus*: an ecological comparison of *M. cupriphilis* with its presumed progenitor, *M. guttatus*. New Phytologist 112: 269–279.

Nagylaki, T. 1976. A model for the evolution of self-fertilization and vegetative reproduction. Journal of Theoretical Biology 58: 55–58.

Ornduff, R. 1969. Reproductive biology in relation to systematics. Taxon 18: 121–133.

Orr, H. A. 1998. The genetics of adaptation: the distribution of factors fixed during adaptive evolution. Evolution 52: 935–949.

Plaistow, S. J., Johnstone, R. A., Colegrave, N., and M. Spencer. 2004. Evolution of alternative mating tactics: conditional versus mixed strategies. Behavioral Ecology 15: 534–42

Ritland, C. and K. Ritland. 1989. Variation of sex allocation among eight taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). American Journal of Botany 76: 1731–1739.

Sakai, S. 1995. Evolutionary stable selfing rates of hermaphroditic plants with competing and delayed selfing modes with allocation to attractive structures. Evolution 49: 557–564.

Scheiner, S. M. 1993. Genetics and evolution of phenotypic plasticity. Annual Review of Ecology and Systematics 24: 35–68.

Schultz, S. T. and J. H. Willis. 1995. Individual variation in inbreeding depression: the roles of inbreeding history and mutation. Genetics 141: 1209–1223.

Scoville, A., Lee, Y. W., Willis, J. H. and J. K. Kelly. 2009. Contribution of chromosomal polymorphisms to the G-matrix of *Mimulus guttatus*. New Phytologist 183: 803–815.

Sicard, A. and M. Lenhard. 2011. The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptations in plants. Annals of Botany 107: 1433–1443.

Stebbins, G. L. 1957. Self-fertilization and population variability in higher plants. American Naturalist 91: 337–354.

Takebayashi, N. and P. L. Morrell. 2001. Is self-fertilization an evolutionary dead end? Revisiting an old hypothesis with genetic theories and a macroevolutionary approach. American Journal of Botany 88: 1143–1150.

Veitia, R. A. and D. Vaiman. 2011. Exploring the mechanistic bases of heterosis from the perspective of macromolecular complexes. FASEB Journal 25: 476–482. doi: 10.1096/fj.10-170639

Willis, J. H. 1999a. The role of genes of large effect on inbreeding depression in *Mimulus guttatus*. Evolution 53: 1678–1691.

Willis, J. H. 1999b. Inbreeding load, average dominance and the mutation rate for mildy deleterious alleles in *Mimulus guttatus.* Genetics 153: 1885–1898.

Winn, A. A., Elle, E., Kalisz, S. K., Cheptou, P-O., Eckert, C. G., Goodwillie, C., Johnston, M. O., Moeller, D. A., Ree, R. H., Sargent, R. D., and M. Vallejo-Maŕın. 2011. Analysis of inbreeding depression in mixed mating plants provides evidence for selective interference and stable mixed mating. Evolution 65: 3339–3359. doi:10.1111/j.1558-5646.2011.01462.x

Wright. S. 1977. Evolution and the Genetics of Populations, vol. 3: Experimental Results and Evolutionary Deductions. University of Chicago Press, Chicago, IL. 662 pages.

Table 2–1. Least squares means and standard deviations are reported for each measured trait of Generation 10. Abbreviations: Corolla Width (CW), Anther-stigma Separation (A/S).

Generation 10		Population				
		No Bee		Bee		Source
Trait		A1	A2	B1	B2	F5M
Days to Flower	mean	27.43	28.76	27.44	25.03	26.24
	st. dev.	1.63	2.46	2.20	1.31	1.38
Avg. CW (mm)	mean	15.02	14.56	15.10	15.36	14.60
	st. dev.	2.49	2.25	2.38	2.21	2.19
Avg. A/S (mm)	mean	0.68	0.99	1.55	1.74	1.82
	st. dev.	0.60	0.76	0.73	0.69	0.67
Self Seed	mean	26.49	29.82	22.19	12.71	7.79
	st. dev.	29.52	32.18	29.03	21.54	14.91
Autogamy	mean	0.88	0.89	0.83	0.68	0.62
	st. dev.	0.33	0.31	0.38	0.47	0.49

Table 2–2. Least squares means and standard deviations for each measured trait of Generation 11. Abbreviations: Corolla Width (CW), Throat Width (TW), Tube Length (TL), Anther-stigma Separation (A/S).

Generation 11		Population				
		No Bee		Bee		Source
Trait		A1	A2	B1	B2	F6M
Days to Flower	mean	27.43	27.34	26.41	26.21	27.19
	st. dev.	3.57	3.35	3.98	3.98	3.29
Avg. CW (mm)	mean	19.97	18.50	18.37	17.84	17.19
	st. dev.	2.95	2.67	2.72	2.59	2.87
Avg. TW (mm)	mean	9.68	9.05	9.18	9.11	8.59
	st. dev.	1.00	0.99	1.02	1.00	0.99
Avg. TL (mm)	mean	12.23	11.94	11.42	11.48	10.73
	st. dev.	1.25	1.10	1.19	1.19	1.17
Avg. A/S (mm)	mean	0.75	1.45	1.89	1.91	2.01
	st. dev.	0.70	0.76	0.87	0.80	0.87
Leaf Width (mm)	mean	19.03	21.34	18.00	17.93	15.41
	st. dev.	4.10	5.14	4.36	4.20	3.84
Internode (mm)	mean	28.38	36.32	31.59	31.42	27.25
	st. dev.	7.92	12.11	7.40	8.28	7.16
Self Seed	mean	33.96	32.06	7.66	8.14	3.74
	st. dev.	42.54	42.57	17.49	19.56	13.55
Autogamy	mean	0.79	0.76	0.42	0.47	0.27
	st. dev.	0.41	0.43	0.49	0.50	0.45
Seed mass (mg)	mean	4.15	2.63	1.45	1.25	0.85
	st. dev.	3.53	2.41	1.83	1.83	1.16
Mass /seed (μg)	mean	5.49	4.12	6.57	5.77	4.82
	st. dev.	1.93	1.93	3.81	2.57	1.88

Table 2–3. Tests for trait mean differences in Generation 11. A) One-way ANOVA with factor population**.** B) GLM ANOVA with factors cohort, treatment, and population nested within treatment. * signifies treatment significance at $\alpha < 0.05$, ** $\alpha < 0.001$. Pop = population, F = f-ratio, P = p-value, LW = Leaf Width, LRT = likelihood ratio test.

	A) Pop		B) Cohort		Treatment		Treatment(Pop)	
Trait	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**P**
Days to Flower	12.26**	<0.001	949.46**	<0.001	17.28	0.065	1.52	0.219
CW	38.37**	<0.001	35.95**	<0.001	1.83	0.354	24.66**	<0.001
TW	41.01**	<0.001	3.73*	0.024	1.76	0.362	25.37**	<0.001
TL	61.89**	<0.001	18.76**	<0.001	19.98*	0.048	5.50*	0.004
A/S	118.35**	<0.001	22.14**	<0.001	4.19	0.193	43.40**	<0.001
LW	59.50**	<0.001	110.06**	<0.001	8.20	0.109	13.09**	<0.001
Internode	40.29**	<0.001	83.55**	<0.001	0.60	0.624	70.11**	<0.001
Seed Mass	33.81**	<0.001	39.24**	<0.001	5.57	0.145	9.93**	<0.001
Mass/Seed	11.70**	<0.001	3.19*	0.075	2.04	0.322	9.99**	<0.001
	LRT	**P**	**LRT**	**P**	**LRT**	**P**	**LRT**	**P**
Autogamy	413.78**	<0.001	58.25**	<0.001	9.58*	0.0083	8.49*	0.0144
Self Seed	513.07**	<0.001	44.99**	<0.001	473.43**	<0.001	1.85	0.3963

Table 2–4. Tukey-Kramer post hoc tests for differences among Generation 11 populations. Populations that share a letter are not significantly different from one another.

Trait	A1	A2	B1	B2	Source
Days to Flower	A	A	B	B	A
CW	A	B	B	B	C
TW	A	B	B	B	C
TL	A	A	B	B	C
A/S	A	B	C	C	C
Leaf Width	B	A	C	C	D
Internode	C	A	B	B	C
Autogamy	A	A	B	B	C
Self Seed	A	A	B	B	B
Seed Mass	A	B	C	C	C
Mass/Seed	B	C	A	AB	BC

Table 2–5. Two-sample t-tests for differences in mean Self Seed between populations of Gen 7 and Gen 11.

A1	N	Mean	St. dev.	SE	Mean diff	95% CI Lower	95% CI Upper	t-stat	p-value	df
Gen 7	237	38.0	51.3	3.3	4.02	-2.83	10.87	1.15	0.250	813
Gen 11	578	34.0	42.5	1.8						
A2										
Gen 7	182	27.4	42.5	3.2	-4.62	-12.20	2.96	1.20	0.232	547
Gen 11	367	32.0	42.6	2.2						
B1										
Gen 7	264	16.4	36.0	2.2	8.70	4.93	12.47	4.53	<0.001	773
Gen 11	511	7.7	17.5	0.77						
B2										
Gen 7	271	9.7	20.6	1.3	1.60	-1.31	4.51	1.08	0.281	808
Gen 11	539	8.1	19.6	0.84						
Source										
Gen 7	245	5.8	14.3	0.92	2.07	0.01	4.13	1.97	0.049	825
Gen 11	582	3.7	13.6	0.56						

Table 2–6. Neutral marker data for genotyped individuals of Generation 10. Unique alleles, defined by length in bp, are listed for each of four markers along with their respective frequency (rounded) in each population. N is the total number of individuals genotyped from each population. H_E is the expected heterozygosity for each locus. H_O is the observed heterozygosity.

MARKER DATA		POPULATION				
	Marker **e461**	**A1**	**A2**	**B1**	**B2**	**Source**
ALLELE	*181*	0.00	0.35	0.06	0.11	0.10
	188	0.23	0.19	0.33	0.45	0.51
	192	0.77	0.46	0.61	0.44	0.40
	N	***187***	***187***	***192***	***185***	***189***
	H_E	0.36	0.63	0.52	0.60	0.58
	H_O	0.05	0.66	0.25	0.30	0.36
	Marker **e523**	**A1**	**A2**	**B1**	**B2**	**Source**
ALLELE	*211*	0.02	0.00	0.14	0.17	0.25
	213	0.97	1.00	0.82	0.81	0.72
	217	0.02	0.00	0.04	0.02	0.03
	N	***180***	***76***	***184***	***190***	***190***
	H_E	0.07	0.00	0.31	0.32	0.42
	H_O	0.02	0.00	0.23	0.23	0.36

Table 2–6. (Continued)

	Marker **e755**	**A1**	**A2**	**B1**	**B2**	**Source**
ALLELE	*479*	0.01	0.32	0.00	0.01	0.03
	481	0.99	0.68	0.90	0.90	0.83
	485	0.00	0.01	0.10	0.09	0.14
	N	***179***	***181***	***189***	***185***	***183***
	H_E	0.02	0.44	0.18	0.18	0.29
	H_O	0.00	0.01	0.13	0.08	0.25
	Marker **e641**	**A1**	**A2**	**B1**	**B2**	**Source**
ALLELE	*316*	0.36	0.10	0.29	0.22	0.24
	393	0.35	0.12	0.46	0.49	0.54
	410	0.29	0.78	0.24	0.29	0.22
	N	***182***	***182***	***189***	***190***	***190***
	H_E	0.66	0.37	0.64	0.63	0.60
	H_O	0.13	0.13	0.55	0.52	0.59

Table 2–7. Genotype counts and allele frequencies at the Drive and Inversion chromosomal polymorphisms for Generation 10.

Drive	dd	Dd	DD	Total	D-type frequency
Source	38	87	67	192	0.576
A1	147	15	25	187	0.174
A2	180	0	1	181	0.006
B1	74	87	30	191	0.385
B2	59	90	39	188	0.447
Inversion	**cc**	**Cc**	**CC**	**Total**	**C-type frequency**
Source	78	83	25	186	0.358
A1	183	0	0	183	0.00
A2	181	0	0	181	0.00
B1	143	40	5	188	0.133
B2	135	44	2	181	0.133

Figure 2–1. (Next page). Trait evolution comparisons between Generation 7 (fully outbred; previous study) and Generation 11. Values are reported as difference of population trait means (delta Z) from the Source population of each respective generation. Error bars are +/- one standard error. Interpretation: Bar length from zero represent absolute trait mean divergence from the Source population grown in the same generation. Comparing bar lengths between generations gives magnitude and direction of change over time. For example, population B2 increased mean CW from the Source population by 1 mm, but CW did not evolve further from the Source population in Generation 11. Population A1 increased mean CW from the Source by 1 mm in Generation 7 and increased CW by 3 mm in Generation 11, a net increase of 2 mm since Generation 6.

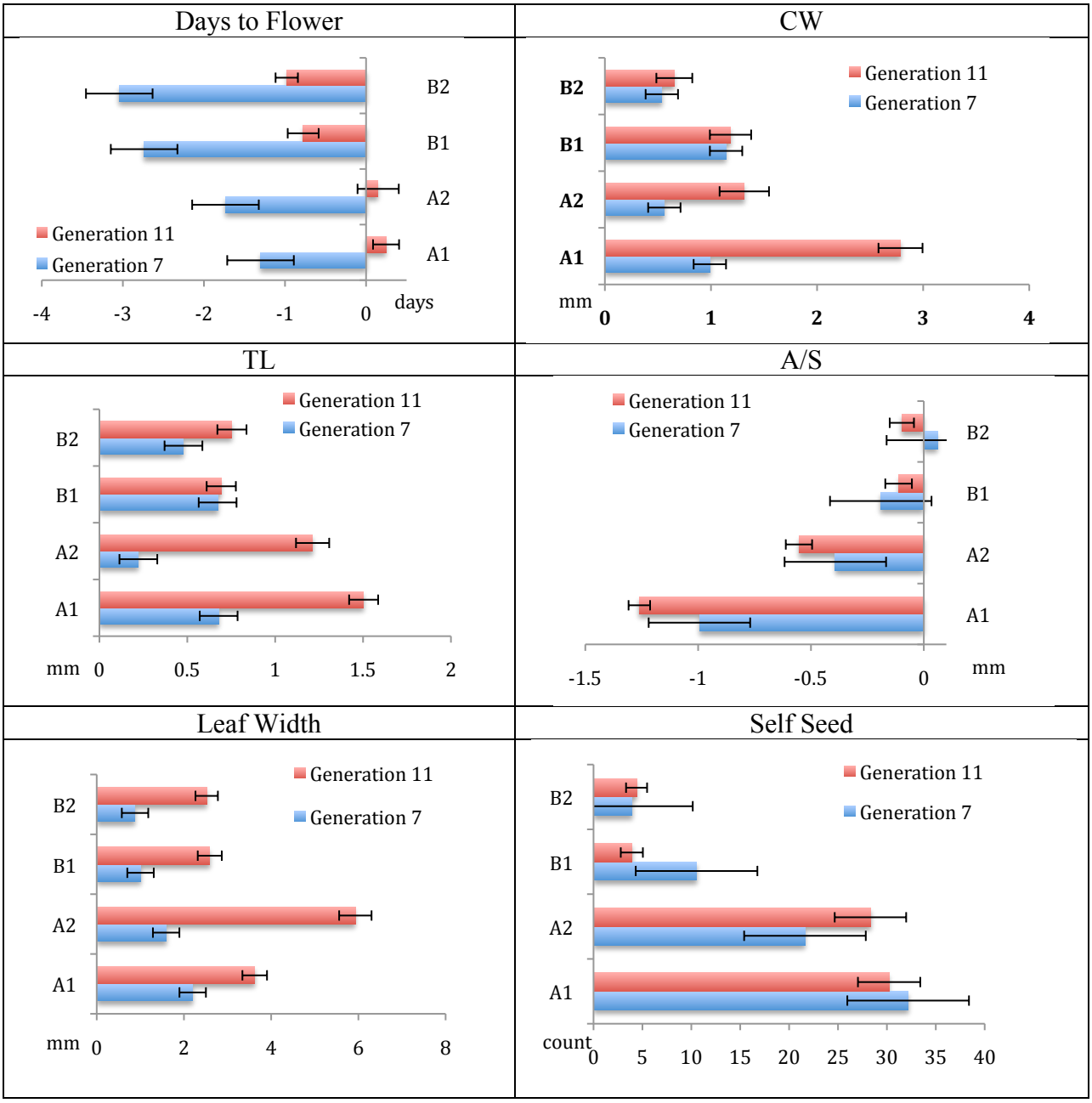

Days to Flower
B2
B1
A2
A1
Generation 11
Generation 7
-4
-3
-2
-1
0
days
CW
B2
B1
A2
A1
Generation 11
Generation 7
mm
0
1
2
3
4
TL
Generation 11
Generation 7
B2
B1
A2
A1
mm
0
0.5
1
1.5
2
A/S
Generation 11
Generation 7
B2
B1
A2
A1
-1.5
-1
-0.5
0
mm
Leaf Width
Generation 11
Generation 7
B2
B1
A2
A1
mm
0
2
4
6
8
Self Seed
Generation 11
Generation 7
B2
B1
A2
A1
count
0
5
10
15
20
25
30
35
40

Figure 2–2. Trait evolution comparisons between Generation 10 and Generation 11. Values are reported as difference of population trait means (delta Z) from the Source population of each respective generation. Error bars are +/- one standard error from the mean. Interpretation: Same as Figure 2–1.

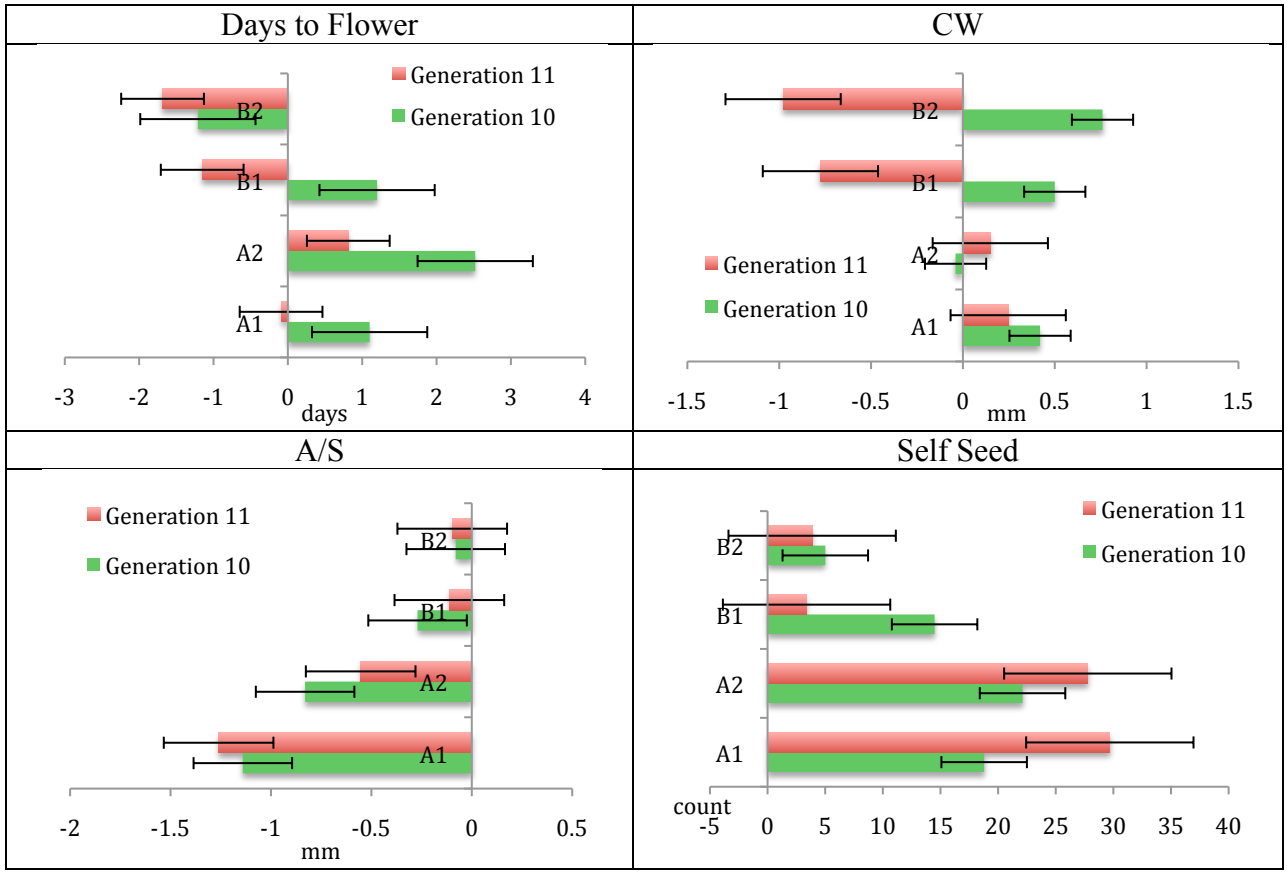
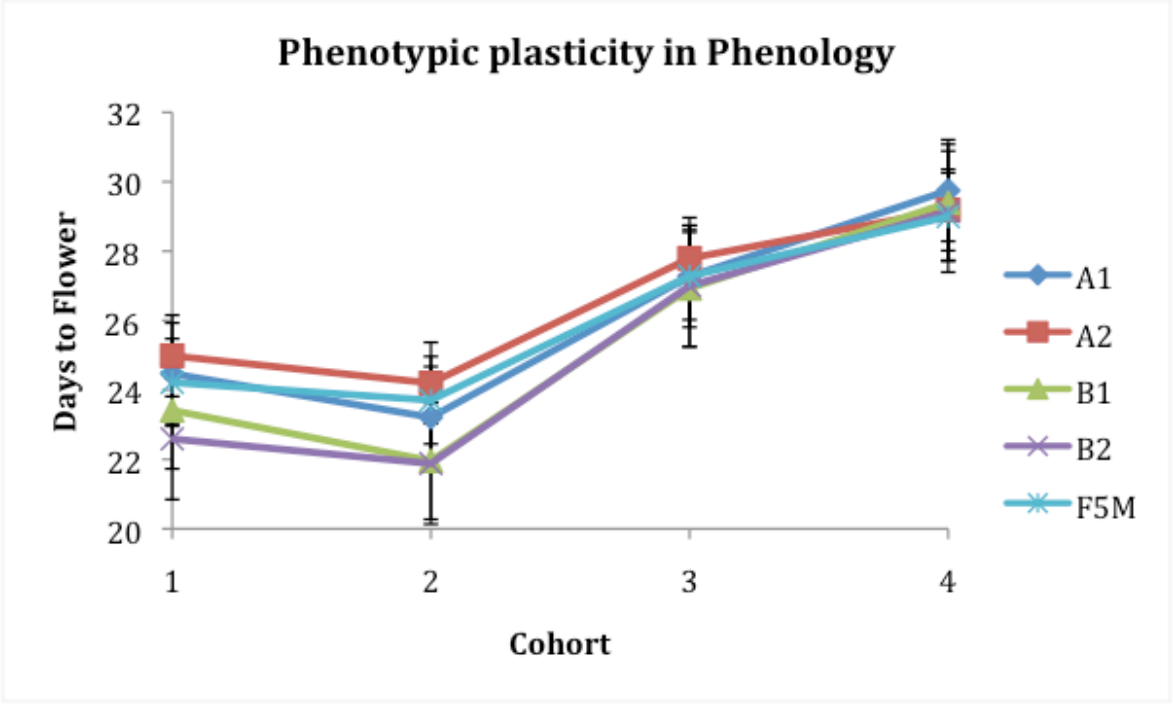
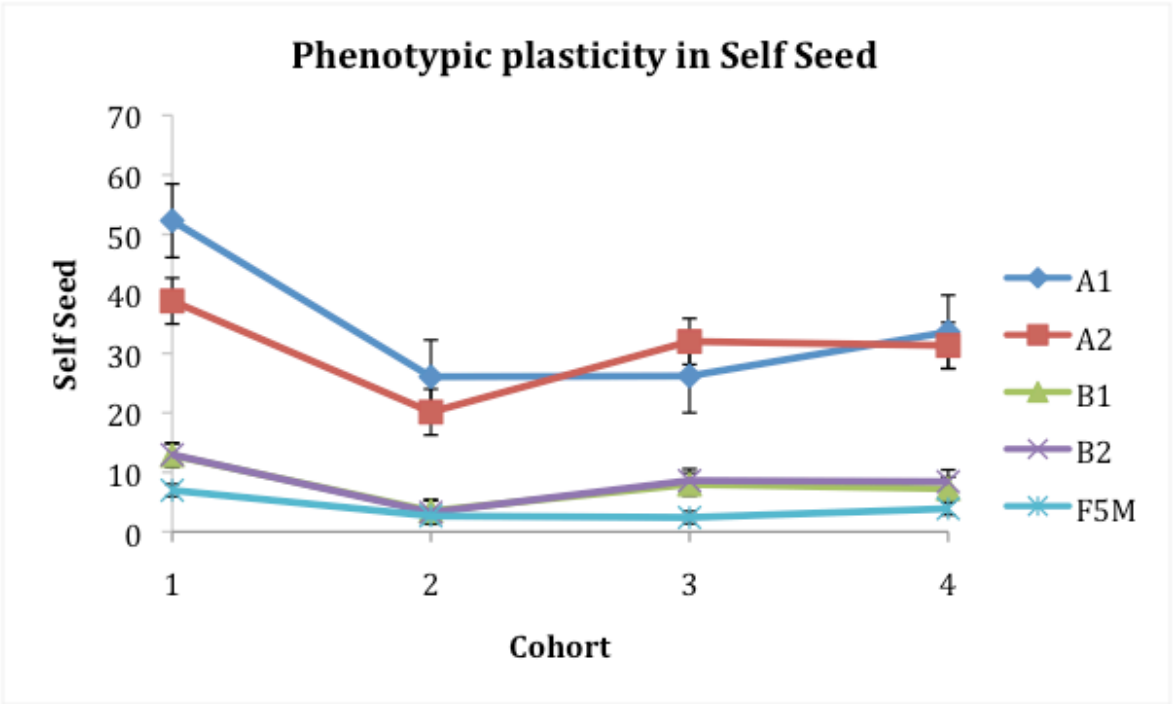


Figure 2–3. Mean trait values for each of four Generation 11 cohorts by population, demonstrating environmentally induced phenotypic plasticity. A) Days to Flower, B) Self Seed.





Chapter 3. Investigating pollinator-plant interactions between *Bombus* and *Mimulus*

ABSTRACT

The immense diversity of floral morphology is often attributed to non-random patterns of visitation by pollinators. Plant traits evolve to maximize transfer of gametes to conspecifics while pollinators evolve behaviors to maximize foraging. I explored floral trait preferences and fidelity of bumblebee (*Bombus*) pollinators when encountering divergent populations of yellow monkeyflower (*Mimulus*). Bees demonstrated clear preference for large flower sizes and discriminated against pale-colored flowers, although these two traits only explained a proportion of the variation in flower visits. Constancy, a measure of pollinator fidelity, was weak, but increased as divergent phenotypes became more distinct. The magnitude of pollinator preference and constancy demonstrated by *Bombus* may be a strong selective force on heritable variation in floral morphology but is unlikely to initiate population divergence and speciation in *Mimulus*.

INTRODUCTION

Understanding the evolution of floral morphology and diversity is a major theme in pollination biology. Pollinator interactions with flowers transfer gametes from one plant to another. Heritable plant characteristics that enhance gamete transfer among plants by influencing pollinator behavior are expected to evolve under natural selection. Such pollinator-driven floral divergence between early plants and insects likely fueled the immense diversification of the angiosperms (Grant 1949; Grant & Grant 1968; Stebbins 1970, Hu et al. 2008). One potential

driver of speciation is ethological isolation whereby innate floral character preferences of different pollinator groups drive divergence between plant populations and maintain isolation, even in sympatry (Grant 1949). Despite the popularity of the ethological isolation hypothesis (reviews in Waser 2001; Fenster et al. 2004; Waser & Campbell 2004), there is little evidence that pollinator preference alone is strong enough to initiate and maintain reproductive isolation between incipient plant species (Waser 1998). In order to understand the degree to which pollinators actively facilitate and maintain divergence between plant populations, we need to determine which plant traits contribute to pollinator preference and how pollinator preference affects fitness across space and time.

Pollinator Preference—Many plant species rely on pollinators for reproduction and have evolved complex phenotypes, coevolving with pollinator sensory systems to enhance reproductive success. Coevolved phenotypic traits include floral architecture, color, scent, ultraviolet patterning, and nectar/pollen rewards (e.g. Ollerton 1996; Waser 1998; Spaethe et al. 2001; Klahre et al. 2011; Leonard et al. 2011). Different pollinator groups appear to select for distinct suites of traits, which may converge in plants of dissimilar evolutionary origins, creating recognizable pollination syndromes (Stebbins 1974; Proctor et al. 1996). Self-fertilizing lineages of flowering plants have greatly reduced floral traits likely unattractive to pollinators, which may discourage pollinator visitation and reinforce selfing (Ornduff 1969; Sicard & Lenhard 2011).

Innate and learned preference for and behavioral response to individual floral characters have been examined using floral manipulation, field observation trials, and artificial flower choice tests. These types of experiments are common, especially in the fields of pollination biology and

animal behavior (e.g. Fenster et al. 2004; Pyke et al. 1988, Real & Rathcke 1991; Hodges 1995), but direct investigations of pollinator selection on individual traits within defined genetic backgrounds are limited (review in Galliot et al. 2006). Modern genetic techniques enable a deeper understanding of the intersection between genes, traits, and pollinator behavior while illuminating the potential of specific genes to persist in populations. A seminal study on pollinator preference analyzed the effects of two specific floral trait QTL on pollinator behavior. A single allele at a floral color QTL was shown to preferentially attract bee visitors to *Mimulus* flowers; another QTL affecting nectar production influenced hummingbird visitation rates even when the gene was introgressed into a different genetic background (Schemske & Bradshaw 1999; Bradshaw & Schemske 2003). This study demonstrated that pollinator preferences can be the source of selection for divergence of floral traits and that single genomic regions can affect evolutionary trajectories. Continued investigation of population-level trait variation, phenotypic and genotypic response to pollinator selection, and trait-level pollinator preference will further clarify how pollinator syndromes are formed and maintained.

Pollinator Constancy—While individuals, species, or aggregate groups of pollinators may have inherent or learned preferences for particular floral traits, preference alone may not be sufficient to create selection pressure on floral traits. Constancy, a measure encapsulating pollinator fidelity for particular variants (i.e. visiting multiple individuals with the same characters consecutively), may reinforce divergence among populations and create stabilizing selection on floral traits within populations (Grant 1950; Harder & Johnson 2009). Inconstant pollination may decrease plant fitness through pollen loss (Campbell & Motten 1985), pollen interference (Thomson et al. 1981), and hybrid formation (Levin 1972). Pollinator constancy is quantified by tracking

individual pollinators as they forage and recording the frequency of transitions between phenotypes. Hopkins and Rausher (2012) documented pollinator-driven reinforcement between sympatric species of *Phlox* with two different color morphs. Reduced hybridization was due to pollinator constancy; pollinators were more likely to continuously forage from flowers within phenotypes of similar pigment intensities than to switch between distinct phenotypes. Across the breadth of field studies of different species and floral morphs, results span the entire spectrum from complete inconstancy (all visit transitions to different phenotypes, *Megapalpus* flies, Ellis & Johnson 2012) to random (butterflies, Pohl et al. 2011) to complete constancy (only visits to one phenotype; bumblebees, Gegear & Thomson 2004).

Both pollinator preference and constancy may be labile traits. Bees have been shown to flip preference and change rates of constancy due to community context (frequency of floral morphs present) and through learned association of traits and rewards. I examined the specific relationship between *Mimulus guttatus* flowers and *Bombus impatiens* (bumblebee) pollinators, asking: 1) Do bumblebees show preference for specific *Mimulus* floral traits? 2) Are plants with a history of self-fertilization less attractive to bumblebees? 3) Are bumblebees constant when pollinating in arrays containing multiple phenotypes?

METHODS

Plants—The annual, self-compatible, western North American wildflower, *Mimulus guttatus* (2n = 28; Phrymaceae) is increasingly used as a model organism for genetic studies of floral variation and the evolution of plant mating systems (Macnair & Cumbes 1989; Ritland & Ritland

1989; Fenster & Ritland 1994; Carr & Fenster 1994; Robertson et al. 1994; Wu et al. 2008). *M. guttatus* has hermaphroditic flowers with multiple adaptations thought to enhance bee pollination, including a touch-sensitive stigma and a wide, showy corolla with a landing platform. The self-fertilizing rate is highly variable among wild *M. guttatus* populations (Ritland & Ganders 1987; Awadalla & Ritland 1997; Sweigart et al. 1999), but the ancestral population of plants used in this study is primarily outcrossing (Willis 1993, 1996, 1999a,b). Plants grown for experiments were various lab-derived populations of *M. guttatus,* originally sourced from a single wild population in Iron Mountain, OR (Willis 1993; Arathi & Kelly 2004). Plants were reared in individual 2 X 2 inch pots under conditions standard for our facility (Arathi & Kelly 2004).

***Pollinators*—**Many pollinator species visit *M. guttutus* but bumblebees (*Bombus*) may be the most important. Iron Mountain populations are visited by *B. edwardsii, B. mixtus*, and *B. melanopygus* (Arathi & Kelly 2004; *B. edwardsii* is recognized as a subspecies of *B. melanopygus* in this region [Owen et al. 2010]). I used *B. impatiens* as the pollinator in the behavioral experiments. In part, this was a practical choice: colonies of *B. impatiens* are commercially available in the Midwest. *B. impatiens* occurs across eastern North America and thus does not pollinate Oregon populations of *M. guttatus*. However, it is closely related to western *Bombus* species (Cameron et al. 2007) and is intermediate in size to the documented visitors at Iron Mountain. *B. impatiens* worker bees are 8.5–16mm long, smaller on average than *B. melanopygus* but larger than *B. mixtus* (Franklin 1912). Both field and greenhouse studies have shown the effectiveness of *Bombus* species as pollinators of *M. guttatus* (Robertson et al. 1999; Arathi & Kelly 2004; Bodbyl Roels & Kelly 2011). I conducted experiments with

bumblebee hives consisting of approximately 20 workers and a queen (Class C, Koppert Biological Services, MI, USA). I used four hives over the duration of the study, two for the Experimental Evolution (EE) experiment and one each for the Flower size and Flower color experiments. I supplemented hives with pollen at the rate of 1 Tbs. every 2–3 days in addition to the *ad libitum* nectar contained in the hive box. Hives experienced greenhouse temperatures ranging between 20 and 30°C.

***Phenotypic measurements*—**Prior to each trial, I assigned a unique ID to plants to track visitation and measured corolla width (CW), anther-stigma separation (A/S—also called herkogamy), throat width (TW), and tube length (TL). I recorded all four floral measures (Figure 3–1) on plants in the EE experiment, but recorded only CW and A/S for the Flower size and Flower color experiments. I also assayed plants in the EE and Flower size experiments for two fitness components: seed production of self-fertilized and pollinated flowers. I marked flowers which received visits from bees during a trial with water-soluble red paint (Bee seed) and then marked the next flower to open on the same plant with blue paint (Self seed). Upon maturation, I harvested seed pods from marked flowers and counted the seeds they contained.

***Experiments*—**

EXPERIMENTAL EVOLUTION (EE):

A previous greenhouse experimental evolution study produced divergent populations of *M. guttatus* via bumblebee pollination and exclusion treatments (Bodbyl Roels & Kelly 2011). Replicate populations A1 and A2 were the result of five consecutive generations of pollinator exclusion, creating highly inbred individuals and effectively selecting for traits that enhance self-

fertilization. Replicate populations B1 and B2 received visits from *B. impatiens* for five consecutive generations, producing primarily outcrossed individuals. I grew 100 individuals from each of these experimental populations to evaluate pollinator preference in populations experiencing a mating system shift. For each trial, I randomly chose 8 flowering plants from a bee excluded population (A1 or A2) and 8 from a bee pollinated population (B1 or B2) and randomly arranged them in a 4 X 4 array.

FLOWER SIZE:

A prior artificial selection study on flower size resulted in three experimental populations with highly divergent corolla width, CW (Kelly 2008). Populations were produced through 12 generations of artificial selection for large (H), and small (L) flower size, grown simultaneously with a randomly crossed control (C) population. I sowed 3–4 seeds from each of 30 randomly chosen genotypes, or lineages, per population and selected 100 mature individuals from each to gauge bee preference for flower size. For each trial, I randomly chose five flowering plants from each population and randomly arranged them in a 5 X 3 array.

FLOWER COLOR:

While using a laboratory cross to investigate epistatic effects of floral size QTL, Kelly and Mojica (2011) discovered a flower color polymorphism: bright vs. pale yellow corolla. The pale phenotype segregated in the progeny of a plant with two floral size QTL (x5b/x8) embedded in a nearly isogenic donor (IM767) genetic background. It is uncertain whether the pale phenotype is due to a novel mutation or from segregating variation present in the founding individuals. I grew 25 individuals with the pale phenotype (P) and 25 normal hued control plants (C) in the

greenhouse and used them to evaluate bumblebee preference for flower color. The control plants also had an IM767 genetic background, but differed from the pale plants at up to four floral size QTL. For each trial, I randomly chose 8 flowering plants from each population and randomly arranged them in a 4 X 4 array.

***Trial procedures*—**For each trial, I placed plants containing a single open flower into experiment specific arrays in an acrylic glass flight chamber. Single flowers were used both to standardize floral display size per plant and to simulate typical field conditions. Wild *M. guttatus* populations at Iron Mountain, OR are early spring ephemerals reliant on moisture from snowmelt and plants typically produce only a single flower prior to desiccation.

The experiment flight chamber was connected directly to the hive with 1.3 cm clear plastic tubing and shutoff valves, allowing for control of bee traffic in and out of the chamber (Figure 3−2). I used only freshly opened, unvisited flowers on unvisited plants for each trial; prior visitation may bias bee preference and behavior (Cameron 1981). To ensure only fresh flowers were presented to the bees, I removed corollas of all unused experimental plants at the end of each day. Each hive acclimated to the flight chamber prior to experiments, with all bees at liberty to enter and exit the chamber. For each trial, a single bumblebee entered the flight chamber and foraged on the flower array. Video recording of the experiment (Sony Handycam, HDR-XR100) began once the bee engaged a flower, defined as first contact with a corolla. I recorded the order, duration, and total number of visits to each flower. I used a stopwatch to record the duration of visit to each flower, defined by first contact to disengagement. I used video playback to verify bee response variables. Trial completion occurred when the bee either voluntarily returned to the

hive or ceased all floral contact for at least two minutes. I performed all trials from each of the three experiments between 9:00 and 11:00 AM to control for potential diurnal foraging patterns.

***Analyses*—**I investigated the relationship between *Mimulus* and *Bombus* pollinators in three distinct ways. First, I quantified phenotypic trait differences among experimental populations. If measured traits vary among populations, they create the potential for pollinator sensory discrimination, which may result in non-random visitation within trials. Similarly, I compared the fitness components Self seed and Bee seed among populations. The experimental populations may intrinsically differ in ability to self or outcross, influencing the genetic composition of offspring independent of pollinator visitation. To evaluate pollinator contribution to seed production, I subtracted Self seed from Bee seed for each plant. This new variable, Bee contribution, reduced bias from naturally occurring variation in plant fecundity within populations.

Second, I evaluated bee response variables (see *Trial procedures)* as indicators of pollinator preference. I defined preference as unequal representation of a bee response variable in a population with respect to the other populations presented in a trial. For example, if 25% of pale flowers and 95% of control flowers received a single pollinator visit, then I considered the bees to have demonstrated a preference for control flowers over the pale flowers. Bee response variables evaluated include: Visit, which equaled 1 if a flower was visited by a bee and 0 if not, the order in which flowers were visited (Standard rank order), total visits to each flower (Total visits), and the total duration a bee spent visiting each flower (Total visit time). I manipulated raw visit order data to create the variable Standard rank order. First, I considered only the

numerical rank of the *initial* visit to a flower. For example, the first flower visited is assigned a rank of 1, the second a 2. If the third visit was a return to the first flower, it still retained the rank of 1, and the next flower visited received a rank of 4. Second, I weighted each individual rank by dividing it by the total number of visits in that trial. The resulting continuous proportional variable, Standard rank order, ranges from $\{0 < X \leq 1\}$ where increasing values indicate flowers visited later in a trial.

Third, I measured pollinator constancy via the sequential order in which each flower was visited in a trial. I calculated constancy using the frequencies of visit transitions within and among populations. For example, in the Flower color experiment, a bee visiting a pale flower (P) could subsequently visit another pale flower (P→P) or a control flower (P→C). Similarly, a bee visiting a control flower (C) could then visit another control flower (C→C) or a pale flower (C→P). I calculated constancy by applying a modified version of Jacobs (1974) constancy index, as proposed by Gegear and Thomson (2004), to the transition frequencies:

$$C_I = (c - e) / (c + e - 2ce)$$

Where c = the proportion of transitions within a population observed in a trial and e = the expected number of transitions within a population. The number of plants available for a bee to visit in a single trial, given the identity of the last flower visited, weighted the expected transitions. For example, in a trial with 8 pale flowers and 8 control flowers, the expected transition frequency [P→C] = 8/15 and [P→P] = 7/15, because the bee is unlikely to turn around and revisit the same flower. For the EE and Flower color experiments, with two populations co-

occurring in equal frequencies in a trial, $e = 2p^2$, where p is the frequency of visits *within* a population (i.e. p = [P→P] = 7/15, modified from Pohl et al. 2011). In the Flower size experiment, $e = 3p^2$, where p is the frequency of bee visits *within* each of the three populations present in each trial (i.e. p = [P→P] = 4/15). Index values range from -1 (maximally inconstant: all transitions between unlike flowers) to +1 (maximally constant: all transitions between like flowers). 0 indicates random transitions between flower types

χ^2 Goodness of Fit analyses further quantified the distribution of flower-to-flower transitions across all trials within an experiment. Thus, expected transition probabilities *within* populations (e.g. p[P→P or C→C]) were (x –1)/(n – 1) where x = number of plants of a particular population that a bee is currently visiting, n = total number of plants in an array. Expected transition probabilities *among* populations (e.g. p[P→C or C→P]) were x/(n – 1) for the EE and Flower color experiments with only two population choices and 2x/(n – 1) for the three population choices in the Flower size experiment.

I used general linear model (GLM) ANOVA on all phenotypic and bee response variables. Factors considered in the models were population and trial. I fit the binary variable, Visit, to a binomial distribution and fit count variables (Self seed, Bee seed, and Total visits) to an overdispersed Poisson distribution. As a proportion, I arcsine square-root transformed Std. rank order prior to analysis. All other variables approximated the normal distribution without further transformation. To determine the extent to which bee behavior could be explained by measured phenotype, I performed additional tests on the bee response variables with floral phenotype measurements CW and A/S as covariates (ANCOVA). Similarly, I used ANCOVA to investigate relationships between phenotype and fitness and among continuous bee response variables. I

performed all statistical analyses using JMP® 8.0.2 (SAS Institute Inc., 2009) and Minitab® 16.1.1 (Minitab Inc., 2010).

RESULTS

EXPERIMENTAL EVOLUTION (EE):

Of the seven phenotypic and fitness variables (CW, TW, TL, A/S, Bee Seed, Self Seed, and Bee contribution—Table 3–1A), only CW and A/S differed significantly among the bee pollinated (B1, B2) and bee excluded (A1, A2) populations (Table 3–2A, ANOVA). I performed twenty-three individual bee choice tests (trials), using a total of 368 plants. Only 27 plants (7%) did not receive at least one visit. The majority (70%) of the unvisited plants were from populations A1 and A2. Unvisited plants had significantly smaller flowers than those that received visits (CW, two-sample t-test, $t = -2.64$, $df = 29$, $p = 0.013$). Although none of the fitness variables differed significantly among populations, flowers visited by bees produced more individual seeds than self-fertilized flowers across all populations (Table 3–1A).

ANOVA for the four bee response variables (Visit, Total visits, Std. rank order, and Total visit time—Table 3–1A) found significant differences among populations for Total visits and Total visit time (Table 3–2A). Bees visited flowers from populations B1 and B2 more often than A1 and A2 (Table 3–1A) and bees spent less time visiting A1 flowers than the other populations (Tukey-Kramer post-hoc tests, Table 3–3). I found no significant interaction terms between factors Population and Trial in any of the ANOVA for EE.

Floral phenotypes (CW and A/S) were significant covariates in EE ANCOVA models for multiple bee response and fitness variables (Table 3–4A). First, CW positively covaried with Visit. Second, CW, A/S, and interaction terms with Population were significant covariates in the model for Total visits. Visit frequency increased with larger CW. Increasing A/S elevated visits in A1 but decreased visits in the other populations. However, Population remained significant in the model, indicating additional factors other than CW and A/S contributed to the observed population-level variation in Total visits. Third, both CW and A/S covaried with Self seed, as either measure increased in size, seed set decreased. Population was significant with the addition of the floral covariates into the model, signifying that additional unmeasured factors contribute to differences among population in this trait (Table 3–4A). ANCOVA models investigating relationships among bee response variables found Std. rank order covaried negatively with Total visit time (Table 3–4A).

EE mean constancy, C_I, was 0.10 (range -0.366 to 0.322). The index was not significantly different than zero ($t = 0.250$, $df = 22$, p-value = 0.810). Thus, visits appeared to be random (Table 3–5A). In a table analysis, bee excluded to bee excluded (N→N) flower transitions were slightly less frequent than expected but overall, bee transition frequencies among flowers did not deviate from expected values ($\chi^2 = 4.09$, $df = 3$, p-value = 0.252, Figure 3–4A).

FLOWER SIZE:

Of 20 bumblebee trials, thirty-four individuals (11%) received no visits; 18 were L, 13 were C, and 3 were H. Floral phenotypic traits CW and A/S and fitness variable Bee seed all significantly

diverged by population (Table 3–2A); H had larger flowers, greater herkogamy, and produced more seeds when visited by bees in comparison with L and C (Table 3–3).

Numerous bee response variables (Visit, Std. rank order, and Total visit time) were significant by Population when examined by a two-factor ANOVA (Table 3–2B). H was more likely to receive a visit, visited earlier in a trial, and visited longer by bees than either L or C (Table 3–3). I found no significant interaction terms between factors Population and Trial in any ANOVA.

As in the EE experiment, floral phenotypes were significant covariates in Flower size ANCOVA models for multiple bee response and fitness variables (Table 3–4B). First, CW, A/S, and their interaction terms with Population positively covaried with Visit. Visit increased along with CW for populations L and C, but no relationship was found between Visit and CW for population H (binary logistic regression, $\chi^2 = 0.02$, p-value = 0.878, Figure 3–3A). Visit decreased with increasing A/S for population C, but no relationship was found between Visit and A/S for populations H or L (binary logistic regression, $\chi^2 = 1.87$, p-value = 0.171; $\chi^2 = 0.12$, p-value = 0.717, Figure 3–3B). Population lost significance in the model; CW and A/S explained population variation in Visit. Second, CW, A/S, and their interaction terms with Population covaried with Total visits (Table 3–4B). CW was a positive covariate (Figure 3–3C), A/S was a negative covariate. Third, CW positively covaried with Total visit time. Population lost significance in the model; CW explained population variation in Total visit time. Fourth, CW covaried with Std. rank order, increased CW corresponded with flowers visited earlier in trials. Population dropped significance in the model; CW explained population variation Std. rank order (Table 3–4B). Fifth, A/S negatively covaried with both fitness variables Bee seed and Bee

contribution. Population lost significance in the model; A/S explained population variation in Bee seed (Table 3–4B).

ANCOVA also clarified relationships among bee response variables. First, Total visits positively covaried with Bee seed (Table 3–4B), as the number of visits increased, so did the number of seeds produced by a flower. Second, Std. rank order negatively covaried with Total visit time; flowers visited later in a trial received less visit time from bees.

Mean C_I for the Flower size experiment was 0.127 (range -0.087 to 0.429, Table 3–5B). Mean constancy was significantly greater than zero ($t = 3.45$, $df = 19$, p-value = 0.0013) but less than 1 ($t = 23.74$, $df = 19$, p-value = <0.001), demonstrating marginally constant movements among populations. Table analysis showed H→H, H→C, and C→H transitions to be more frequent than expected and L→L transitions less frequent than expected given a the null model ($\chi^2 = 32.47$, $df = 8$, p-value < 0.001, Figure 3–4B).

FLOWER COLOR:

I performed only three trials comparing Flower color populations due to limited plant availability. Of the 48 experimental plants, 25 (52%) flowers received no visits by a bee. Only 5 of the unvisited flowers were controls, so the majority (75%) of pale flowers did not receive a visit by a bee. Pale flowers had smaller CW than control flowers but no difference was observed in A/S (Table 3–2A).

Bee response variables Std. rank order, Visit, Total visits, and Total visit time were significant by Population in two-factor ANOVA (Table 3–2B). Control flowers were more likely to be visited, visited first, receive more visits, and be visited longer by bees than pale flowers.

Floral phenotypes were significant covariates in Flower color ANCOVA models for only one bee response variable (Table 3–4C). CW and A/S, along with their interactions with Population, covaried with Total visits. CW was a positive covariate, meaning bee visits increased along with flower size. A/S was a negative covariate; bee visits increased as A/S decreased in size. I found no significant covariates in ANCOVA models among bee response variables (Table 3–4C).

Mean C_I for the flower color experiment was 0.333 (n = 3, range -0.316 to 0.677, Table 3–5C), not significantly different from zero (t = 1.02, df = 2, p-value = 0.412). Within the three trials, two bees tended towards constant visitation of control flowers while the third was marginally inconstant. Table analysis showed bees transitioning from control to control (C→C) flowers more often than expected and from pale (P→P, P→C) flowers less than expected (χ^2 = 30.72, df = 3, p-value < 0.001, Figure 3–4C).

DISCUSSION

Numerous field and greenhouse studies have documented pollinator preference for various floral traits (see Kessler & Baldwin 2011 for general review; Chittka & Raine 2006 for bees). Pollinator behavior is often influenced by flower size (Elle & Carney 2003) with bumblebees preferring intermediate (Aigner 2004) to large corolla sizes (Galen 1989; 1996). In *Mimulus,*

flower size may indicate reward amount; flower size typically is positively correlated with pollen load (Ritland and Ritland 1989; Robertson et al. 1994; Fenster and Carr 1997). In my experiments, flower size (CW) was the most important factor determining bee visitation. Mean CW of unvisited flowers from all three experiments was lower than flowers that received at least one visit (Table 3–6). Preference for large corolla sizes may be innate, learned, or reflect physical constraint. Laboratory experiments with naïve bumblebees have demonstrated innate preference for large floral sizes (Lunau 1991). Associative learning, by linking rewards with floral size, has also been demonstrated in bumblebees (Blarer et al. 2002). Physical constraint, or lack of mechanical fit, occurs when pollinators ineffectively transfer pollen due to some aspect of floral morphology (e.g., Nilsson 1988; Cresswell 2000; Armbruster et al. 2004).

Physical constraint may have presented the greatest challenge to bees foraging in my experiments. I observed bees often fell off of small flowers as the flowers bent under their weight and the lower corolla 'landing pad' appeared too small to grip well. Despite not being able to grasp small flowers well, bees were still able to extract pollen from small flowers without physical constraint caused by a narrow corolla tube. The mean head width of *B. impatiens* workers is 2.67 mm (del Castillo & Fairbairn 2012), and the pollen containing tube width (TW) of the smallest flower in my trials was 7.84 mm.

Anther-stigma separation (A/S), or herkogamy, is typically decreased in species that regularly self-fertilize (Grant 1949; Ornduff 1969; Ushimaru & Nakata 2002). Previously, a strong relationship was found between A/S and the ability to produce self-fertilized seeds in the EE populations; the bee excluded populations (A1, A2) evolved decreased A/S as a direct response

to selection for increased selfing capacity (Bodbyl Roels & Kelly 2011). Thus the significant relationship found between A/S and the fitness traits in the experiments is probably caused by the effect of A/S on the production of self seed. Although the intent of the variable Bee Seed was to capture variation in seed set influenced by bee visits, we did not verify that seed pods visited by bees contained exclusively outcrossed seed. Therefore, it is possible that Bee seed pods produced some self-fertilized seed, which could explain the persistence of A/S as a covariate with Bee Seed. The significant relationship found between A/S and the bee response variable Total visits in all three experiments is less intuitive. A/S is the physical difference separating the anthers from the stigma. Both anther and stigma exertion are considered pollination traits and vary widely across flowers with different pollination syndromes and mating systems (e.g. Fenster et al. 2004). Anther exertion, relative to the opening of the corolla, affects pollen removal by pollinators (Wolfe & Barret 1989; Conner et al. 1995). Stigma exertion influences the extent of stigma contact with the body of a pollinator, affecting receipt of pollen (e.g. Fenster et al. 2004). Thus, bee behavior may be influenced directly by some aspect of A/S or may simply be a result of correlation with another bee preference trait not measured in the study.

Flower color has been considered a primary mechanism for signaling between plants and pollinators for more than a century (Darwin 1876; Kugler 1943). Bees prefer flower colors in the blue-violet and yellow range of the visual spectrum (Sutherland & Vickory 1993; Schemske & Bradshaw 1999; Fenster et al. 2004) and rarely visit red flowers (Bradshaw et al. 1995, but see Chittka & Waser 1997). Color preference may be contingent on available flowers for foraging or may be learned through association with rewards (Jones & Reithel 2001), but Heinrich et al. (1977) demonstrated that innate color preferences can also exist independent of reward. In my

flower color experiment, both pale and control flowers were yellow, but the pale phenotype had little pigment compared to the vibrant yellow of the control flowers. Bees readily distinguished among the phenotypes and strongly preferred control plants, although I cannot be certain that flower color alone was the basis of bee preference. Flower size was significantly different between populations (Table 3–2C), confounding the ability to disentangle bee preference for flower color with flower size. The flower color experiment, with differences between populations due to the effects of genes associating with only a few floral size QTL, provides support for previous work in which few loci significantly impact pollinator behavior (Schemske & Bradshaw 1999; Bradshaw & Schemske 2003).

Prior *Mimulus* research has found significant amounts of heritable variation in numerous floral traits, including development rate, flower color, corolla width (CW) and length (TL), anther and stigma lengths (A/S), ovary size and number, pollen load, and self seed (Carr & Fenster 1994; Robertson et al. 1994; Bradshaw & Schemske 2003; Kelly & Arathi 2003; van Kleunen & Ritland 2004; Scoville et al. 2009). Due to high correlations among floral traits, much of this variation can be grouped in a 'flower size' indicator (Robertson et al. 1994). Bumblebees foraging on *Mimulus* flowers in my experiment demonstrated strong preference among floral size and color variants. Due to the heritable nature of these traits, this suggests that *Bombus* may be a significant selective force on floral morphology in naturally outcrossing *M. guttatus* populations.

Mating system evolution—The evolution from outcrossing to self-fertilization is one of the most frequent transitions in the history of the angiosperms (Stebbins 1974). Selfing plants are typically characterized by reduced floral traits such as substantially smaller corollas, less herkogamy, less pollen/nectar per flower, and longer tube length (Grant 1949; Ornduff 1969). These 'selfing

syndrome' (Ornduff 1969) characteristics may provide reinforcement of self-fertilization with respect to pollinator behavior (Ivey & Carr 2005). Floral morphology associated with inbreeding and lack of nutritional rewards may discourage pollinators. In my study, the smallest flowers were the most likely to not receive any visits. Flowers of selfing *Mimulus* species are substantially smaller than any of the flowers presented in my arrays and thus are unlikely to be visited by bumblebees in nature. I was unable to quantify pollen loads in my experimental plants, but other studies have shown that bumblebees can assess floral rewards prior to contact with flowers and will visit perceived low reward flowers less often (Cresswell & Robertson 1994; Rasheed & Harder 1997a,b). Inbreeding often decreases pollen production and quality in *M. guttatus* (Carr & Dudash 1996), reducing visitation by pollinators (Robertson et al. 1999).

The EE experiment directly assayed the ability of bumblebees to distinguish among populations of plants that experienced five consecutive generations of differential mating system selection. Five generations was sufficient to create divergence in floral morphology between the outcrossed (B1 and B2) and selfing (A1 and A2) populations (see Bodbyl Roels & Kelly 2011) and bumblebees demonstrated preference for the outcrossed populations. This preference was indicated by significant effects of population in bee response variables measuring duration of flower visits and number of visits to each flower (Table 3−2B). The ability of bumblebees to discriminate among plants solely divergent by mating system has intriguing implications for mating system evolution in nature; particularly maintenance of mixed-mating. Employment of self-fertilization as reproductive assurance (Baker 1967; Kalisz et al. 2004) may ultimately discourage future visits by pollinators if traits facilitating selfing are unattractive. The trade-offs

between pollinator attraction and self-fertilization in floral traits are unclear and warrant further investigation.

***Constancy*—**Plant species benefit from pollinator constancy; visits only to conspecifics minimize pollen loss to heterospecifics. However, constancy is not necessarily beneficial for pollinators; strict constancy limits foraging opportunity and increases search times for recognizable phenotypes, contradicting predictions of optimal foraging theory (MacArthur & Pianka 1966; Kacelnik et al. 1986; Chittka et al. 1999). Constancy has been described in many insect groups including honeybees, bumblebees, stingless bees, solitary bees, beetles, flies, beeflies, and butterflies (Grant 1950; Free 1963, 1970; Waser 1986; Slaa et al. 1998; Gross 1992; Goulson et al. 1997; Goulson & Wright 1998; Gegear & Laverty 2005; Raine & Chittka 2005; Mico & Galante 2005; Ellis & Johnson 2012; Pohl et al. 2011; Hopkins & Rausher 2012). Constancy estimates vary widely for each group of pollinators and may be contingent on factors such as reward volume, distance between flowers, and flower color (Hill et al. 2001; Gegear & Thomson 2004; Hopkins & Rausher 2012). Estimates of constancy are variable for foraging bumblebees but average 0.40 (Free 1970; Heinrich 1976; Bowers 1985; Waser 1986; Dramstad 1996; Saville et al. 1997; Gegear & Thomson 2004; Chip Taylor, pers. comm.) In my experiments, estimates of bee constancy in flower visits ranged from random (mean $C_I = 0$) to moderately constant (mean $C_I = 0.33$). Constancy is predicted to increase as phenotypes become more easily discernable to pollinators (Gegear & Laverty 2001) and my results are consistent with this prediction. The ability of the bees to distinguish between populations was most apparent in the flower size and flower color experiments. Bees showed a clear preference for the high and control populations versus the low population in the flower size experiment, and

preferred control flowers over pale flowers. As predicted mean constancy values were higher for these two experiments than in the EE experiment, where population differences were subtle (Table 3−5B,C). However, mean constancy estimates were not as high as I would have expected given the extent of bee preference observed in the experiments. The low constancy values in the experiments are likely due to the relatively subtle distinctions between populations presented to the bumblebees. Most constancy measures involve visitation recorded among species, rather than among variants within species. Additionally, Leebens-Mack and Milligan (1998) found that hybrid plants present in their behavioral arrays functioned as 'bridges,' facilitating pollinator foraging between divergent types. In the flower size experiments, my control population may have operated in this manner, blending the available phenotypes into a continuum along which transitions from one population to another were less obvious.

***Bumblebee behavior*—**Pollinators decide which flowers to forage upon by melding innate preferences and individual, learned experience. In the EE experiment, bees demonstrated significant population preference (indicated by $p < 0.05$) for particular flowers they were in contact with (Total visit time) and flowers that they returned to (Total visits). In other words, bee preference for populations manifested itself only when the bees already had some information or experience with a particular flower. In contrast, there was no evidence for preference demonstrated through Std. rank order ($p = 0.92$) and only weak evidence for preference by Visit ($p = 0.08$). These response variables addressed bee behavior in situations where bees had less information about flowers, that is, before they made physical contact. If an increase in phenotypic divergence among populations enhances discriminatory ability among pollinators, I would expect to see greater evidence of pollinator preference in the Flower size and Flower color

experiments. Indeed, all bee response variables (except Total visit in the Flower size experiment) were significant by population in these two experiments. The increased phenotypic divergence among populations in the Flower size and Flower color experiments provided bees with more information prior to contact with a flower. This contrast in behavioral response among experiments provides insight into the limitations of bees to recognize preferred populations despite demonstrated preference once in contact with flowers.

***Implications for ethological speciation*—**Pollinator involvement in plant speciation is enigmatic. Pollinator preferences, previously thought to be fixed (e.g. bee specialization on blue/yellow flowers, hummingbirds on red), have instead been found to be much more general and labile (Ollerton 1996; Aigner 2001; Waser 2001). Correspondingly, flower visitation is much less constant than earlier believed (e.g. Ellis & Johnson 2012, but see Jones & Reithel 2001). Despite strong preference for flower size and color, bees were not constant in my experiments. Thus, constancy is unlikely to play a role in ethological speciation unless differences among populations are very pronounced. Constancy is perhaps more likely post-speciation if plants and pollinators have previously developed highly specialized coevolutionary relationships or if populations re-entering sympatry are already highly divergent in phenotype.

How then do plant species converge on pollination syndromes and evolve specialized morphology without strong preference by a single group of pollinators? Gegear and Burns (2007) used a combination of pollinator choice tests and computer simulations to determine the effects of pollinator preference on ethological speciation. Simulated allopatric populations visited by one pollinator required only weak color preference to fix for a single flower color.

However, simulated sympatric populations, each with its own pollinator, required strong color preference/fidelity by both pollinators to maintain reproductive isolation between the populations. The strong color preferences required by the models to maintain population isolation were not observed in trials with live pollinators (but see Hopkins & Rausher 2012). The study concluded that adaptive divergence in floral color due to pollinator preference is possible, but is not strong enough to induce ethological speciation. However, pollination syndromes contain suites of traits that may be genetically linked (Bradshaw et al. 1995; Hodges et al. 2002) and specific trait combinations have been found to confer higher fitness than others (Armbruster 1990; Cresswall & Galen 1991). Similarly, pollinator preference increases when flowers differ in multiple floral characteristics than when they differ in a single characteristic (Gegear & Laverty 2001, 2005; Raguso & Willis 2002; Gegear 2005). Whether pollinators select on individual traits linked to other floral characters or on preferred combinations of traits is unknown (O'Connell & Johnston 1998; Medel et al. 2003).

My results suggest that pollinators use multiple cues when foraging. The EE and Flower size experimental plants harbored substantial phenotypic and genetic variation both within and across populations. Overall, flower size was the most important morphological trait in determining which plants bees visited; however, flower size only explained a fraction of the variation in bee response variables. Other unmeasured traits, such as pollen load, which may or may not be linked with floral size, likely contributed to bee visitation choices within trials. The flower color experiment reduced trait variability within and among populations. Pale phenotype plants only differed from control plants at a few flower size QTL with the remainder of the genome isogenic. Flower color populations were divergent in both flower size, although size differences were not

striking, and color. Bees clearly discriminated against pale flowers, with much less visitation (only 25% of plants overall) than expected if considering differences in flower size alone. Strong discrimination against the combination of small floral size and pale coloration would likely eliminate the pale phenotype in nature within only a few generations.

CONCLUSION

Pollinator preference for specific *Mimulus* floral traits was demonstrated by patterns of non-random visitation by bumblebees among divergent populations. Multiple cues influenced foraging behavior but flower size and color explained the largest fraction of the variation in bee visits to flowers. Signatures of pollinator preference and constancy increased as trait divergence among populations became more extreme. My results suggest *Bombus* may be a significant selective force upon floral morphology in outcrossing populations and may contribute to pollinator-mediated ethological isolation.

LITERATURE CITED

Aigner, P. A. 2001. Optimality modeling and fitness trade-offs: when should plants become pollinator specialists? Oikos 95: 177–184.

Aigner, P. A. 2004. Floral specialization without trade-offs: optimal corolla flare in contrasting pollination environments. Ecology 85: 2560−2569.

Arathi, H. S., and J. K. Kelly. 2004. Corolla morphology facilitates both autogamy and bumblebee pollination in *Mimulus guttatus*. International Journal of Plant Science 165: 1039−1045.

Armbruster, W. S. 1990. Estimating and testing the shapes of adaptive surfaces: the morphology and pollination of *Dalechampia* blossoms. American Naturalist 135: 14−31.

Armbruster, W. S., Pelabon, C., Hansen, T. F. and C. P. H. Mulder. 2004. Floral integration, modularity, and accuracy: distinguishing complex adaptations from genetic constraints. Pp. 23–49. *In* M. Pigliucci and K. A. Preston, eds. Phenotypic integration: studying the ecology and evolution of complex phenotypes. Oxford University Press, Oxford.

Awadalla, P., and K. Ritland. 1997. Microsatellite variation and evolution in the *Mimulus guttatus* species complex with contrasting mating systems. Molecular Biology and Evolution 14: 1023–1034.

Baker, H. G. 1967. Support for Baker's Law-as a rule. Evolution 21: 853–856.

Blarer, A., Keasar, T. and A. Shmidaa. 2002. Possible mechanisms for the formation of flower size preferences by foraging bumblebees. Ethology 108: 341–351.

Bodbyl Roels, S. A. and J. K. Kelly. 2011. Rapid evolution caused by pollinator loss in *Mimulus guttatus*. Evolution 65: 2541–2552. doi:10.1111/j.1558-5646.2011.01326.x

Bowers, M. A. 1985. Bumblebee colonization, extinction, and reproduction in subalpine meadows in Northeastern Utah. Ecology 66: 914–927.

Bradshaw, H. D., Wilbert, S. M., Otto, K. G. and D. W. Schemske. 1995. Genetic-mapping of floral traits associated with reproductive isolation in monkeyflowers (*Mimulus*). Nature 376: 762–765.

Bradshaw, H. D., Jr. and D. W. Schemske. 2003. Allele substitution at a flower color locus produces a pollinator shift in two monkeyflower species (*Mimulus*). Nature 426: 176–178.

Cameron, S. A. 1981. Chemical signals in bumble bee foraging. Behavioral Ecology and Sociobiology 9: 257–260.

Campbell, D. R. and A. F. Motten. 1985. The mechanisms of competition for pollination between two forest herbs. Ecology 66: 554–563.

Carr, D. E., and M. R. Dudash. 1996. Inbreeding depression in two species of *Mimulus* (Scrophulariaceae) with contrasting mating systems. American Journal of Botany 83: 586−593.

Carr, D. E., and C. B. Fenster. 1994. Levels of genetic variation and covariation for *Mimulus* (Scrophulariaceae) floral traits. Heredity 72: 606−618.

Chittka, L., and N. M. Waser. 1997. Why red flowers are not invisible to bees. Israel Journal of Plant Sciences 45: 169–183.

Chittka, L., Thomson, J. D. and N. M. Waser. 1999. Flower constancy, insect psychology, and plant evolution. Naturwissenschaften 86: 361–177.

Chittka, L. and N. E. Raine. 2006. Recognition of flowers by pollinators. Current Opinion in Plant Biology 9: 428–435.

Conner, J. K., Davis, R., and S. Rush. 1995. The effect of wild radish floral morphology on pollination efficiency by four taxa of pollinators. Oecologia 104: 234–245.

Cresswall, J. E. and C. Galen. 1991. Frequency-dependent selection and adaptive surfaces for floral character combinations: The pollination of *Polemonium viscosum.* American Naturalist: 138: 13–42

Cresswell, J. E., and A. W. Robertson. 1994. Discrimination by pollen-collecting bumblebees among differentially rewarding flowers of an alpine wildflower, *Campanula rotundifolia* (Campanulaceae). Oikos 69: 304–308.

Cresswell, J. E. 2000. Manipulation of female architecture in flowers reveals a narrow optimum for pollen deposition. Ecology 81: 3244–3249.

Darwin, C. 1876. The effects of cross- and self-fertilization in the vegetable kingdom. John Murray, London, UK. 482 pages.

del Castillo, R. C. and D. J. Fairbairn. 2012. Macroevolutionary patterns of bumblebee body size: detecting the interplay between natural and sexual selection. Ecology and Evolution 1: 46–57. doi: 10.1002/ece3.65

Dole. J. A. 1992. Reproductive assurance mechanisms in three taxa of the *Mimulus guttatus* complex (Scrophulariaceae). American Journal of Botany 79: 650−659.

Dramstad, W. E. 1996. Do bumblebees (Hymenoptera, Apidae) really forage close to their nests? Journal of Insect Behavior 9: 163–182.

Elle, E. and R. Carney. 2003. Reproductive assurance varies with flower size in *Collinsia parviflora* (Scrophulariaceae). American Journal of Botany 90: 888−896.

Ellis, A. G., and S. D. Johnson. 2012. Lack of floral constancy by bee fly pollinators: implications for ethological isolation in an African daisy. Behavioral Ecology, advanced access, first published online February 23, 2012. doi:10.1093/beheco/ars019

Fenster, C. B., and K. Ritland. 1994. Evidence for natural selection on mating system in *Mimulus* (Scrophulariaceae). International Journal of Plant Science 155: 588−596.

Fenster, C. B. and D. E. Carr. 1997. Genetics of sex allocation in *Mimulus* (Scrophulariaceae). Journal of Evolutionary Biology 10: 641–661.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., and J. D. Thomson. 2004. Pollination syndromes and floral specialization. Annual Review of Ecology, Evolution, and Systematics 35: 375–403.

Free J. B. 1963. The flower constancy of honeybees. Journal of Animal Ecology 32: 119–131.

Free J. B. 1970. The flower constancy of bumblebees. Journal of Animal Ecology 39: 395–402.

Galen, C. 1989. Measuring pollinator-mediated selection on morphometric floral traits: Bumblebees and the alpine skypilot, *Polemonium viscosum.* Evolution 43: 882–890.

Galen, C. 1996. Rates of floral evolution: Adaptation to Bumblebee pollination in an alpine wildflower, *Polemonium viscosum*. Evolution 50: 120–125.

Galliot, C., Hoballah, M. E., Kuhlemeier, C., and J. Stuurman. 2006. Genetic control of flower size and nectar volume in *Petunia* pollination syndromes. Planta 225: 203–212.

Gegear, R. J. and T. M. Laverty. 2001. The effect of variation among floral traits on the flower constancy of pollinators. Pp. 1–20. *In:* L. Chittka and J. D Thomson, eds. Cognitive Ecology of Pollination: Animal Behaviour and Floral Evolution. Cambridge University Press, Cambridge, UK.

Gegear, R. J. and J. D. Thomson. 2004. Does the flower constancy of bumblebees reflect foraging economics? Ethology 110: 793–805.

Gegear, R. J. 2005. Multicomponent floral signals elicit selective foraging in bumblebees. Naturwissenschaften 92: 269–271.

Gegear, R. J. and T. M. Laverty. 2005. Flower constancy in bumblebees: a test of the trait variability hypothesis. Animal Behavior 69: 939–949. doi:10.1016/j.anbehav.2004.06.029

Gegear, R. J. and J. G. Burns. 2007. The birds, the Bees, and the virtual flowers: can pollinator behavior drive ecological speciation in flowering plants? American Naturalist 170: 551–566.

Goulson, D., Ollerton, J. and C. Sluman. 1997. Foraging strategies in the small skipper butterfly, *Thymelicus flavus*: when to switch? Animal Behavior 53: 1009–1016.

Goulson, D. and N. P. Wright. 1998. Flower constancy in the hoverflies *Episyrphus balteatus* (Degeer) and *Syrphus ribesii* (L.) (Syrphidae). Behavioral Ecology 9: 213–219.

Grant, V. 1949. Pollination systems as isolating mechanisms in Angiosperms. Evolution 3: 82−97.

Grant, V. 1950. The flower constancy of bees. Botanical Review 16: 379–398.

Grant, K. A., and V. Grant. 1968. Hummingbirds and their flowers. Journal of Ecology 58: 318–319.

Gross, C. L. 1992. Floral traits and pollinator constancy: foraging by native bees among three sympatric legumes. Australian Journal of Ecology 17: 67–74.

Harder, L. D. and S. D. Johnson. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. New Phytologist 183: 530-545.

Heinrich, B. 1976. The foraging specializations of individual bumblebees. Ecological Monographs 46: 105–128.

Heinrich, B., Mudge, P. R. and P. G. Deringis. 1977. Laboratory analysis of flower constancy in foraging bumblebees: *Bombus ternarius* and *Bombus terricola*. Behavioral Ecology and Sociobiology 2: 247–265.

Hill, P. S. M., Hollis, J. and H. Wells. 2001. Foraging decisions in nectarivores: unexpected interactions between flower constancy and energetic rewards. Animal Behaviour 62: 729–737.

Hodges, S. A. 1995. The influence of nectar production on hawkmoth behavior, self pollination and seed production in *Mirabilis multiflora* (Nyctaginaceae). American Journal of Botany 82: 197−204.

Hodges, S. A., Whittall, J. B., Fulton, M. and J. Y. Yang. 2002. Genetics of floral traits influencing reproductive isolation between *Aquilegia formosa* and *Aquilegia pubescens.* American Naturalist 159: S51–S60.

Hopkins, R. and M. D. Rausher. 2012. Pollinator-mediated selection on flower color allele drives reinforcement. Science 335: 1090–1092.

Hu, S., Dilcher, D. L., Jarzen, D. M., and D. W. Taylor. 2008. Early steps of angiosperm–pollinator coevolution. Proceedings of the National Academy of Sciences 105: 240–245.

Ivey, C. T., and D. E. Carr. 2005. Effects of herbivory and inbreeding on the pollinators and mating system of *Mimulus guttatus* (Phrymaceae). American Journal of Botany 92: 1641−1649.

Jacobs, J. 1974. Quantitative measurement of food selection: a modification of the forage ratio and Ivlev's Electivity Index. Oecologia 14: 413–417.

Jones, K. N. and J. S. Reithel. 2001. Pollinator-mediated selection on a flower color polymorphism in experimental populations of *Antirrhinum* (Scrophulariaceae). American Journal of Botany 88: 447–454.

Kacelnik, A., Houston, A. I. and P. Schmidt-Hempel. 1986. Central place foraging in honey bees: the effect of travel time and nectar flow on crop filling. Behavioral Ecology and Sociobiology 19: 19–24. doi:10.1007/BF00303838.

Kalisz, S., Vogler, D. W., and K. M. Hanley. 2004. Context-dependent autonomous self-fertilization yields reproductive assurance and mixed mating. Nature 430: 884–887.

Kelly, J. K. 2008. Testing the rare alleles model of quantitative variation by artificial selection. Genetica 132:187−198.

Kelly, J. K. and H. S. Arathi. 2003. Inbreeding and the genetic variance of floral traits in *Mimulus guttatus.* Heredity 90: 77−83.

Kelly, J. K. and J. P. Mojica. 2011. Interactions among flower size QTLs of *Mimulus guttatus* are abundant but highly variable in nature. Genetics 189: 146–171.

Kessler, D. and I. T. Baldwin. 2011. Back to the past for pollination biology. Current Opinion in Plant Biology 14: 429–434.

Klahre, U., Gurba, A., Hermann, K., Saxenhofer, M., Bossolini, E., Guerin, P. M., and C. Kuhlemeier. 2011. Pollinator choice in *Petunia* depends on two major genetic loci for floral scent production. Current Biology 21: 730–739.

Kugler, H. 1943. Hummeln als Blutenbesucher. Ein Beitrag zur experimentellen Blutenokologie. Ergebn Biologie 19: 143–323.

Leebens-Mack, J. H. and B. G. Milligan. 1998. Pollination biology in hybridizing *Baptisia* (Fabaceae) populations. American Journal of Botany 85: 500–507.

Leonard, A. S., Dornhaus, A., and D. R. Papaj. 2011. Flowers help bees cope with uncertainty: signal detection and the function of floral complexity. Journal of Experimental Biology 214: 113–121.

Levin, D. A. 1972. The adaptedness of corolla colour and outline in experimental and natural populations of *Phlox drummondii.* American Naturalist 106: 57–70.

Lunau, K. 1991. Innate flower recognition in bumblebees (*Bombus terrestris, B. lucorum*; Apidae): optical signals from the stamens as landing reaction releasers. Ethology 88: 203–214.

Macnair, M. R., and Q. J. Cumbes. 1989. The genetic architecture of interspecific variation in *Mimulus.* Genetics 122: 211−222.

MacArthur, R. H. and E. R. Pianka. 1966. On the optimal use of a patchy environment. American Naturalist 100: 603–609.

Medel, R., Botto-Mahan, C., and M. Kalin-Arroyo. 2003. Pollinator-mediated selection on the nectar guide phenotype in the Andean monkey flower, *Mimulus luteus*. Ecology 84: 1721–1732.

Mico, E., and E. Galante. 2005. Behavior of Cetonid beetles (Coleoptera: Scarabaeoidea: Cetoniidae) on the Mediterranean shrub *Cistus albidus*. Elytron 19: 89–98.

Nilsson, L. A. 1988. The evolution of flowers with deep corolla tubes. Nature 334: 147–149.

O'Connell, L. M., and M. O. Johnston. 1998. Male and female pollination success in a deceptive orchid, a selection study. Ecology 79: 1246–1260.

Ollerton, J. 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-pollinator systems. Journal of Ecology 84:767–769.

Ornduff, R. 1969. Reproductive biology in relation to systematics. Taxon 18:121–133.

Owen, R. E., Whidden, T. L. and R. C. Plowright. 2010. Genetic and morphometric evidence for the conspecific status of the bumble bees, *Bombus melanopygus* and *Bombus edwardsii*. Journal of Insect Science 10: 1−18.

Pohl, N. B., Van Wyk, J. and D. R. Campbell. 2011. Butterflies show flower colour preferences but not constancy in foraging at four plant species. Ecological Entomology 36: 290–300. doi: 10.1111/j.1365-2311.2011.01271.x

Proctor, M., Yeao, P., and A. Lack. 1996. The Natural History of Pollination. Harper Collins Publishers. 479 pages.

Pyke, G. H., Day, L. P. and K. A.Wale. 1988. Pollination ecology of Christmas Bells (*Blandfordia nobilis* Sm.): effects of adding artificial nectar on pollen removal and seed set. Australian Journal of Ecology 13: 279–284.

Raguso, R. A., and M. A. Willis. 2002. Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Manduca sexta*. Animal Behaviour 64: 685–695.

Raine, N. E., and L. Chittka. 2005. Comparison of flower constancy and foraging performance in three bumblebee species (Hymenoptera: Apidae: *Bombus*). Entomologia Generalis 28: 81–89.

Rasheed, S. A., and L. A. Harder. 1997a. Economic motivation for plant species preferences of pollen-collecting bumble bees. Ecological Entomology 22: 209–219.

Rasheed, S. A., and L. A. Harder. 1997b. Foraging currencies for non-energetic resources: pollen collection by bumblebees. Animal Behavior 54: 911–926.

Real, L. A. and B. J. Rathcke. 1991. Individual variation in nectar production and its effect on fitness in *Kalmia latifolia*. Ecology 72: 149–155.

Ritland, K., and F. R. Ganders. 1987. Covariation of selfing rates with parental gene fixation indexes within populations of *Mimulus guttatus*. Evolution 41: 760–771.

Ritland, C., and K. Ritland. 1989. Variation of sex allocation among 8 taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). American Journal of Botany 76: 1731−1739.

Robertson, A. W., Diaz, A. and M. R. MacNair. 1994. The quantitative genetics of floral characters in *Mimulus guttatus*. Heredity 72: 300−311.

Robertson, A. W., Mountjoy, C., Faulkner, B. E., Roberts, M. V. and M. R. Macnair. 1999. Flower choice by bumblebees collecting pollen from *Mimulus guttatus* (Scrophulariaceae): the effects of pollen quality and reward depletion. Ecology 80: 2594−2606.

Saville, N. M., Dramstad, W. E., Fry, G. L. A. and S. A. Corbet. 1997. Bumblebee movement in a fragmented agricultural landscape. Agriculture, Ecosystems and Environment 61: 145−154.

Schemske, D. W. and H. D. Bradshaw. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). Proceedings of the National Academy of Science 96: 11910–11915.

Scoville, A., Lee, Y. W., Willis, J. H. and J. K. Kelly. 2009. Contribution of chromosomal polymorphisms to the G-matrix of *Mimulus guttatus*. New Phytologist 183: 803–815.

Sicard, A. and M. Lenhard. 2011. The selfing syndrome: a model for studying the genetic and evolutionary basis of morphological adaptations in plants. Annals of Botany 107: 1433–1443.

Slaa, E. J., Cevaal, A., and M. J. Sommeijer. 1998. Floral constancy in *Trigona* stingless bees foraging on artificial flower patches: a comparative study. Journal of Apicultural Research 37: 191–198.

Spaethe, J., Tautz, J., and L. Chittka. 2001. Visual constraints in foraging bumblebees: flower size and colour affect search time and flight behaviour. Proceedings of the National Academy of Science 98: 3898–3903.

Stebbins, G. L. 1970. Adaptive radiation in angiosperms. I. Pollination mechanisms. Annual Review of Ecological Systematics 1: 307–326.

Stebbins, G. L. 1974. Flowering plants: evolution above the species level. Harvard University Press, Cambridge, MA. 399 pages.

Sutherland, S. D., and R. K. Vikkery, Jr. 1993. On the relative importance of floral color, shape and nectar rewards in attracting pollinators to *Mimulus*. Great Basin Naturalist 53: 107–117.

Sweigart, A., Karoly, K., Jones, A. and J. H. Willis. 1999. The distribution of individual inbreeding coefficients and pairwise relatedness in a population of *Mimulus guttatus*. Heredity 83: 625–632.

Thomson, J. D., Andrews, B. J. and R. C. Plowright. 1981. The effect of a foreign pollen on ovulae development in *Diervilla lonicera* (Caprifoliaceae). New Phytologist 90: 777–783.

Ushimaru, A., and K. Nakata. 2002. The evolution of flower allometry in selfing species. Evolutionary Ecology Research 4: 1217–1227.

van Kleunen, M. and K. Ritland. 2004. Predicting the evolution of floral traits associated with mating system in a natural plant population. Journal of Evolutionary Biology 17: 1389–1399.

Waser, N. M. 1986. Flower constancy: definition, cause and measurement. American Naturalist 127: 593–603.

Waser, N. M. 1998. Pollination, angiosperm speciation, and the nature of species boundaries. Oikos 82:198–201.

Waser, N. M. 2001. Pollinator behavior and plant speciation: looking beyond the "ethological isolation" paradigm. Pp. 318–335. *In* L. Chittka and J. D. Thomson, eds. Cognitive ecology of pollination. Cambridge University Press, Cambridge, UK.

Waser, N. M., and D. R. Campbell. 2004. Ecological speciation in flowering plants. Pp. 264–277. *In* U. Dieckmann, H. Metz, M. Doebeli, and D. Tautz, eds. Adaptive speciation. Cambridge University Press, Cambridge, UK.

Willis, J. H. 1993. Partial self fertilization and inbreeding depression in two populations of *Mimulus guttatus*. Heredity 71: 145−154.

Willis, J. H. 1996. Measures of phenotypic selection are biased by partial inbreeding. Evolution 50: 1501–1511.

Willis, J. H. 1999a. Inbreeding load, average dominance, and the mutation rate for mildly deleterious alleles in *Mimulus guttatus*. Genetics 153: 1885–1898.

Willis, J. H. 1999b. The role of genes of large effect on inbreeding depression in *Mimulus guttatus*. Evolution 53: 1678–1691.

Wolfe, L. M., and S. C. H. Barrett. 1989. Patterns of pollen removal and deposition in tristylous *Pontederia cordata* L. (Pontederiaceae). Biological Journal of the Linnaean Society 36: 317–329.

Wu, C. A., Lowry, D. B., Cooley, A. M., Wright, K. M., Lee, Y. W. and J. H. Willis. 2008. *Mimulus* is an emerging model system for the integration of ecological and genomic studies. Heredity 100: 220–230.

Table 3–1. Floral trait measures and bee response variables for A) Experimental evolution (EE) trials, B) Flower size trials, and C) Flower color trials. Values reported are LS means. Standard deviations are given in parentheses. CW = corolla width, TW = throat width, TL = tube length, A/S = anther-stigma separation, LW = leaf width. Bee Contr. = Bee seed – Self seed.

A) EE

Pop	CW (mm)	TW (mm)	TL (mm)	A/S (mm)	Self Seed	Bee Seed	Bee Contr.	Visit	Std. Rank Order	Total Visits	Total Visit Time (s)
A1	15.98 (3.41)	8.56 (1.24)	11.19 (1.49)	1.17 (1.06)	6.69 (14.99)	34.13 (50.82)	26.57 (54.51)	0.91 (0.29)	0.38 (0.27)	1.80 (1.14)	11.99 (8.90)
A2	16.68 (3.87)	8.88 (1.21)	10.76 (1.45)	1.62 (1.13)	12.81 (25.98)	31.88 (49.01)	20.05 (56.8)	0.89 (0.32)	0.36 (0.24)	1.85 (1.3)	19.60 (20.33)
B1	17.31 (3.36)	8.81 (1.20)	11.09 (1.61)	1.85 (1.01)	9.10 (23.02)	31.65 (38.45)	17.08 (37.73)	0.95 (0.23)	0.37 (0.26)	1.99 (1.15)	21.10 (18.14)
B2	16.28 (2.83)	8.82 (1.01)	11.27 (1.12)	1.88 (0.93)	3.23 (11.43)	41.85 (55.30)	38.54 (57.53)	0.97 (0.17)	0.36 (0.24)	2.25 (1.17)	20.00 (20.68)

B) Flower size

Pop	CW (mm)	A/S (mm)	Self Seed	Bee Seed	Bee Contr.	Visit	St. Rank Order	Total Visits	Total Visit Time (s)
C	17.36 (2.78)	1.65 (0.92)	3.65 (10.68)	30.38 (38.38)	31.63 (40.52)	0.87 (0.34)	0.41 (0.31)	2.16 (1.72)	13.01 (15.8)
H	26.11 (4.24)	1.90 (0.98)	5.75 (18.35)	40.24 (55.93)	42.82 (61.94)	0.97 (0.17)	0.30 (0.26)	2.89 (1.79)	35.87 (49.39)
L	12.46 (2.40)	1.32 (0.80)	2.60 (7.49)	24.45 (28.87)	26.52 (30.11)	0.82 (0.39)	0.44 (0.28)	1.52 (1.28)	7.94 (11.84)

C) Flower color

Pop	CW (mm)	A/S (mm)	Visit	Std. Rank Order	Total Visits	Total Visit Time (s)
Control	14.50 (1.64)	1.44 (0.46)	0.79 (0.41)	0.45 (0.29)	1.08 (0.72)	20.04 (23.13)
Pale	12.26 (2.14)	1.33 (0.57)	0.25 (0.44)	0.72 (0.29)	0.25 (0.44)	2.88 (7.81)

Table 3–2. GLM ANOVA results for A) plant phenotypes and B) bee response variables of EE, flower size, and flower color experiments. Likelihood ratio tests (LRT) are reported from GLM models. Significant p-values are followed by a *.

A) *Phenotype*	Factor			Factor		
Trait	**Population**			**Trial**		
EE	*LRT*	*df*	*p-value*	*LRT*	*df*	*p-value*
CW	14.34	3	0.0025*	101.17	21	<.0001*
TW	4.67	3	0.1970	57.74	21	<.0001*
TL	3.92	3	0.2700	57.64	21	<.0001*
A/S	19.74	3	0.0002*	20.17	21	0.5099
Self Seed	4.68	3	0.1963	35.57	21	0.0020*
Bee Seed	3.36	3	0.338	45.2	21	<.0001*
Bee Contr.	5.67	3	0.1286	42.86	21	0.0002*
Flower size						
CW	466.10	2	<.0001*	57.52	19	<.0001*
A/S	21.01	2	<.0001*	20.43	19	0.3688
Self Seed	3.46	2	0.1766	25.58	19	0.1422
Bee Seed	8.47	2	0.0145*	51.76	19	<.0001*
Bee Contr.	5.23	2	0.0729	33.67	19	0.0201*
Flower color						
CW	24.82	1	<.0001*	30.22	2	<.0001*
A/S	0.61	1	0.4329	8.25	2	0.0162*

Table 3–2. (Continued)

B)

Bee response variables	Factor			Factor		
Traits	**Population**			**Trial**		
EE	*LRT*	*df*	*p-value*	*LRT*	*df*	*p-value*
Std. Rank Order	0.36	3	0.9480	19.29	21	0.5663
Total Visit Time	11.55	3	0.0091*	85.07	21	<.0001*
Visit	6.75	3	0.0802	30.30	21	0.0861
Total Visits	35.66	3	<.0001*	206.35	21	<.0001*
Flower size						
Std. Rank Order	16.02	2	0.0003*	30.47	19	0.0460
Total Visit Time	49.32	2	<.0001*	42.07	19	0.0017*
Visit	15.88	2	0.0004*	52.17	19	<.0001*
Total Visits	3.46	2	0.1766	25.58	19	0.1422
Flower color						
Std. Rank Order	4.63	1	0.031*	0.66	2	0.7192
Total Visit Time	17.11	1	<.0001*	24.33	2	<.0001*
Visit	15.40	1	<.0001*	1.68	2	0.4311
Total Visits	26.69	1	<.0001*	12.22	2	0.0022*

Table 3–3. Tukey-Kramer post hoc tests for differences among populations for EE and Flower size experiments. Only variables significant by population in two-factor ANOVA are included (Table 3–2). Letters that are shared by populations are not significantly different from one another. A) EE, B) Flower size

A) EE

Trait	A1	A2	B1	B2
CW	B	AB	A	AB
A/S	B	A	A	A
Total Visits	A	A	A	B
Total Visit Time	B	A	A	A

B) Flower size

Trait	Control	High	Low
CW	A	B	C
A/S	A	A	B
Bee Seed	A	B	B
Std. Rank Order	A	B	A
Visit	AB	A	B
Total Visit Time	A	B	A

Table 3–4. (Following 3 pages) ANCOVA. A) EE, B) Flower size, C) Flower color. Each variable was tested with a full GLM model, including interaction terms between population and covariates (Factors: Trial, Population, CW, A/S, Population*CW, Population*A/S). Non-significant population by covariate interactions were dropped from the models. Significant p-values are followed by a *.

A) EE				
Source	**Variable**	**df**	**LRT**	**p-value**
Bee response variables				
Trial	Visit	21	32.65	0.050
Population		3	5.13	0.162
CW		1	9.29	0.002*
A/S		1	1.70	0.191
Trial	Total visits	21	198.75	<.0001*
Population		3	36.91	<.0001*
CW		1	30.39	<.0001*
A/S		1	12.78	0.0003*
Pop*CW		3	52.95	<.0001*
Pop*A/S		3	31.76	<.0001*
Trial	Total visit time	21	4.354	<.0001*
Population		3	3.054	0.0287*
CW		1	2.428	0.1202
A/S		1	0.239	0.6253
Trial	Std. rank order	21	0.807	0.711
Population		3	0.109	0.954
CW		1	3.615	0.058
A/S		1	0.055	0.814
Fitness variables				
Trial	Self seed	15	1233.12	<.0001*
Population		3	71.79	<.0001*
CW		1	53.65	<.0001*
A/S		1	176.35	<.0001*
Trial	Bee seed	15	45.38	<.0001*
Population		3	2.94	0.4006
CW		1	0.32	0.5680
A/S		1	6.93	0.0085*
Pop*CW		3	8.96	0.0298*
Trial	Bee contr.	15	2.788	0.0006*
Population		3	1.633	0.1827
CW		1	0.547	0.4603
A/S		1	1.386	0.2404
Bee response inter-relationships				
Trial	Bee contr.	15	41.49	0.0003*
Population		3	3.53	0.3159
Total visits		1	1.09	0.2948
Trial	Total visit time	21	105.53	<.0001*
Population		3	12.44	0.0060*
Std. rank order		1	49.48	<.0001*
Pop*Std. rank order		3	9.55	0.0227*

B) Flower size				
Source	**Variable**	**df**	**LRT**	**p-value**
Bee response variables				
Trial	Visit	19	33.37	0.0217*
Population		2	1.63	0.4414
CW		1	16.09	<.0001*
A/S		1	2.79	0.0947
Pop*CW		2	16.70	0.0002*
Pop*A/S		2	10.89	0.0043*
Trial	Total visits	19	359.69	<.0001*
Population		2	20.56	<.0001*
CW		1	61.33	<.0001*
A/S		1	11.16	0.0008*
Pop*CW		2	49.38	<.0001*
Pop*A/S		2	19.22	<.0001*
Trial	Total visit time	19	2.40	0.0011*
Population		2	1.76	0.1731
CW		1	22.49	<.0001*
A/S		1	2.36	0.1252
Trial	Std. rank order	19	1.67	0.0413*
Population		2	1.13	0.3219
CW		1	12.74	0.0004*
A/S		1	0.84	0.3577
Fitness variables				
Trial	Self seed	19	24.61	0.1734
Population		2	2.70	0.2584
CW		1	0.90	0.3417
A/S		1	0.32	0.5657
Trial	Bee seed	19	44.90	0.0007*
Population		2	0.81	0.6664
CW		1	1.51	0.2176
A/S		1	6.08	0.0137*
Trial	Bee contr.	19	28.72	0.0704
Population		2	0.60	0.7396
CW		1	1.61	0.2041
A/S		1	3.10	0.0779
Bee response inter-relationships				
Trial	Bee contr.	19	1.88	0.0178*
Population		2	0.87	0.4221
Total visits		1	5.41	0.0210*
Trial	Total visit time	2	1.92	0.0131*
Population		19	0.99	0.3699
Std. rank order		1	16.07	<0.001*
CW		1	14.71	0.0002*

C) Flower color				
Source	**Variable**	**df**	**LRT**	**p-value**
Bee response variables				
Trial	Visit	2	1.16	0.5590
Population		1	7.81	0.0052*
CW		1	0.60	0.4370
A/S		1	2.77	0.0958
Trial	Total visits	2	12.04	0.0024*
Population		1	15.20	<.0001*
CW		1	8.96	0.0028*
A/S		1	6.12	0.0133*
Pop*CW		1	8.22	0.0041*
Pop*A/S		1	3.97	0.0463*
Trial	Total visit time	2	21.52	<.0001*
Population		1	8.03	0.0046*
CW		1	1.52	0.2165
A/S		1	0.002	0.9606
Pop*CW		1	7.69	0.0055*
Trial	Std. rank order	2	0.14	0.8679
Population		1	1.35	0.2592
CW		1	0.28	0.6028
A/S		1	0.15	0.7009
Bee response inter-relationships				
Trial	Total visit time	1	1.06	0.3022
Population		2	23.65	<.0001*
Std. rank order		1	2.29	0.1301

Table 3–5. Transition frequencies and constancy between experimental populations within trials. A) EE: N = bee excluded populations (A1, A2), B = bee pollinated populations (B1, B2). B) Flower size: H = high, L = low, and C = control. C) Flower color: P = pale phenotype, C = Control.

A) Experimental Evolution (EE)

Experiment	N→N	N→B	B→N	B→B	# transitions	C_I
1	0.08	0.35	0.31	0.27	26	-0.185
2	0.19	0.26	0.23	0.32	31	0.162
3	0.14	0.29	0.29	0.29	21	-0.013
4	0.25	0.25	0.30	0.20	20	0.030
5	0.25	0.30	0.30	0.15	20	-0.072
6	0.12	0.20	0.20	0.48	25	0.322
7	0.29	0.24	0.29	0.19	21	0.083
8	0.17	0.21	0.24	0.38	29	0.230
9	0.16	0.37	0.37	0.11	19	-0.366
10	0.23	0.29	0.29	0.19	31	-0.032
11	0.32	0.23	0.27	0.18	22	0.130
12	0.19	0.23	0.26	0.32	31	0.162
13	0.14	0.27	0.27	0.32	37	0.049
14	0.16	0.32	0.36	0.16	25	-0.241
15	0.31	0.24	0.28	0.17	29	0.096
16	0.23	0.27	0.23	0.27	30	0.130
17	0.18	0.29	0.26	0.26	34	0.013
18	0.32	0.27	0.29	0.12	41	0.008
19	0.15	0.27	0.27	0.31	26	0.054
20	0.13	0.34	0.38	0.16	32	-0.326
21	0.21	0.26	0.29	0.24	42	0.035
22	0.29	0.29	0.29	0.14	35	-0.013
23	0.07	0.30	0.30	0.33	27	-0.057
Mean:	**0.20**	**0.27**	**0.28**	**0.24**	**28.43**	**0.010**

Table 3–5. (Continued)

B) Flower size

Experiment	H→H	L→L	C→C	H→L	H→C	L→H	L→C	C→H	C→L	# trans	C_I
1	0.15	0.03	0.06	0.03	0.18	0.09	0.12	0.18	0.15	33	-0.007
2	0.14	0.07	0.07	0.24	0.03	0.14	0.14	0.14	0.03	29	0.080
3	0.11	0.09	0.06	0.09	0.20	0.09	0.09	0.20	0.09	35	0.032
4	0.04	0.07	0.11	0.11	0.15	0.04	0.19	0.19	0.11	27	-0.064
5	0.10	0.05	0.10	0.12	0.14	0.17	0.10	0.07	0.17	42	-0.019
6	0.12	0.07	0.10	0.12	0.12	0.07	0.14	0.14	0.12	42	0.104
7	0.11	0.09	0.11	0.14	0.11	0.17	0.09	0.06	0.11	35	0.171
8	0.13	0.07	0.17	0.13	0.10	0.07	0.13	0.13	0.07	30	0.282
9	0.11	0.03	0.11	0.16	0.11	0.16	0.11	0.14	0.08	37	-0.005
10	0.06	0.11	0.11	0.06	0.22	0.17	0.06	0.11	0.11	18	0.085
11	0.26	0.04	0.13	0.04	0.17	0.04	0.04	0.22	0.04	23	0.407
12	0.19	0.06	0.19	0.06	0.13	0.13	0.06	0.13	0.06	16	0.411
13	0.19	0.04	0.04	0.07	0.19	0.15	0.07	0.11	0.15	27	0.038
14	0.25	0.05	0.05	0.15	0.15	0.20	0.05	0.05	0.05	20	0.248
15	0.15	0.10	0	0.15	0.15	0.10	0.10	0.20	0.05	20	0.013
16	0.15	0.04	0.04	0.04	0.27	0.12	0.04	0.19	0.12	26	-0.039
17	0.28	0.03	0.14	0.10	0.14	0.07	0.07	0.14	0.03	29	0.429
18	0.14	0	0.07	0.07	0.21	0.14	0.07	0.14	0.14	14	-0.087
19	0.20	0.05	0.10	0.15	0.05	0.10	0.15	0.10	0.10	20	0.248
20	0.22	0.11	0	0	0.33	0.11	0	0.11	0.11	9	0.213
Mean:	**0.15**	**0.06**	**0.09**	**0.10**	**0.16**	**0.12**	**0.09**	**0.14**	**0.09**	**27**	**0.127**

C) Flower color

Experiment	P→P	P→C	C→P	C→C	# transitions	C_I
1	0.11	0.11	0.11	0.67	9	0.639
2	0	0.29	0.43	0.29	7	-0.316
3	0	0.07	0.13	0.80	15	0.677
Mean:	**0.04**	**0.15**	**0.22**	**0.58**	**10**	**0.333**

Table 3–6. Two-sample T-tests for flower size differences between plants visited at least once in a trial and those unvisited. A) EE, B) Flower size, C) Flower color.

A)

Visit	N	Mean	Stdev	SE	Mean diff	95% CI Lower	Upper	t-stat	p-value	df
0	27	14.81	3.72	0.72	1.95	0.44	3.46	2.64	0.013	29
1	341	16.77	3.38	0.18						

B)

Visit	N	Mean	Stdev	SE	Mean diff	95% CI Lower	Upper	t-stat	p-value	df
0	34	13.55	5.05	0.87	5.74	3.83	7.65	6.04	<0.001	47
1	266	19.29	6.39	0.39						

C)

Visit	N	Mean	Stdev	SE	Mean diff	95% CI Lower	Upper	t-stat	p-value	df
0	23	12.55	2.48	0.51	1.58	0.33	2.81	2.58	0.014	37
1	25	14.13	1.60	0.32						

Figure 3–1. Phenotypic floral measurements.

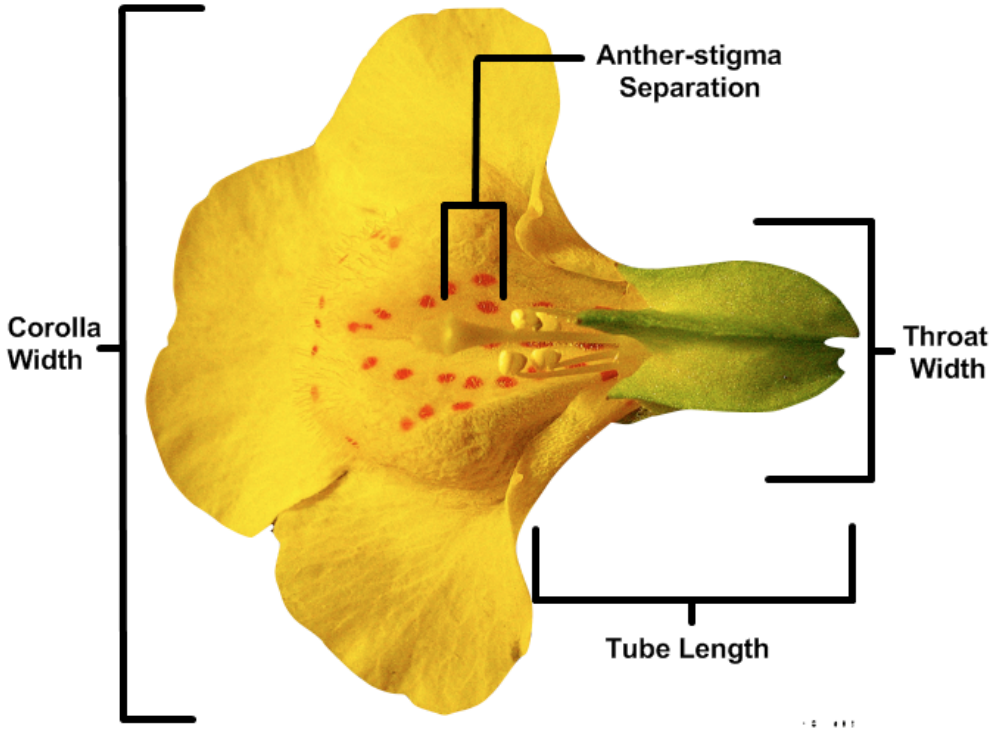


Figure 3–2. Schematic of the acrylic glass flight chamber used for bumblebee experiments.

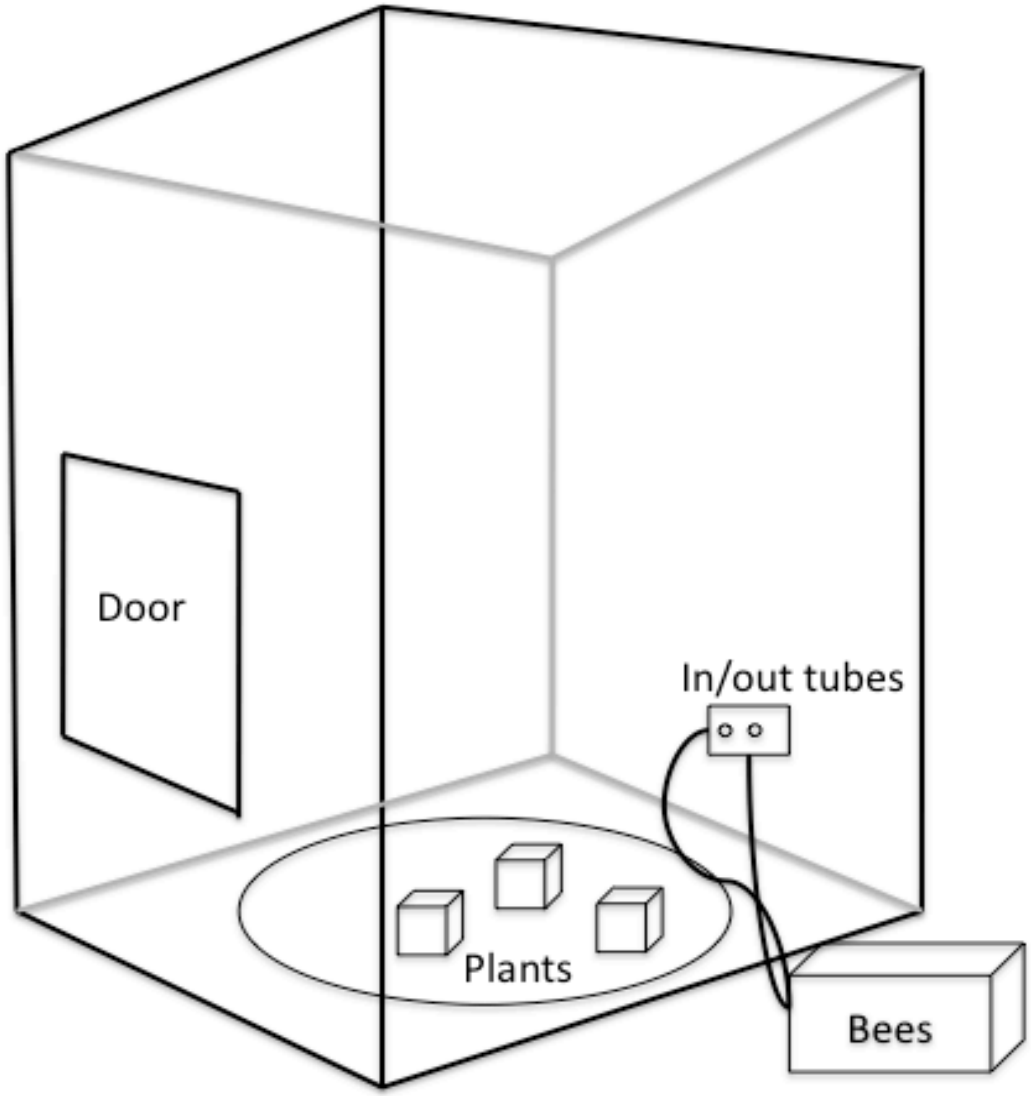


Figure 3–3. Scatterplots from select ANCOVA models from the Flower size experiment where the covariate (x-axis) had a significant interaction with Population.

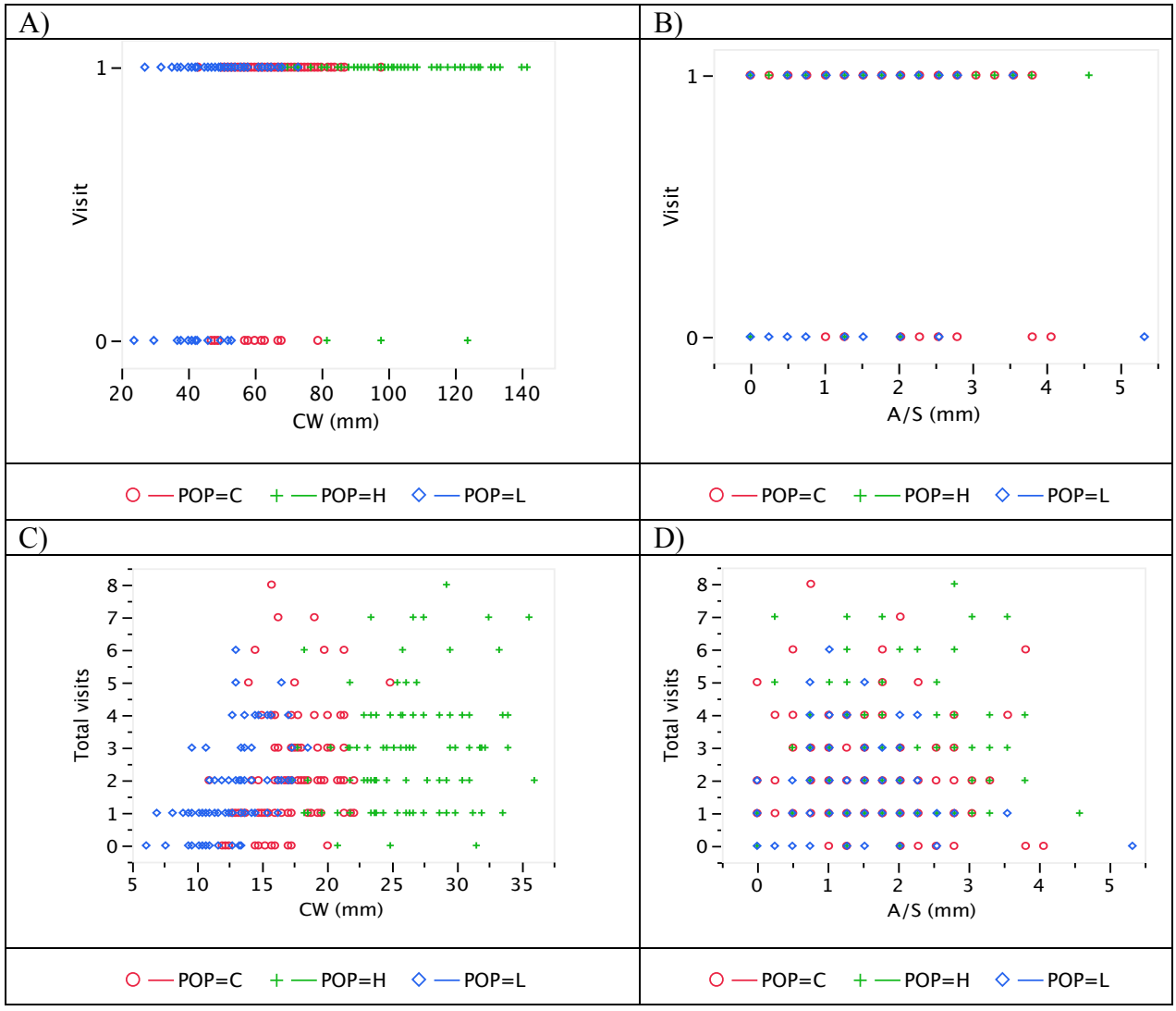


Figure 3–4. Expected and observed counts of transitions between flower types for each experiment. A) EE: N = No Bee populations, B = Bee. B) Flower size: H = High population, C = Control, and L = Low. C) Flower color: P = Pale population, C = Control.

A)

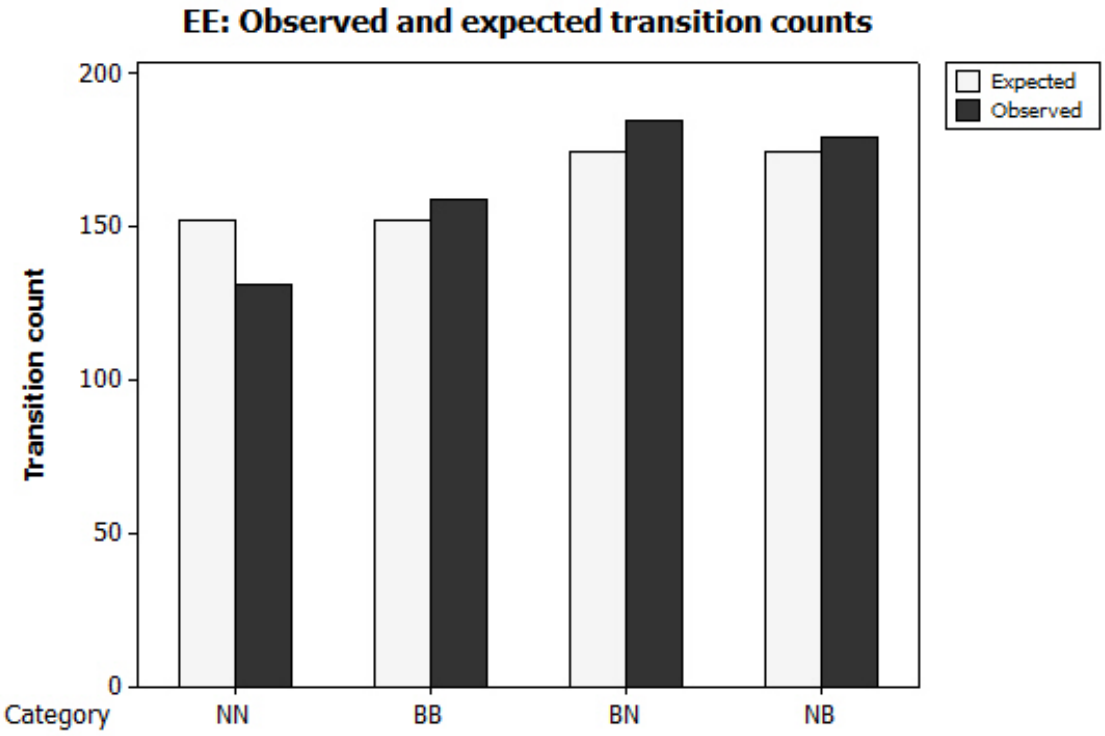
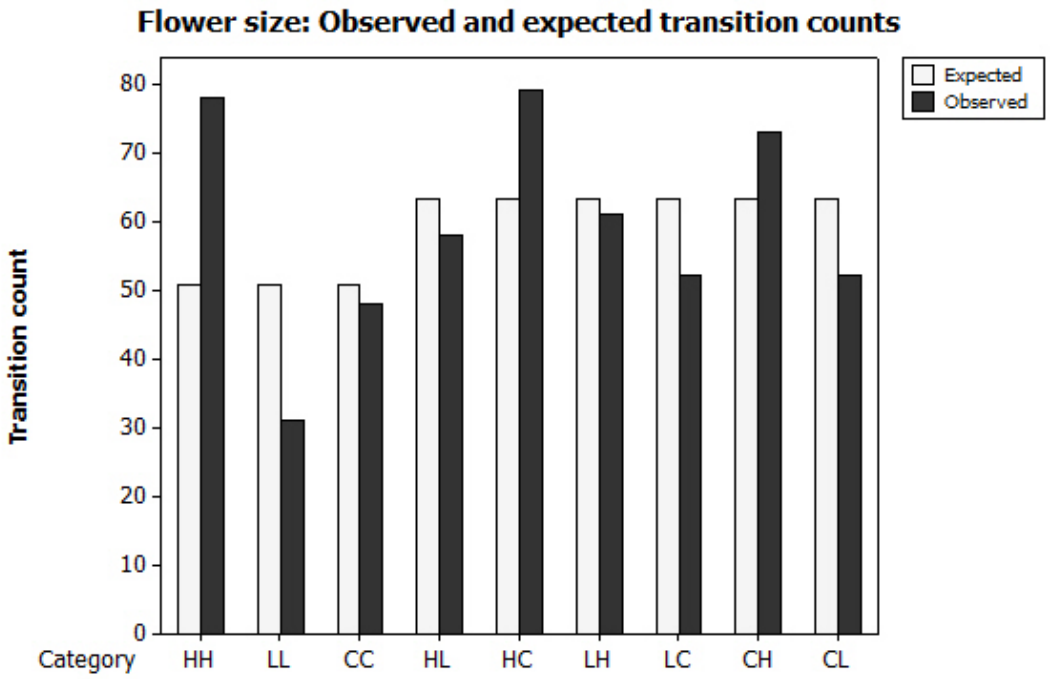
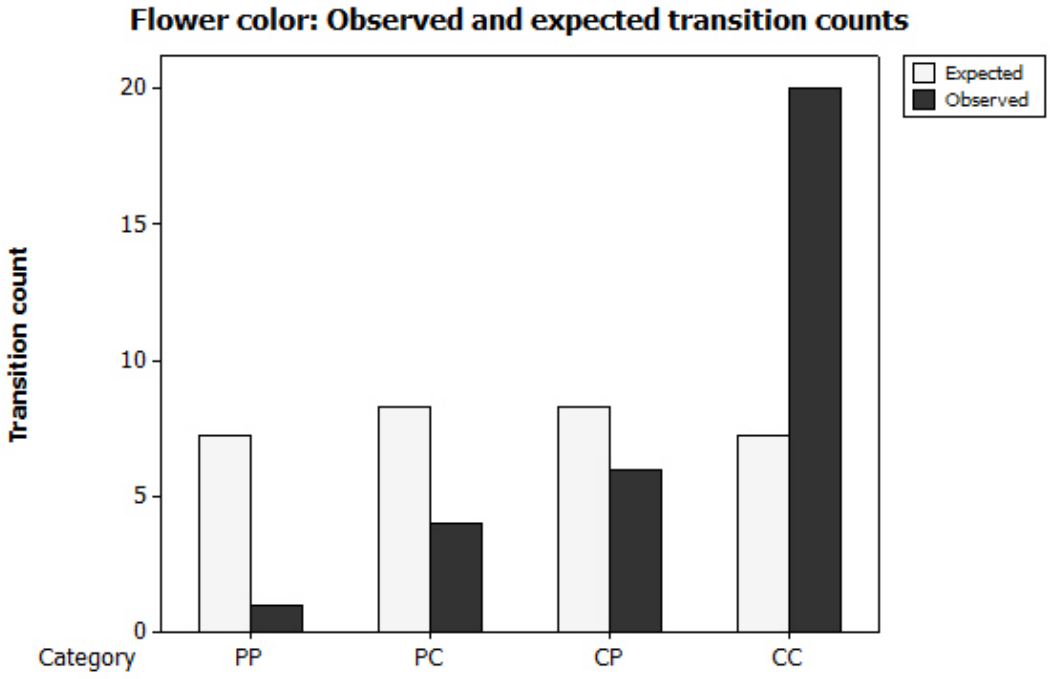


Figure 3–4. (Continued)
B)



C)



Chapter 4. Characterization of floral ultraviolet patterning in *Mimulus*

ABSTRACT

Flowers function as a signaling mechanism between plants and pollinators; pollinators rely on multiple floral cues to optimize foraging. Many floral traits such as shape, size, and color have been thoroughly investigated in the literature but others are cryptic to human observers and are often overlooked. I use four independent methods to document floral ultraviolet (UV) patterning in populations of *Mimulus guttatus* and *M. nasutus,* including UV photography, optical reflectance spectrometry, pigment extract spectrometry, and SEM imagery. Optical reflectance spectra were shown to be ineffective diagnostic tools for recording UV patterns. UV patterns were found to be variable both within and among populations, both in extent of petal coverage and in chemical composition. UV patterns appeared to be primarily due to variable concentrations of UV absorbing chemicals within the floral tissue, and were not affected by epidermal cell shape.

INTRODUCTION

The diversity of angiosperm floral morphology is an intriguing result of evolutionary forces. Most floral displays function to mediate interactions between plants and pollinators. Some plants produce generic floral shapes that attract generalist pollinators (Waser et al. 1996), while others evolve complex morphologies signifying obligate mutualistic coevolutionary relationships with a single pollinator (e.g. Bradshaw et al. 2010). Numerous studies have shown strong pollinator

preferences for particular floral traits (Muller 1883; Knuth 1906; Baker 1963; Grant & Grant 1965; Ollerton 1996; Waser 1998) including size, scent, color, orientation, and many others (Chittka & Menzel 1992; Spaethe et al. 2001; Bradshaw & Schemske 2003; Spaethe & Chittka 2003; Fenster et al. 2009; Klahre et al. 2011). Pollinator trait preference may contribute to the formation of pollination syndromes, where floral morphology predicts assemblages of pollinators (reviews in Fenster et al. 2004; Waser et al. 1996; Ollerton 1998). Floral adaptation to suites or individual pollinators is also expected to promote diversification and speciation through reproductive isolation (Bradshaw et al. 1998; Bradshaw & Schemske 2003; Hodges & Arnold 1994; Ippolito et al. 2004).

Concerning the evolution of floral traits in response to pollinators, most studies have been limited to traits that humans can naturally perceive. This limitation is a significant bias given that pollinators experience floral displays differently than humans. For instance, insect, avian, and even bat pollinators perceive light in the near ultraviolet (UV) spectrum (200–400 nm, Church et al. 1998; Muller et al. 2009), wavelengths that cannot be detected by the human eye. Most flowers either fully reflect or absorb UV light, but at least 7% create contrasting patterns of UV reflectance and absorbance upon the floral surface (Guldberg & Atsatt 1975). UV patterns usually do not replicate any other visual patterns on the floral surface, creating a unique signal. UV patterning within a single flower or inflorescence focuses pollinator attention on the reproductive parts, functioning as a nectar guide and contrasting with background foliage (Richtmyer 1923; Lutz 1924). For example, black-eyed susan (*Rudbekia hirta*, Compositae) absorb UV light throughout the center of the flower and down the proximal petal surface; this region contrasts highly with a reflecting band that extends to the distal petal tips (see Figure 1 of

Thompson et al. 1992). Bees exhibit an instinctive feeding response when they encounter a UV absorbing region of a flower. This instinct clearly demonstrates the effectiveness of UV patterning in attracting pollinators (Manning 1956). Studies have shown that hummingbirds also have the capacity to see UV light (Goldsmith 1980), but the importance of floral UV patterns in hummingbird pollination is unknown.

Although many pollinators see and respond to UV spectra, few studies have been devoted to describing UV patterning in flowers (Daumer 1956, 1958; Kugler 1963; Richtmyer 1923; Lutz 1924, 1933; Lotmar 1933; Guldberg & Atsatt 1975), and even fewer have investigated its ecological and evolutionary significance (Chittka & Menzel 1992; Chittka et al. 1994; Gronquist et al. 2001). Many previous studies incorporating full-spectrum analyses of floral color have relied on optical fiber spectrometers to produce standardized light reflectance spectra (see Arnold et al. 2010). However, standard spectral measurements encompass only a small region of the floral surface and are often averaged across measurements, masking the signal for UV patterning.

Once source of UV patterning is chemical compounds within the floral tissue. Chemical color, or pigment-based color, results from compounds absorbing various wavelengths of light. Unabsorbed wavelengths reflected back to the viewer create the perception of color. Pigment-based flower color is due to unique combinations of cholorphylls, carotenoids, xanthophylls, flavonoids, and anthoxanthins distributed among petal tissues (Weevers 1952; Thompson et al. 1972; Horovitz & Cohen 1972; Eisner et al. 1973). The chemical basis of UV patterning has been assigned to a group of secondary plant metabolites called flavonoids, and more recently, to

dearomatized isoprenylated phloroglucinols (DIPs) (Gronquist et al. 2001). Flavonoids occur in almost all plant tissues; one estimate posits that 2% of all carbon photosynthesized by plants is converted into flavonoids (Smith 1972). Floral flavonoids are widely distributed across taxa (Geissman 1963) and appear yellow in the visible spectrum. The first study to characterize the chemical basis of UV nectar guides in flowers found high concentrations of UV absorbing flavonoids in the petal bases of *Rudbekia* (Thompson et al. 1972). Other flavonoid glycosides found to absorb UV light in flowers are anthochlors (including chalcones and aurones) and flavones (Reiseberg & Shilling 1985). Scanning electron microscopy (SEM) localized UV absorbing flavonoid compounds to vacuoles of petal epidermal cells in *Viguie* (Reiseberg & Shilling 1985).

UV patterns can also be caused by floral structural components. Microstructures alter wavelength perception by selectively reflecting some, but not all wavelengths, from a substance. Unlike pigment-based colors, structural color can be perceived to shift wavelengths when viewed from different angles, termed iridescence. Some epidermal cell morphologies are known to influence light capture and reflection in flower petals (Kevan & Backhaus 1998). For example, diffraction grating caused by floral cuticular striations produce UV iridescence in *Tulipa* sp. (Liliaceae), *Hibiscus trionum* (Malvaceae), and *Mentzelia lindleyi* (Loasaceae—Whitney et al. 2009a, b). Similarly, bract hairs of *Leontopodium* (Compositae) have parallel striations that act as a photonic crystal, absorbing UV light (Vigneron et al. 2005). Most angiosperm flowers contain papillate or conical cells, rising upward from the plane of tissue (Figure 4–1); their geometry contrasts greatly from the smooth, flat cells found in other plant tissues and may also influence UV patterning. Conical cells have been described in 75–80% of flowers examined thus

far (Kay et al. 1981; Christensen & Hansen 1998) and are usually found on floral surfaces that interact with pollinators. Conical cells function in a variety of capacities to facilitate pollination: they help pollinators grip the flower (Kevan & Lane 1985; Whitney et al. 2009a, b), keep petals dry (Whitney et al. 2011), may affect petal temperature (Whitney et al. 2011), influence petal reflexing (van Houwlingen et al. 1998) and help focus or scatter wavelengths of light (Kay et al. 1981; Gorton & Vogelmann 1996). Structural components and pigments may act in concert to create UV patterning (Reiseberg & Shilling 1985; Noda et al. 1994; Glover & Whitney 2010). In *Viguiera* (Asteraceae), UV patterning may be influenced by the observed cuticular striations on the conical cells, which are also filled with UV absorbing flavonoids (Figures 2, 4 in Reiseberg & Shilling 1985).

UV patterns in plants are important for both display and defense. The intended recipients of UV displays are pollinators. The defense is to protect the flower from high levels of UV radiation and prevent herbivory (Koti et al. 2004, Gronquist et al. 2001). The UV absorbing properties of flavonoids have been experimentally shown to protect floral tissues (Middleton & Teramura 1993; Tevini et al. 1991; Day et al. 1993) and DNA from induction of UV radiation damage (Stapleton & Walbot 1994). UV absorbing DIPs found in high concentration in plant ovary walls have also been shown to be toxic to herbivores (Gronquist et al. 2001).

Mating system transitions from insect pollination to bird pollination or self-fertilization have occurred thousands of times in the history of the angiosperms, and correlated evolution of numerous traits accompanies these transitions (review in Karron et al. 2012). These transitions raise several questions about UV patterning. First, does UV patterning, as a pollinator-specific

trait, evolve along with mating system? Second, is UV patterning lost when plants are no longer reliant on pollinators? I address these questions by assaying inter- and intra-specific variation in UV patterns among related species with divergent mating systems. Finally, what causes UV patterning? Are regions of UV absorbance/reflectance due solely to differential chemical concentrations in petal tissue or does the shape of epidermal cells interact with incoming wavelengths of light to create patterns? I address these questions through spectral analyses of floral photopigments and structural component analysis using SEM. Using *Mimulus* (Phrymaceae) as a model system, I investigate the relationship between UV patterning and mating system. Since members of the genus range from outcrossing to exclusively selfing lineages, I am able to study the evolutionary consequences of variable pollinator dependency on UV patterning.

Mimulus is an excellent system for studying plant-pollinator relationships due to immense diversity in floral coloration, morphology, and life history (Grant 1924). *Mimulus* is a model system in ecology and evolution biology and has a rapidly expanding presence in functional genomics (Wu et al. 2008). The genus has numerous attributes that invite ecological study including tremendous phenotypic variation, high genetic diversity, occupation of numerous habitats, and mating systems ranging from completely outcrossing to obligately selfing to obligately asexual (Wu et al. 2008). *Mimulus* is easily propagated in the greenhouse; all species are self compatible, easily emasculated and hand pollinated, and most have only a 6–12 week generation time under experimental conditions (Wu et al. 2008). However, evolutionary trends in UV patterning and resulting relationships with pollinators in the genus are completely unknown.

I seek to document ultraviolet patterning in populations of the primarily outcrossing *Mimulus guttatus* and primarily selfing *Mimulus nasutus*.

METHODS

Mimulus study populations—I surveyed 12 different *Mimulus* populations for UV patterning and possible mechanisms underlying patterning. To determine if UV patterns would be lost under immediate pollinator-limiting conditions, I surveyed five populations (F5M, A1, A2, B1, and B2) of *M. guttatus* resulting from a previous experimental evolutionary study (see Bodbyl Roels & Kelly 2011). Replicate populations A1 and A2 were the result of nine consecutive generations of pollinator exclusion (four generations extended beyond Bodbyl Roels & Kelly 2011), creating highly inbred individuals and effectively selecting for traits that enhance self-fertilization. Replicate populations B1 and B2 received visits from pollinating bumblebee *Bombus impatiens* (Apidae) for all nine generations, producing primarily outcrossed individuals. Population F5M represented the original source population of the experiment, derived from wild plants collected at Iron Mountain, Linn County, Oregon (Willis 1993a; Arathi and Kelly 2004). To look for intra-species variation, I also surveyed five other laboratory propagated *M. guttatus* lines. Two originated from Iron Mountain, OR (IM 62, IM 767, Willis 1993b; Willis 1999; Holeski 2007), another two from Point Reyes National Seashore, Marin County, California (PRE1 and PRE5, Holeski 2007), and finally one was a wild-collected population (VSS) from Oswald State Park, Tillamook County, OR (collector: Vanessa Koelling, 2005). To investigate differences in UV patterning between closely related species with different mating systems, I surveyed two wild-collected populations of *M. nasutus* (BCCER1 and BCCER2) from Big Chico

Creek Reserve, Butte County, CA (collector: Vanessa Koelling & Cory Wallace, 2005). *M. nasutus* is self-fertilizing species thought to be derived from *M. guttatus* (Fenster and Ritland 1994). In some analyses, the geographically similar populations of *M. guttatus* (PRE1 and PRE5) were pooled together and referred to as PRE. Similarly, the two *M. nasutus* (BCCER1 and BCCER2) populations were pooled and referred to as NSBC when results were indistinguishable from one another. Ten plants from each population were greenhouse reared in 2" x 2" pots under identical light conditions. All plants were reared in identical light conditions in the greenhouse because increased UV light exposure can increase production of flavonoids in floral tissue (Gorton & Vogelmann 1996).

Ultraviolet photography— Visible and UV-only digital photographs (VIS/UV) of *Mimulus* flowers were taken with a UV-capable camera, an Olympus E–510 digital SLR with M42 Novoflex Noflexar 3.5/35 mm Exa Exakta lens. UV photographs were taken with the addition of a 330WB80 18 mm Excite Florescence UV pass filter (specifications: center wavelength 330nm ± 5 nm, HBW bandwidth 60nm ± 6 nm, Size 18 mm ±0.25 mm, Transmission > 80%), which only transmits UV wavelengths of 290–390 nm to the camera sensor. UV light was supplemented by two Vivitar 285HV Zoom Thyristor flash units modified to directly expose the flash bulbs and positioned ~15 mm forward and to either side of the flower being photographed. All photographs were taken at ISO 400, WB Auto, F-stop 5,6 with only shutter speed varying between shots. Average shutter speeds were 1/60" for visible shots and 1/8" for UV. Addition of the UV pass filter created a large focus shift, which was problematic because the flowers were not visible through the viewfinder once the filter was in place. To compensate, flowers were positioned for visible shots at a lens focal length of 0.4 m, and positioned at 0 m (shortest

position) for UV shots. VIS/UV photographs were taken of the first flower produced by each plant on the day of anthesis. One flower from each population was also photographed in VIS/UV daily from anthesis to corolla shedding to assess if UV patterning changed over floral duration.

***Optical spectrometry*—**Full reflectance spectra were produced from first-day flowers by taking readings at three locations on each flower (Figure 4–2). Spectra were recorded at 1 nm intervals from 200–850 nm using a Red Tide UV-VIS spectrometer (Ocean Optics) with an optical fiber attachment. Measurements were taken at a 45° angle from the floral surface, illuminated with a full-spectrum Xenon bulb, and recorded with Logger Pro 3.8.4 (Vernier Software & Technologies). All acquired spectra were standardized by a Xenon transmission spectrum such that reflected values are represented as a percentage of available light. These spectra were uploaded to FReD, a public-access floral reflectance database (Arnold et al. 2010). For display, spectra were summarized by averaging values every 5nm and trimmed to a range of 300–700nm.

***Photopigment extracts*—**An additional flower from each plant was used to extract pigments for spectral analysis. Five mm diameter samples, from each of the three floral locations measured for optical spectrometry (Figure 4–2), were extracted in a 95% methanol/3% HCl solution for 30 minutes. Extracts were placed in optically clear cuvettes and absorbance spectra from 200–850 nm (1 nm increments) were taken using the aforementioned spectrometer, calibrated by a blank of the extraction solution. Values are displayed in the same manner as the optical spectra.

***SEM*—**One flower from each population was used for SEM evaluation of epidermal cell structure. Whole flowers were fixed with glutaraldehyde and then transferred through a graded

ethanol series to dehydrate tissue (Appendix). Fixed whole flowers were critical point dried for 20 minutes. One sample from four target regions on each flower (Figure 4–2) was mounted and sputter-coated with gold. The fourth region, not evaluated in the optical reflectance and pigment extract assays, was added to SEM analyses after UV photography clarified that the lower medial corolla does not always absorb UV wavelengths. In contrast, the upper medial of the lower corolla (fourth region) absorbed UV light in all assayed plants. All SEM images were taken with an SE2 scanner at a 45° angle, at 2.97 KX magnification, EHT = 10 kV, and a working distance of 15 mm.

RESULTS

Photographs— A vibrant UV pattern was observed in all surveyed populations. A large UV absorbing patch, originating within the corolla tube and extending down to the distal margin of the lower medial corolla lobe contrasted with UV reflectance throughout the rest of the flower, including highly reflective anthers and stigmatic surfaces (Figure 4–3A). This UV pattern only occurred on the forward facing petal surfaces, the rear surfaces of petals were uniformly UV reflecting. Variation in the UV pattern was observed among and within the representative ten individuals surveyed for each population. PRE and VSS individuals had incomplete UV absorption patches that did not extend all the way to the end of the lower medial lobe (Figure 4–3A). One A2 individual also had truncated UV absorbing patch that ended well before the distal margin of the medial lower corolla (Figure 4–3B). Two A1 individuals (A1–4, A1–6) completely lacked a UV pattern, both flowers entirely reflected UV, with the exception of the dark red nectar guides in the center of the lower corolla (Figure 4–3B). All flowers produced by

these two plants similarly lacked the typical UV pattern. One A1 plant also had a floral mutation that left streaks of non-pigmented, UV reflecting tissue on the corolla surface (Figure 4–3B). Individuals of the selfing species, *M. nasutus*, displayed the full UV pattern observed in *M. guttatus* populations F5M, A1, A2, B1, B2, IM62, and IM767. Flowers in all populations withered after four days of anthesis. Individual flowers photographed for four consecutive days did not show changes in UV pattern over the life of the flower (Figure 4–3C).

Optical spectrometry— Optical reflectance spectra were *not* indicative of differential UV absorbing vs. UV reflecting regions of the corolla. Spectra from each measured location on the corolla were nearly identical (Figure 4–4A) and little variation was observed either within or among populations (Figure 4–4A,B). The spectra showed floral tissue absorbing the majority of incoming UV light (200–400 nm). From 480 to 700 nm, tissue reflected most incoming visible light, with major spectral peaks located at 543, 611, 590, and 660 nm, in order of decreasing magnitude. The overall shape of the reflected spectra places *Mimulus* within the 'typical yellow' categorization of flowers, stimulating both red and green receptors in human vision, but stimulating only the green receptor in bees (Chittka et al. 1994). In a comparison among all populations, differences observed in the spectra were due solely to intensity, or magnitude of reflectance (Figure 4–4B). From 530 to 700 nm, the population with the highest reflectance values (IM62) reflected 10–19% more of the available light than the population with the lowest reflectance values (B1).

Pigment extraction— Absorption spectra of pigment extracts showed contrast between UV absorbing vs. UV reflecting regions of the flower (Figure 4–5A). Most populations (A1, A2, B1,

B2, IM767, NSBC, PRE and VSS) absorbed more UV light from 220–430 nm in the LC (lower central) region than the other two locations (LL-lower lateral, UL-upper lateral). Spectra from F5M and IM62 were less differentiated. UL pigments absorbed either equivalent amounts of incoming light (B1, B2, IM767, NSBC, PRE, VSS), slightly more (A1, A2, IM62), or slightly less (F5M) than LL pigments, though differences are likely not meaningful. At wavelengths larger than 500nm, extracted tissue pigments from all populations recorded little to no absorption. The basic shape of the absorption spectra also varied among populations. The most prominent feature had 4 maxima in most populations, located at 332, 339, 418, and 444 nm, with 418 nm usually the highest peak. The first minor peak on the left shoulder of the feature (332nm) was less well defined in IM767, PRE, and VSS. Most populations displayed a secondary feature peaking at 273 nm, but it was poorly defined in PRE and VSS. Instead, those populations displayed a different peak at 225 nm (Figure 4–5A). NSBC produced uniformly undefined spectra, which may be an artifact of producing tiny flowers that lacked sufficient tissue for the extractions.

The two A1 individuals that did not display a photographed UV pattern (A1–4 and A1–6) had reduced UV absorption compared to other A1 individuals (Figure 4–5B). Specifically, the absorption peaks of the LC tissue of A1–4 and A1–6 were 44–53% reduced in absorption magnitude relative to other flowers in that population. The absorption magnitude of A1–6 tissue UL was also unusually elevated compared to the other A1 individuals.

SEM— SEM images recorded conical cells on the petal surface of all flowers assayed except for LC and UC (upper central) of *M. nasutus* population BCCER1, which lacked any relief

altogether (Figure 4–6). Conical cell shape varied widely among populations, although my interpretation is tentative given samples were from only a single flower per population. Samples from the UC region tended to have larger, denser, and sharper collections of peaks; the LC region shared these characteristics to a lesser degree. The LL and UL regions contained conical cells that had less relief, were smaller, and more widely spaced than UC and LC. Cell patterns from each individual formed natural groups that reflected their evolutionary relatedness based on taxonomy and geographic proximity. One group contained individuals from F5M, A1, A2, B1, B2, IM62, and IM767, which were nearly indistinguishable from one another. IM767 was the most divergent in this group, with conical cells from LC shaped more like finger projections than like the triangular cones of the rest of the group. Individual A1–6 had conical cell shapes in each image region that were indistinguishable from F5M, which was notable because A1-6 did not display a UV pattern in UV photographs while F5M had a full pattern (Figure 4–3B). PRE5 and VSS form the next group; both contain large, wide-based peaks in LC and UC (Figure 4–6). Curiously, PRE1 had a novel conical cell shape not seen in any of the other populations. Conical cells of PRE1 were rounded and were densely packed together, entirely lacking peaks. The apparent deflation of cells in the UL PRE1 image was likely the result of damage that occurred during the fixation protocol. *M. nasustus* BCCER1 and BCCER2 form the third group, characterized by reduced conical cell morphology. When they occurred, NSBC conical cells were smaller and spaced wider apart than in *M. guttatus* populations. As noted earlier, BCCER1 completely lacked conical cells in LC and UC regions. No cuticular striations, which could indicate structural iridescence, were observed on any of the cell structures.

DISCUSSION

UV absorption patterns in flowers were first proposed as potential cues for pollinators over 80 years ago (Richtmyer 1923; Lutz 1924). Current documentation of UV patterning in flowers is often undertaken via optical spectral reflectance; a method which is shown here to be ineffective for differentiating UV absorbing and reflecting regions of a flower (but see Ollerton et al. 2003). For instance, Cooley et al. (2008) concluded that no UV patterning was present in bumblebee pollinated Chilean *Mimulus luteus* var *luteus, M. l. variegates, M. naiandinus,* or *M. cupreus,* because their optical spectrometry data did not produce any reflectance at UV wavelengths. My optical reflectance spectra also did not record reflectance at UV wavelengths, yet I was able to document striking reflectance/absorbance patterns with UV photography. My results suggest that the Cooley et al. conclusions and those of other studies relying solely on optical reflectance spectra be reevaluated with UV photography. Interpretation of optical spectral reflectance data can be challenging because reflectance spectra only provide data on the amount of available light reflected back to the sensor. Which wavelengths, if any, are being absorbed by the sample is difficult to determine from these data. Apparent absorbing regions can be due to true absorption by the sample, lack of available light at those wavelengths, or scattering of reflected light off the sample that is not recorded by the sensor. Similarly, spectral absorption data, as from extracted tissue pigments in this study, also can not be used to infer reflectance. For these reasons, optical reflectance spectra and spectrometry of extracted pigments are methods best reserved for summarizing the interaction between light and plant tissue over the entire UV/visual spectrum. When the goal is characterizing UV patterning, UV photography is the most direct and effective method.

Few studies have recorded variation in floral UV patterning among populations of the same species. One example is in *Chlorogalum* (Liliaceae); UV photography revealed slight variations in UV patterning for multiple populations of five different species (Jernstedt 1980). Even fewer studies have documented variation within a single population. One such study of *Brassica rapa* L. (Brassicaceae) found variable UV patterning within populations dependent on genotype, suggesting the observed variation was heritable (Yoshioka et al. 2005). Another study in progress is quantifying the extent of variability in UV patterning on the petal surface of multiple populations of *Argentina anserina* (Rosaceae, Matthew Koski, pers. comm.). My study simultaneously documented variation in UV patterning among and within populations of *M. guttatus*. I found two deviant UV patterns, one in which the UV absorbing region did not extend out to the tip of the lower central corolla and the other lacking UV absorbance entirely. Both types were found in the A1 and A2 populations of a previous experimental evolution study (Bodbyl Roels & Kelly 2011). Both of these populations had been repeatedly self-fertilized for 9 generations, while their counterparts, B1 and B2, had been pollinated by bumblebees, producing primarily outcrossed individuals. The selfing regime experienced by A1 and A2 probably contributed to the phenotypic expression of variation in UV pattern present in the populations; inbreeding increases homozygosity, exposing the effects of alleles masked at heterozygous sites. Further tests could be conducted to clarify the origin of UV pattern variation in the selfed populations by surveying outcrossed individuals of A1 and A2. No UV pattern variations were found in B1, B2, or the original source population F5M, suggesting that the dominant UV pattern in the original, wild source population (also ancestral to IM62 and IM767) may be a full UV absorbing patch extending to the petal tip. The other *M. guttatus* populations (PRE & VSS)

displayed the incomplete UV absorbing variant, with no additional variation detected within those populations. Further sampling of wild populations would clarify the extent of natural variation in UV patterning.

Curiously, the self-fertilizing species, *M. nasutus*, thought to be derived from a *M. guttatus*-like ancestor, contained the full UV absorbing patch. If UV patterning functions primarily for pollinator attraction, then it would be expected to be lacking in selfing lineages, whose traits typically maximize self-fertilization capabilities and discourage pollinator visits. The continuing presence of a UV pattern in *M. nasutus* suggests either relictual persistence or other fitness benefits selectively maintaining UV pattern expression despite mating system change. Further examination of UV patterning in *Mimulus* is warranted, especially in additional selfing species closely related to *M. guttatus*. Potential candidates include *M. laciniatus*, *M. pardalis*, *M. longulus*, *M. clementinus*, *M. micranthus*, *M. platycalyx*, and *M. cupriphilus* (Grant 1924; Vickery 1978; Macnair et al. 1989; Ritland & Ritland 1989; Sweigart & Willis 2003).

Tissue pigment extract spectrometry showed elevated levels of UV absorbing chemicals present in the lower central corolla of all surveyed *Mimulus* populations, matching the UV absorbing regions documented photographically (Figure 4–5). The elevated compounds were likely flavonoids and DIPs, which are the primary chemical contributors to UV patterning in flowers (Thompson et al. 1972; Reiseberg & Shilling 1985; Gronquist et al. 2001). UV absorbing compounds in plant tissue, particularly flavonoids, have been found in numerous studies to protect plant tissues and DNA from damage caused by UV radiation (Robberecht & Caldwell 1978, 1983; Tevini & Teramura 1989; Day et al. 1993; Stapleton & Walbot 1994; Ferreyra et al.

2010). UV absorbing regions, when present in flowers, radiate outward from the reproductive structures, which are sensitive to UV damage (Koti et al. 2004). The sunscreening effect of these UV absorbing compounds may be a key function of UV patterning in flowers, explaining UV pattern persistence in some selfing species that no longer receive pollinator services. A test of sunscreen hypothesis in *Mimulus* would be to expose UV-absorption lacking mutant flowers, found in populations A1 and A2, to UV radiation and document any resulting reproductive sensitivity. However, *M. nasutus* produces primarily cleistogamous flowers, which are not directly exposed to the sun, negating the need for sunscreening pigments. Another hypothesis for the maintenance of UV patterning in selfing species is protection from herbivory. If DIPs and flavonoids are toxic to herbivores then it may be advantageous for them to be concentrated in and around the reproductive parts of a flower. Research gives some credence to this hypothesis; DIPs can be lethal to *Utetheisa ornatrix* moths (Gronquist et al. 2001) and flavonoids also act as herbivore antifeedants (e.g. Echeverri et al. 1991; Mullin et al. 1991; Russell et al. 2000, but see Sisi & Mullin 1999). A test of the anti-herbivory hypothesis would be to extract the UV-absorbing pigments from both *M. guttatus* and *M. nasutus* flowers and investigate their toxicity on common *Mimulus* herbivores.

UV-absorbing flavonoids (and DIPs) of multiple classes are abundant throughout angiosperm taxa, and any number, type, or concentration of these compounds may cause UV patterns in flowers. DIPs have previously been found in high concentrations in anthers and ovarian walls (Gronquist et al. 2001) and thus are unlikely to have been observed in the spectra of *Mimulus* petal tissue extracts. Extracts contained peaks indicating the presence of mixed flavonoids. Flavonoids typically have two absorption maxima occurring at 300–550 nm (Band I) and

240–285 nm (Band II, Markham 1982); Band I peaks in *Mimulus* ranged from 332–444 nm and the Band II peak was located at 273 nm. Some variation in the spectra was observed among *M. guttatus* populations, suggesting that PRE and VSS may contain different identities or concentrations of UV-absorbing chemicals. Determining the identity and concentrations of UV-absorbing chemicals was outside the scope of this study, but further investigation using high performance liquid chromatography (HPLC) on the extracts of both petal and reproductive tissues could quantify the various flavonoids and DIPs segregating among populations (see Markham 1982; Gronquist et al. 2001). NSBC produced rather undefined spectra without clear peaks. Further investigation of *M. nasutus* floral pigment extracts could be performed by pooling tissue samples from multiple flowers on a plant to increase sample volume.

Floral epidermal cell geometry interacts with incoming wavelengths of light in numerous ways. Some plants have specialized leaf epidermal cells that focus incoming light onto the mesophyll, maximizing photosynthetic capacity in low-light situations (Haberlandt 1914; Martin et al. 1989; Poulson & Vogelmann 1990; Myers et al. 1994; Broderson & Vogelmann 2007). Previous work (Brehm & Krell 1975; Noda et al. 1994) suggested that epidermal cell shapes may influence UV patterning in flowers, but few studies have directly investigated the relationship. Gorton & Vogelmann (1996) were the first to quantify light focusing capabilities of petal epidermal shapes in *Antirrhinum* (Plantaginaceae). Using the *mixta* mutant of *Antirrhinum majus* (Noda et al. 1994), they found that cell shape (conical vs. flat) did affect the focal length of penetrating UV wavelengths; however, the presence of flavonoids in the epidermal cell vacuoles absorbed almost all UV light, negating the effect of cell shape (Gorton & Vogelmann 1996). I compared SEM images from four floral regions to determine if epidermal cell shape correlated with regions of

UV-absorption. Regions that absorbed UV light in the photographic and spectral analyses (always UC, sometimes LC) did appear to contain different epidermal cell shapes, containing more numerous and pointed conical cells, than UV reflecting regions (LL, UL). However, the aberrant individual A1–6 that uniformly reflected UV across the floral surface (Figure 4–3B) had conical cells that appeared identical in size, shape, and density to the other Iron Mountain-derived populations displaying the full UV pattern. Additionally, a full UV pattern was found in *M. nasutus*, despite a lack of conical cells in the SEM images of both UV absorbing regions in BCCER1 (Figure 4–6). Cell shape has been found to influence visible light characteristics (Kay et al. 1981); for example, mutant *Antirrhinum* flowers lacking conical cells appeared paler and less textured than those with normal conical cells (Gorton & Vogelmann 1996). My studies in *Mimulus* support Gorton and Vogelmann's conclusions that conical cells do not significantly influence UV patterning in flowers.

Floral conical cells have been implicated to serve multiple functions independent of light (see Introduction). Perhaps the most compelling hypothesis is that they function as pollinator traction enhancers, or 'bee grippers' (Whitney et al. 2009c). The tactile properties of conical cells act as cues for pollinators and increase foraging efficiency; bees actively prefer conical cells and discriminate against flat cells on flowers (Glover & Martin 1998; Comba et al. 2000; Whitney et al. 2009c; Whitney et al. 2011; Rands et al. 2011; Alcorn et al. 2012). The patterns observed in my SEM images of *Mimulus* are consistent with this hypothesis. The most prominent conical cells were located in the LC and UC regions of the flower, which make up the pollinator 'landing pad' of the corolla. The LL and UL regions, located on lobes that do not usually make physical contact with pollinators, had smaller and fewer conical cells. The *M. nasutus* populations had the

smallest and least dense conical cells of all surveyed, completely lacking conical cells in the central region of the corolla. Selfing species are predicted to lose pollinator attractive traits no longer actively maintained by selection, thus the missing conical cells in *M. nasutus* may indicate trait loss as a result of mating system evolution.

CONCLUSION

Bold ultraviolet patterning of flowers in multiple populations of *M. guttatus* and *M. nasutus* was documented using four independent methods: UV photography, optical reflectance spectrometry, pigment extract spectrometry, and SEM imagery. Optical reflectance spectrometry was ineffective for differentiating variable UV reflectance and absorbance regions within the flower. UV patterns varied both within and among surveyed populations. Naturally self-fertilizing species maintained UV patterns but inbreeding of a naturally outcrossing population resulted in occasional UV pattern aberrants, suggesting selection upon UV patterning is not entirely linked to that of pollinator attraction. Chemical components within floral tissue, likely flavonoids and DIPs, were found to be the primary mechanism creating UV patterns. Variation observed in petal epidermal cell shape did not appear to be related to the formation of UV patterns, but supported previous work on tactile pollinator signaling.

LITERATURE CITED

Alcorn, K., Whitney, H. and B. Glover. 2012. Flower movement increases pollinator preference for flowers with better grip. Functional Ecology. Early view published May 28, 2012. doi: 10.1111/j.1365-2435.2012.02009.x

Arathi, H. S., and J. K. Kelly. 2004. Corolla morphology facilitates both autogamy and bumblebee pollination in *Mimulus guttatus*. International Journal of Plant Science 165: 139–145.

Arnold, S. E., Faruq, S., Savolainen, V., McOwan, P. W., and L. Chittka. 2010. FreD: The Floral Reflectance Database – a web portal for analyses of flower colour. PLoS One 5: e14287.

Baker, H. G. 1963. Evolutionary mechanisms in pollination biology. Science 139: 877–883.

Bodbyl Roels, S. A. and J. K. Kelly. 2011. Rapid evolution caused by pollinator loss in *Mimulus guttatus*. Evolution 65: 2541–2552.

Bradshaw, H. D., Otto, K. G., Frewen, B. E., McKay, J. K., and D. W. Schemske. 1998. Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). Genetics 149: 367–382.

Bradshaw, H. D., and D. W. Schemske. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. Nature 426: 176–178.

Bradshaw, E., Rudall, P., Devey, D.,Thomas, M., Glover, B. J., and R. M. Bateman. 2010. Comparative labellum micromorphology of the sexually deceptive temperate orchid genus *Ophrys:* diverse epidermal cell types and multiple origins of structural colour. Botanical Journal of the Linnean Society 162: 504–540.

Brehm, B. G. and D. Krell. 1975. Flavonoid localization in epidermal papillae of flower petals: a specialized adaptation for ultraviolet absorption. Science 190: 1221–1223.

Brodersen, C. R. and T. C. Vogelmann. 2007. Do epidermal lens cells facilitate the absorptance of diffuse light? American Journal of Botany 94: 1061–1066.

Chittka L, and R. Menzel. 1992. The evolutionary adaptation of flower colours and the insect pollinators' colour vision. Journal of Comparative Physiology A 171: 171–181. doi:10.1007/BF00188925

Chittka, L., Shmida, A., Troje, N. and R. Menzel. 1994. Ultraviolet as a component of flower reflections, and the colour perception of Hymenoptera. Vision Research 34: 1489–1508.

Christensen, K., and H. Hansen. 1998. SEM-studies of epidermal patterns of petals in the Angiosperms. Opera Botanica 135: 1–91.

Church, S. C., Bennett, A. T. D., Cuthill, I. C. and J. C. Partridge. 1998. Ultraviolet cues affect the foraging behavior of blue tits. Proceedings of the Royal Society B: Biological Sciences 265: 1509–1514.

Comba, L., Corbet, S. A., Hunt, H., Outram, S., Parker, J. S., and B. J. Glover. 2000. The role of genes influencing the corolla in pollination of *Antirrhinum majus*. Plant, Cell, and Environment 23: 639–647.

Cooley, A. M., Carvallo, G., and J. H. Willis. 2008. Is floral diversification associated with pollinator divergence? Flower shape, flower colour and pollinator preference in Chilean *Mimulus*. Annals of Botany 101: 641–650. doi:10.1093/aob/mcn014

Daumer, K. 1956. Reizmetrische untersuchung des farbensehens der bienen. Zeitschrift für vergleichende Physiologie 38: 413–478.

Daumer, K. 1958. Blumenfarben: wie side die bienen sehen. Zeitschrift für vergleichende Physiologie 41: 49–110.

Day, T. A., Martin, G. and T. C. Vogelmann. 1993. Penetration of UV-B radiation in foliage: evidence that the epidermis behaves as a non-uniform filter. Plant, Cell, and Environment 16: 735–741.

Echeverri, F., Cardona, G., Torres, F., Pelaez, C., Quinones, W. and E. Renteria. 1991. Ermanin: An insect deterrent flavonoid from *Passiflora foetida* resin. Phytochemistry 30: 153–155.

Eisner, T., Silberglied, R. F., Aneshansley, D., Carrel, D., and H. C. Howland. 1969. Ultraviolet video-viewing: the television camera as an insect eye. Science 155: 1172–1174.

Fenster, C. B. and K. Ritland. 1994. Evidence for natural selection on mating system in *Mimulus* (Scrophulariaceae). International Journal of Plant Science 155: 588–596.

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R. and J. D. Thomson. 2004. Pollination syndromes and floral specialization. Annual Review of Ecology, Evolution, and Systematics 35: 375–403. doi:10.1146/annurev.ecolsys.34.011802.132347

Fenster, C. B., Armbruster, W. S., and M. R. Dudash. 2009. Specialization of flowers: is floral orientation an overlooked first step? New Phytologist 183: 502–506. doi:10.1111/j.1469-8137.2009.02852.x

Ferreyra, M. L., Rius, S., Emiliani, J., Pourcel, L., Feller, A., Morohashi, K., Casati, P. and E. Grotewold. 2010. Cloning and characterization of a UV-B-inducible maize flavonol synthase. Plant Journal 62: 77–91.

Geissman, T. A. 1963. Pp. 213–250. *In* Comparative Biochemistry M. Florkin and E. H. Stotz, eds. Elseviers, New York, New York. Vol. 9.

Glover, B. J. and C. Martin. 1998. The role of petal cell shape and pigmentation in pollination success in *Antirrhinum majus*. Heredity 80: 778–784.

Glover, B. J. and H. M. Whitney. 2010. Structural colour and iridescence in plants: the poorly studied relations of pigment colour. Annals of Botany 105: 505–511. doi:10.1093/aob/mcq007

Goldsmith, T. H. 1980. Hummingbirds see near ultraviolet light. Science 207: 786–788.

Gorton, H. L., and T. C. Vogelmann. 1996. Effects of epidermal cell shape and pigmentation on optical properties of *Antirrhinum* petals at visible and ultraviolet wavelengths. Plant Physiology 112: 879–888.

Grant, V. and K. A. Grant. 1965. Flower pollination in the phlox family. New York: Columbia University Press. 180 pages.

Grant, A. L. 1924. A monograph of the genus *Mimulus*. Annals of the Missouri Botanical Garden 11: 99–398.

Gronquist, M., Bezzerides, A., Attygalle, A., Meinwald, J., Eisner, M. and T. Eisner. 2001. Attractive and defensive functions of the ultraviolet pigments of a flower (*Hypericum calycinum*). Proceedings of the National Academy of Science 98: 13745–13750.

Guldberg, D. and P. R. Atsatt. 1975. Frequency of reflection and absorption of ultraviolet light in flowering plants. American Midland Naturalist 93: 35–43.

Haberlandt, G. 1914. Physiological Plant Anatomy. Macmillan, London. 777 pages.

Hodges, S. A. and M. L. Arnold. 1994. Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. Proceedings of the National Academy of Sciences 91: 2493–2496.

Holeski, L. M. 2007. Within and between generation phenotypic plasticity in trichome density of *Mimulus guttatus*. Journal of Evolutionary Biology 20: 2092–2100.

Horovitz, A. and Y. Cohen. 1972. Ultraviolet reflectance characteristics in flowers of crucifers. American Journal of Botany 59: 706–713.

Ippolito, A., Fernandes, G. W. and T. P. Holtsford. 2004. Pollinator preferences for *Nicotiana alata, N. forgetiana*, and their F1 hybrids. Evolution 58: 2634–2644.

Jernstedt, J. A. 1980. Ultraviolet absorption by flowers of *Cholorogalum* (Liliaceae). Bulletin of the Torrey Botanical Club 107: 163–171.

Karron, J. D., Ivey, C. T., Mitchell, R. J., Whitehead, M. R., Peakall, R. and A. L. Case. 2012. New perspectives on the evolution of plant mating systems. Annals of Botany 109: 493–504. doi: 10.1093/aob/mcr319

Kay, Q. O. N., Daoud, H. S., and C. H. Stirton. 1981. Pigment distribution, light reflection and cell structure in petals. Botanical Journal of the Linnean Society 83: 57–84.

Kevan, P. G., and M. A. Lane. 1985. Flower petal microtexture is a tactile cue for bees. Proceedings of the National Academy of Sciences 82: 4750–4752.

Kevan, P. G., and W. G. K. Backhaus. 1998. Colour vision: ecology and evolution in making the best of the photic environment. Pp. 163–183. *In* W. G. K. Backhaus, R. Kliegl, and J. S. Werner, *eds*. Colour vision – perspectives from different disciplines. Berlin: De Gruyter.

Klahre, U., Gurba, A., Hermann, K., Saxenhofer, M., Bossolini, E., Guerin, P. M., and C. Kuhlemeier. 2011. Pollinator choice in *Petunia* depends on two major genetic loci for floral scent production. Current Biology 21: 730–739.

Knuth, P. 1906. Handbook of flower pollination. Oxford: Clarendon Press. 382 pages.

Koti, S., Reddy, K. R., Kakani, V. G., Zhao, D. and V. R. Reddy. 2004. Soybean (*Glycine max*) pollen germination characteristics, flower and pollen morphology in response to enhanced ultraviolet-B radiation. Annals of Botany 94: 855–864. doi:10.1093/aob/mch212

Kugler, H. 1963. UV-musterung auf bluten und ihr Zustandekommen. Planta 59: 296–329.

Lotmar, R. 1933. Neue untersuchungen iber den farbensinn der bienen, mit besonderer berucksichtigung des ultravioletts. Zeitschrift für vergleichende Physiologie 19: 673–723.

Lutz, F. E. 1924. Apparently non-selective characters and combinations of characters including a study of ultraviolet in relation to the flower-visiting insects. Annals of the New York Academy of Science 29: 181–283.

Lutz, F. E. 1933. "Invisible" colors of flowers and butterflies. Journal of the American Museum of Natural History 33: 565–576.

Macnair, M. R., Macnair, V. E. and B. E. Martin. 1989. Adaptive speciation in *Mimulus*: an ecological comparison of *Mimulus cupriphilus* with its presumed progenitor *Mimulus guttatus*. New Phytologist 112: 269–279.

Manning, A. 1956. The effect of honey-guides. Behaviour 9: 114–139.

Markham, K. R. 1982. Techniques of Flavonoid Identification. Academic Press, New York. 113 pages.

Martin, G., Josserand, S. A., Bornman, J. F., and T. C. Vogelmann. 1989. Epidermal focussing and light microenvironment within leaves of *Medicago sativa*. Plant Physiology 76: 485–492.

Middleton, E. M. and A. H. Teramura. 1993. The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage. Plant Physiology 103: 741–752.

Muller, N. H. 1883. The fertilization of flowers. London: Macmillan. 669 pages.

Muller, B., Glosmann, M., Peichl, L., Knop, G., Hagemann, C., and J. Ammermuller. 2009. Bat eyes have ultraviolet-sensitive cone photoreceptors. PLoS One 4: e6390.

Mullin, C. A., Alfatafta, A. A., Harman, J. L., Everett, S. L. and A. Serino. 1991. Feeding and toxic effects of floral sesquiterpene lactones, diterpenes, and phenolics from sunflower (*Helianthus annuus* L.) on western corn rootworm. Journal of Agricultural and Food Chemistry 39: 2293–2299.

Myers, D. A., Vogelmann, T. C., and J. F. Bornman. 1994. Epidermal focussing and effects on light utilization in *Oxalis acetosella*. Plant Physiology 91: 651–656.

Noda, K., Glover, B. J., Linstead, P. and C. Martin. 1994. Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor. Nature 369: 661–664. doi: 10.1038/369661a0

Ollerton, J. 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant–pollinator systems. Journal of Ecology 84: 767–769.

Ollerton, J. 1998. Sunbird surprise for syndromes. Nature 394: 726–727.

Ollerton, J., Johnson, S. D., Cranmer, L., and S. Kellie. 2003. The pollination ecology of an assemblage of grassland Asclepiads in South Africa. Annals of Botany 92: 807–834. doi:10.1093/aob/mcg206

Poulson, M. E., and T. C. Vogelmann. 1990. Epidermal focussing and effects upon photosynthetic light-harvesting in leaves of *Oxalis*. Plant, Cell, and Environment 13: 803–811.

Rands, S. A., Glover, B. J. and H. M. Whitney. 2011. Floral epidermal structure and flower orientation: getting to grips with awkward flowers. Arthropod-Plant Interactions 5: 279–285.

Richtmyer, F. K. 1923. The reflection of ultraviolet by flowers. Journal of the Optical Society of America 7: 151–168.

Rieseberg, L. H. and E. E. Schilling. 1985. Floral flavonoids and ultraviolet patterns in *Viguiera* (Compositae). American Journal of Botany 72: 999–1004.

Ritland, C., and K. Ritland. 1989. Variation of sex allocation among 8 taxa of the *Mimulus guttatus* species complex (Scrophulariaceae). American Journal of Botany 76: 1731–1739.

Robberecht, R., and M. M. Caldwell. 1978. Leaf epidermal transmittance of ultraviolet radiation and its implications for plant sensitivity to ultraviolet-radiation induced injury. Oecologia 32: 277–287.

Robberecht, R., and M. M. Caldwell. 1983. Protective mechanisms and acclimation to solar ultraviolet-B radiation in *Oeniothera stricta*. Plant, Cell, and Environment 6: 477–485.

Russell, G. R., Bowers, W. S., Keesing, V., Niemeyer, H. M., Sevenet, T., Vasanthaverni, S. and S. D. Wratten. 2000. Patterns of bioactivity and herbivory on *Nothofagus* species from Chile and New Zealand. Journal of Chemical Ecology 26: 41–56.

Sisi, L. and C. A. Mullin. 1999. Lipid, polyamide, and flavonol phagostimulants for adult western corn rootworm from sunflower (*Helianthus annuus* L.) pollen. Journal of Agricultural and Food Chemistry 47: 1223–1229. doi: 10.1021/jf980858w

Smith, H. 1972. The photocontrol of flavonoid biosynthesis. Pp. 433–437. *In* Phytochrome. K. Mitrakos and W. Shropshire, eds. Academic Press, London.

Spaethe, J., and L. Chittka. 2003. Inter-individual variation of eye optics and single object resolution in bumblebees. Journal of Experimental Biology 206: 3447–3453.

Spaethe, J., Tautz, J., and L. Chittka. 2001. Visual constraints in foraging bumblebees: flower size and colour affect search time and flight behaviour. Proceedings of the National Academy of Science 98: 3898–3903.

Stapleton, A. E. and V. Walbot. 1994. Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. Plant Physiology 105: 881–889.

Sweigart, A. L., and J. H. Willis. 2003. Patterns of nucleotide diversity are affected by mating system and asymmetric introgression in two species of *Mimulus*. Evolution 57: 2490–2506.

Tevini, M., and A. H. Teramura. 1989. UV-B effects on terrestrial plants. Photochemistry and Photobiology 50: 479–487.

Tevini, M., Braun, J. and G. Fieser. 1991. The protective function of the epidermal layer of rye seedlings against ultraviolet-B radiation. Photochemisty and Photobiology 53: 329–333.

Thompson, W. R., Meinwald, J., Aneshansley, D., and T. Eisner. 1972. Flavonols: pigments responsible for ultraviolet absorption in nectar guide of flower. Science 177: 528–530.

van Houwelingen, A., Souer, E., Spelt, K., Kloos, D., Mol, J., and R. Koes. 1998. Analysis of flower pigmentation mutants generated by random transposon mutagenesis in *Petunia hybrida*. Plant Journal 13: 39–50.

Vickery, R. K. 1978. Case studies in the evolution of species complexes in *Mimulus*. Journal of Evolutionary Biology 11: 405–507.

Vigneron, J. P., Rassart, M., Ve ´rtesy, Z., Kertész, K., Sarrazin, M., Biró, L. P., Ertz, D. and V.

Lousse. 2005. Optical structure and function of the white filamentary hair covering the edelweiss bracts. Physics Review E 71: 011906.

Waser, N. M. 1998. Pollination, angiosperm speciation, and the nature of species boundaries. Oikos 81: 198–201.

Waser, N. M., Chittka, L., Price, M. V., Williams, N. M. and J. Ollerton. 1996. Generalization in pollination systems, and why it matters. Ecology 77: 1043–1060.

Weevers, T. 1952. Flower colours and their frequency. Acta botanica Neerlandica 1: 81–92.

Whitney, H. M., Kolle, M., Andrew, P., Chittka, L., Steiner, U., and B. J. Glover. 2009a. Floral iridescence, produced by diffractive optics, acts as a cue for animal pollinators. Science 323: 130–133.

Whitney, H. M., Kolle, M., Alvarez-Fernandez, R., Steiner, U., and B. J. Glover. 2009b. Contributions of iridescence to floral patterning. Communicative and Integrative Biology 2: 230–232.

Whitney, H. M., Chittka, L., Bruce, T. J. A., and B. J. Glover. 2009c. Conical epidermal cells allow bees to grip flowers and increase foraging efficiency. Current Biology 19: 948–953. doi: 10.1016/j.cub.2009.04.051

Whitney, H. M., Bennett, K. M. V., Dorling, M., Sandbach, L., Prince, D., Chittka, L., and B. J. Glover. 2011. Why do so many petals have conical epidermal cells? Annals of Botany 108: 609–616.

Willis, J. H. 1993a. Partial self fertilization and inbreeding depression in two populations of *Mimulus guttatus*. Heredity 71: 145–154.

Willis, J. H. 1993b. Effects of different levels of inbreeding on fitness components in *Mimulus guttatus*. Evolution 47: 864–876.

Willis, J. H. 1999. The role of genes of large effect on inbreeding depression in *Mimulus guttatus*. Evolution 53: 1678–1691.

Wu, C. A., Lowry, D. B., Cooley, A. M., Wright, K. M., Lee, Y-W., and J. H. Willis. 2008. *Mimulus* is an emerging model system for the integration of ecological and genomic studies. Heredity 100: 220–230.

Yoshioka, Y., Horisaki, A., Kobayashi, K., Syafaruddin, S., Niikura, S., Ninomiya, S., and R. Ohsawa. 2005. Intraspecific variation in the ultraviolet colour proportion of flowers in *Brassica rapa* L. Plant Breeding 124: 551–556.

Figure 4–1. SEM image of conical cells from the lower central (LC) UV absorbing region of the corolla of *Mimulus guttatus*, population F5M.

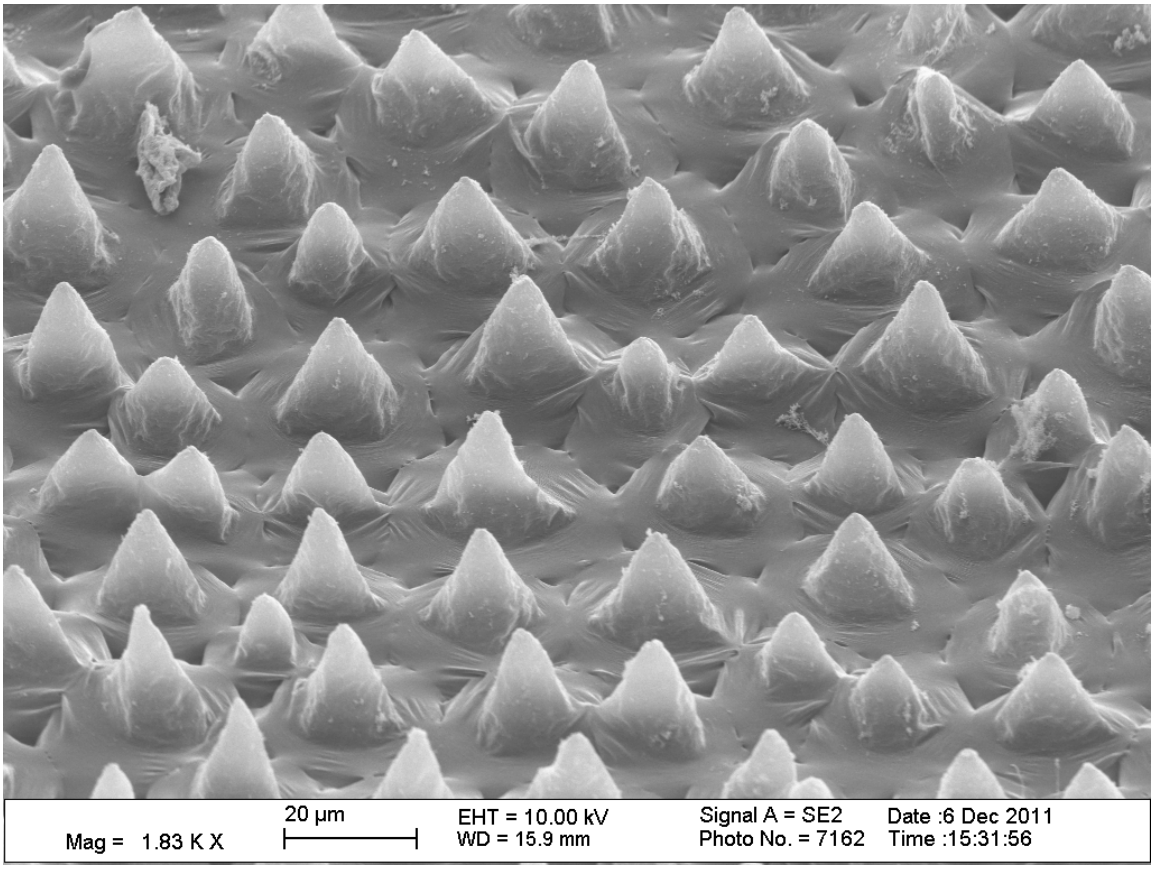


Figure 4–2. Front view of a *Mimulus guttatus* flower. Circles denote sampling sites for optical spectrometry, pigment extraction, and SEM. Sites are labeled as: LC = lower central, UC = upper central, LL = lower lateral, UL = upper lateral. UC was only used for SEM.

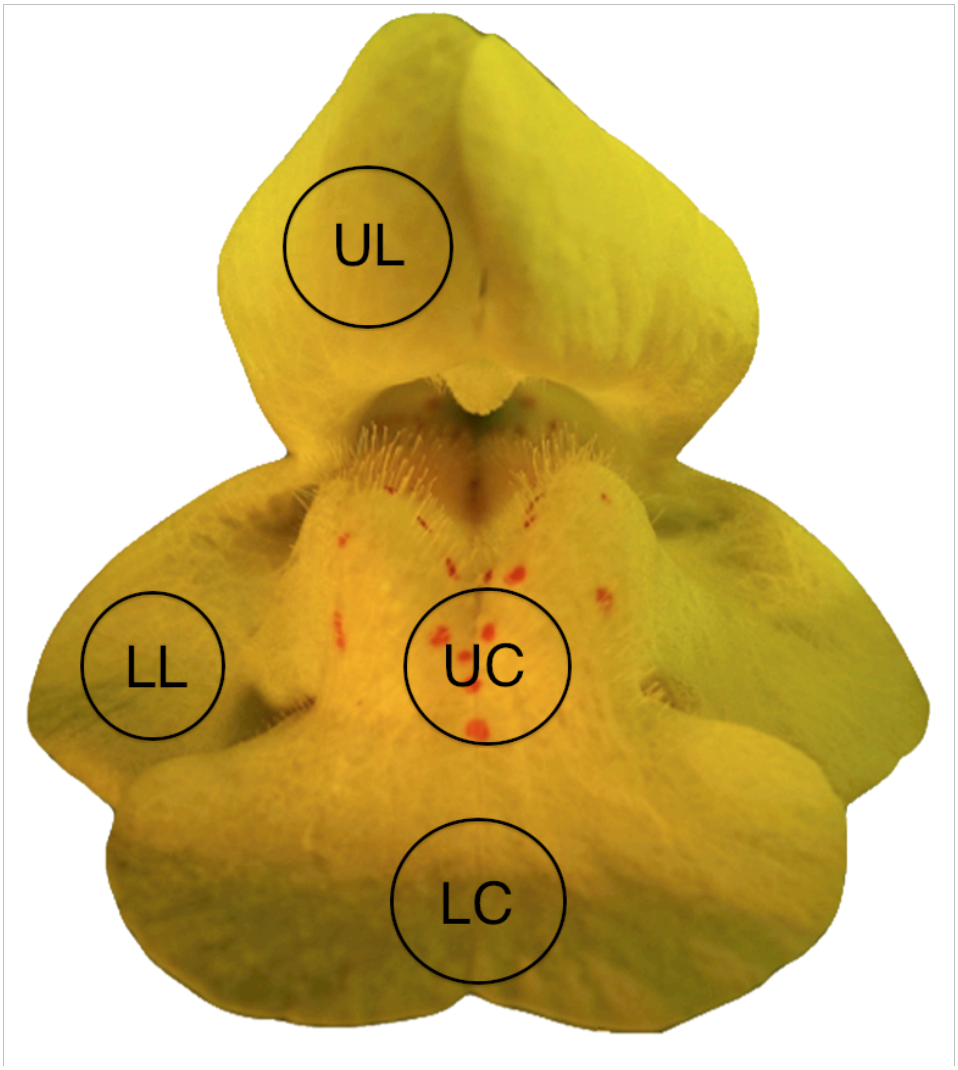
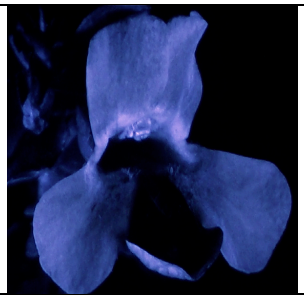
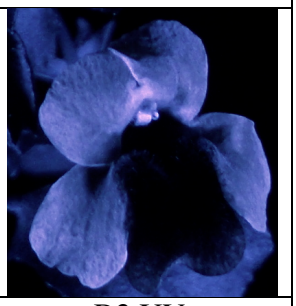
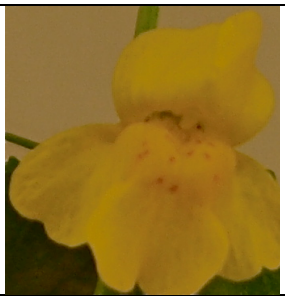
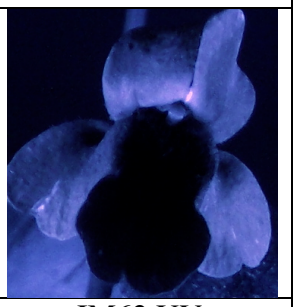
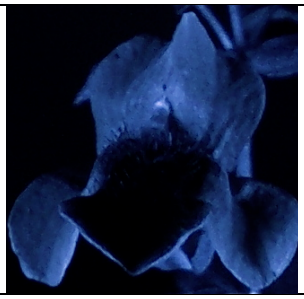
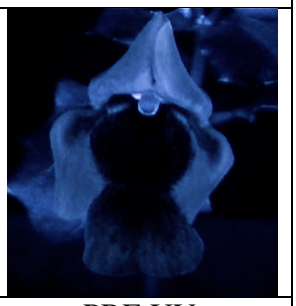
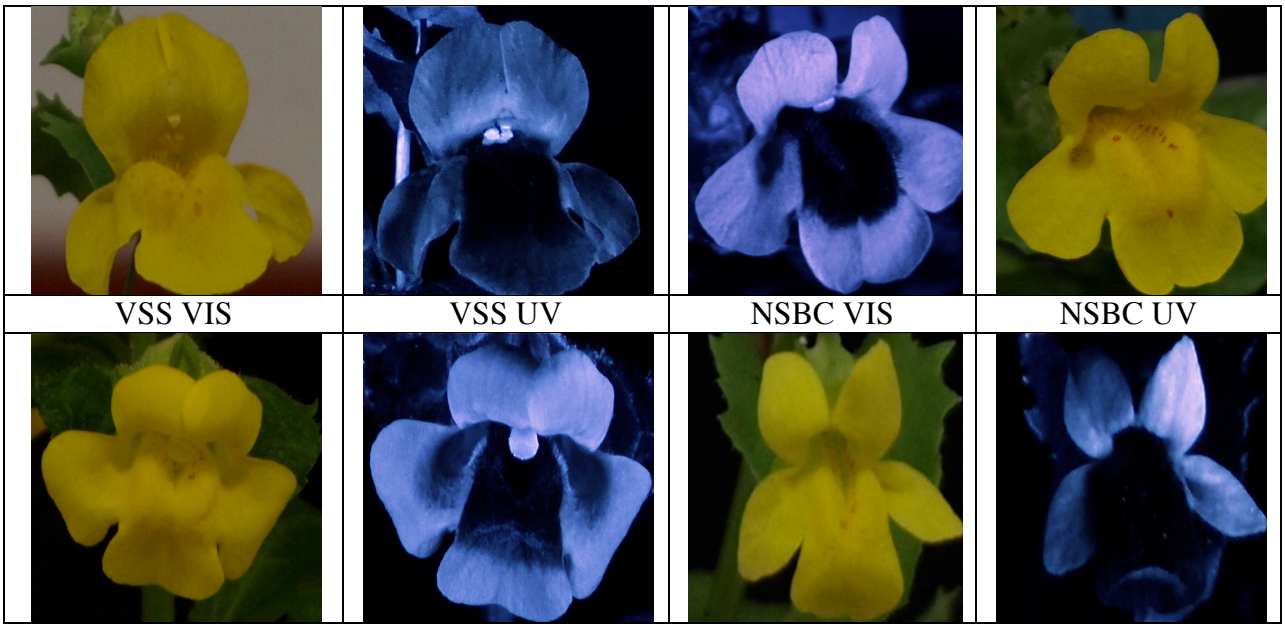


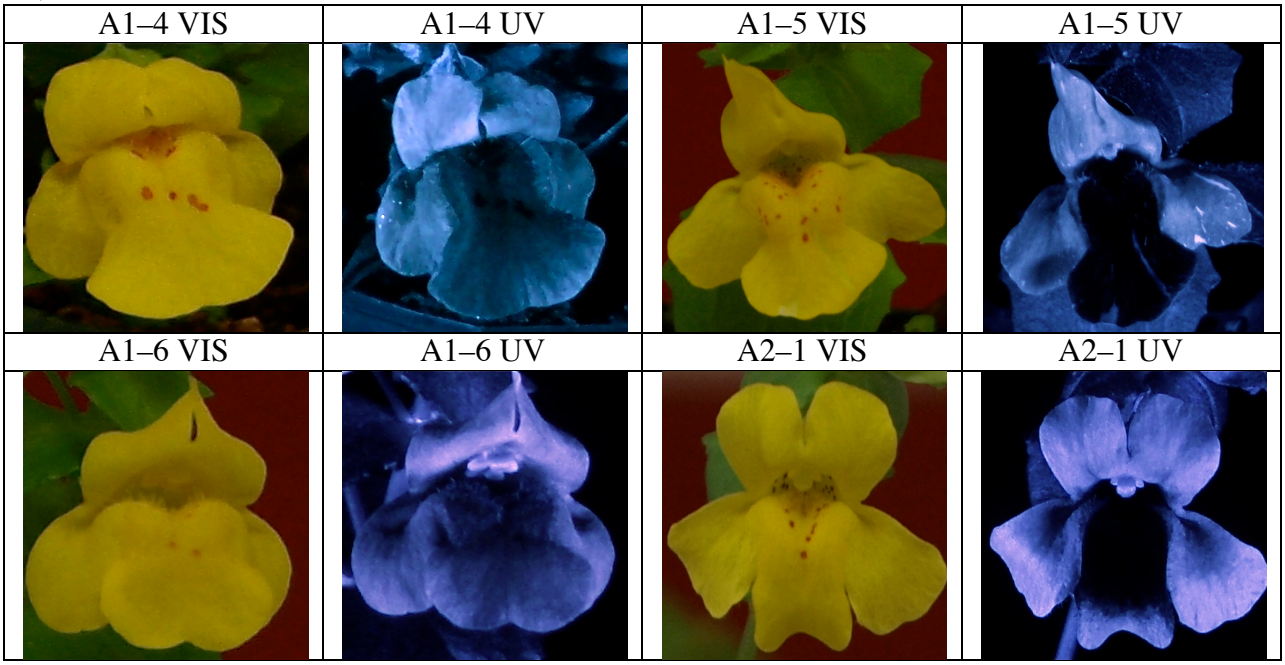
Figure 4–3. Visible (VIS) and ultraviolet (UV) photography characterizing floral patterns in *Mimulus*. Panel A: A single representative was chosen to display the typical floral pattern observed in each population. Panel B: Unusual observations. Two A1 individuals (A2–4 & A2–6) did not absorb UV light in the middle lower corolla, one A1 individual (A1–5) had a petal color mutation, and one A2 individual (A2–1) displayed a truncated UV pattern more similar to the PRE and VSS populations than to other A2 individuals. Panel C: UV patterning did not change of the duration of the floral lifespan. Individual PRE1–1 photographed over a four day period. All UV photos are displayed in false color.

A)

A1 VIS	A1 UV	A2 VIS	A2 UV
			
B1 VIS	B1 UV	B2 VIS	B2 UV
			
F5M VIS	F5M UV	IM62 VIS	IM62 UV
			
IM767 VIS	IM767 UV	PRE VIS	PRE UV


VSS VIS
VSS UV
NSBC VIS
NSBC UV

B)


A1–4 VIS
A1–4 UV
A1–5 VIS
A1–5 UV
A1–6 VIS
A1–6 UV
A2–1 VIS
A2–1 UV

C)

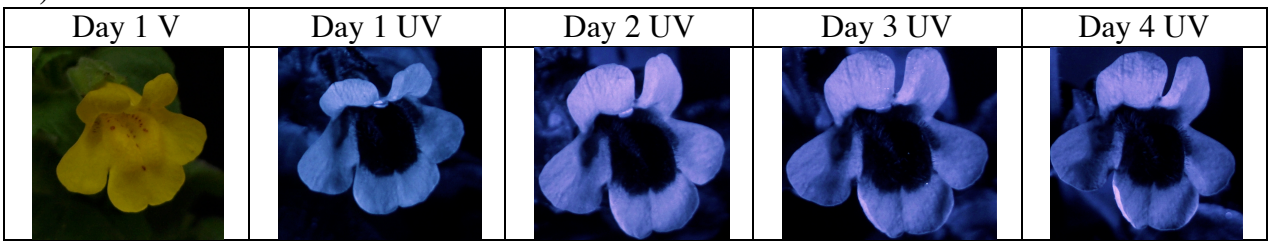

Day 1 V
Day 1 UV
Day 2 UV
Day 3 UV
Day 4 UV

Figure 4–4. Optical reflectance spectra. A) *M. guttatus*, population F5M, at three locations on each flower. LC refers to lower central (UV absorbing), LL is lower lateral (UV reflecting), and UL is upper lateral (UV reflecting—see Figure 4–3). B) Averaged spectra for all populations. Wavelength (nm) is displayed on the x-axis, reflectance (proportion of available light) on the y-axis.

A)

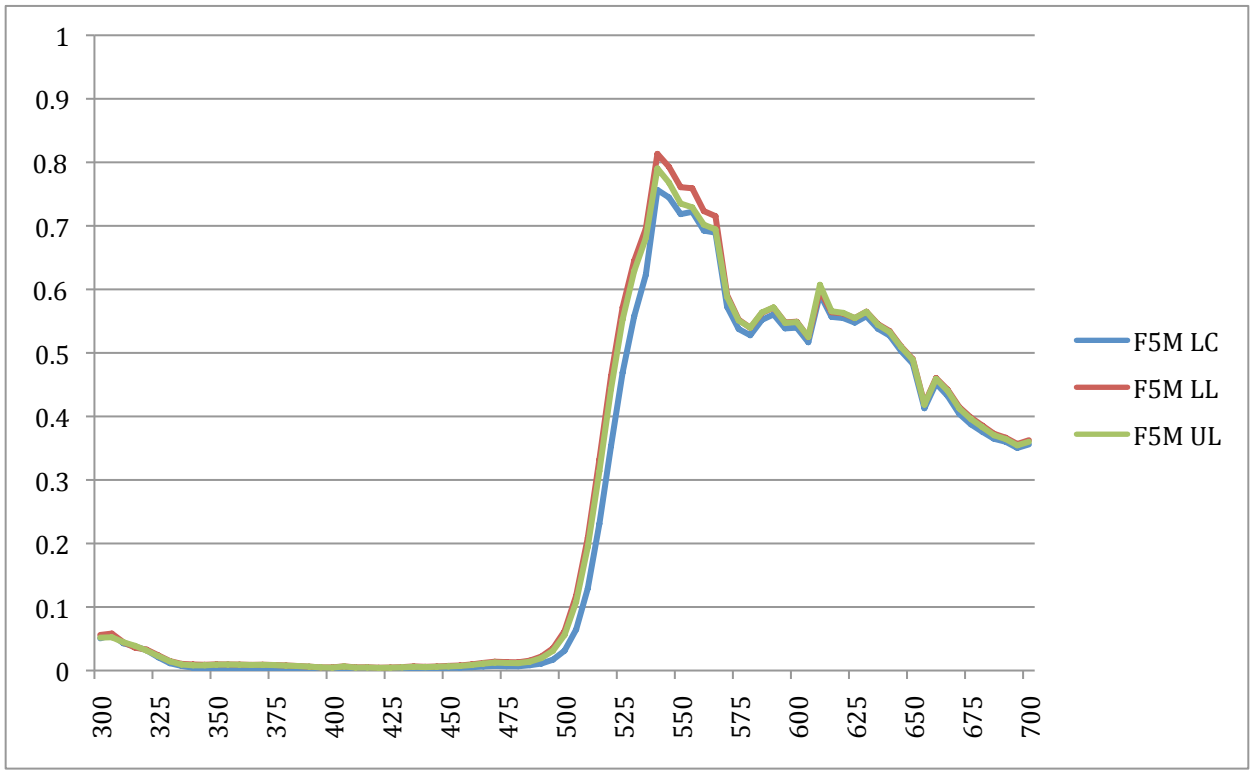


Figure 4–4. (Continued)
B)

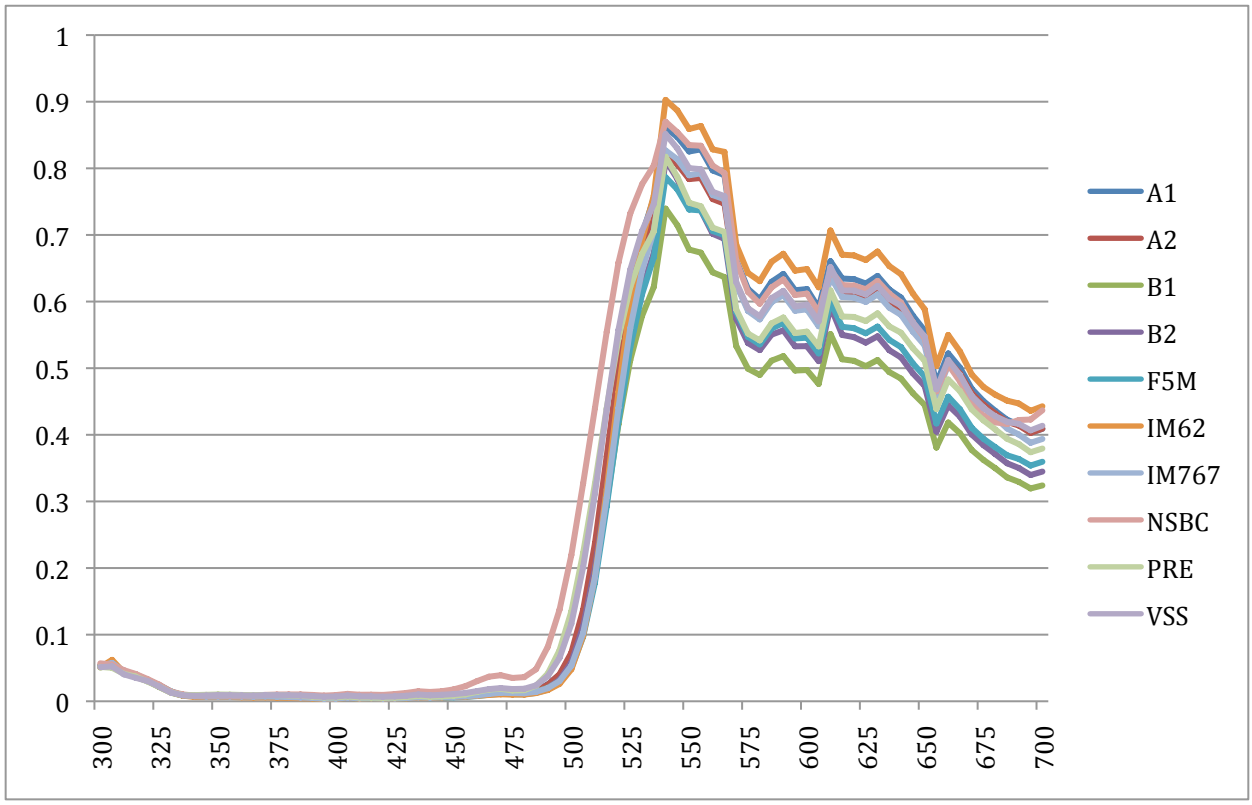
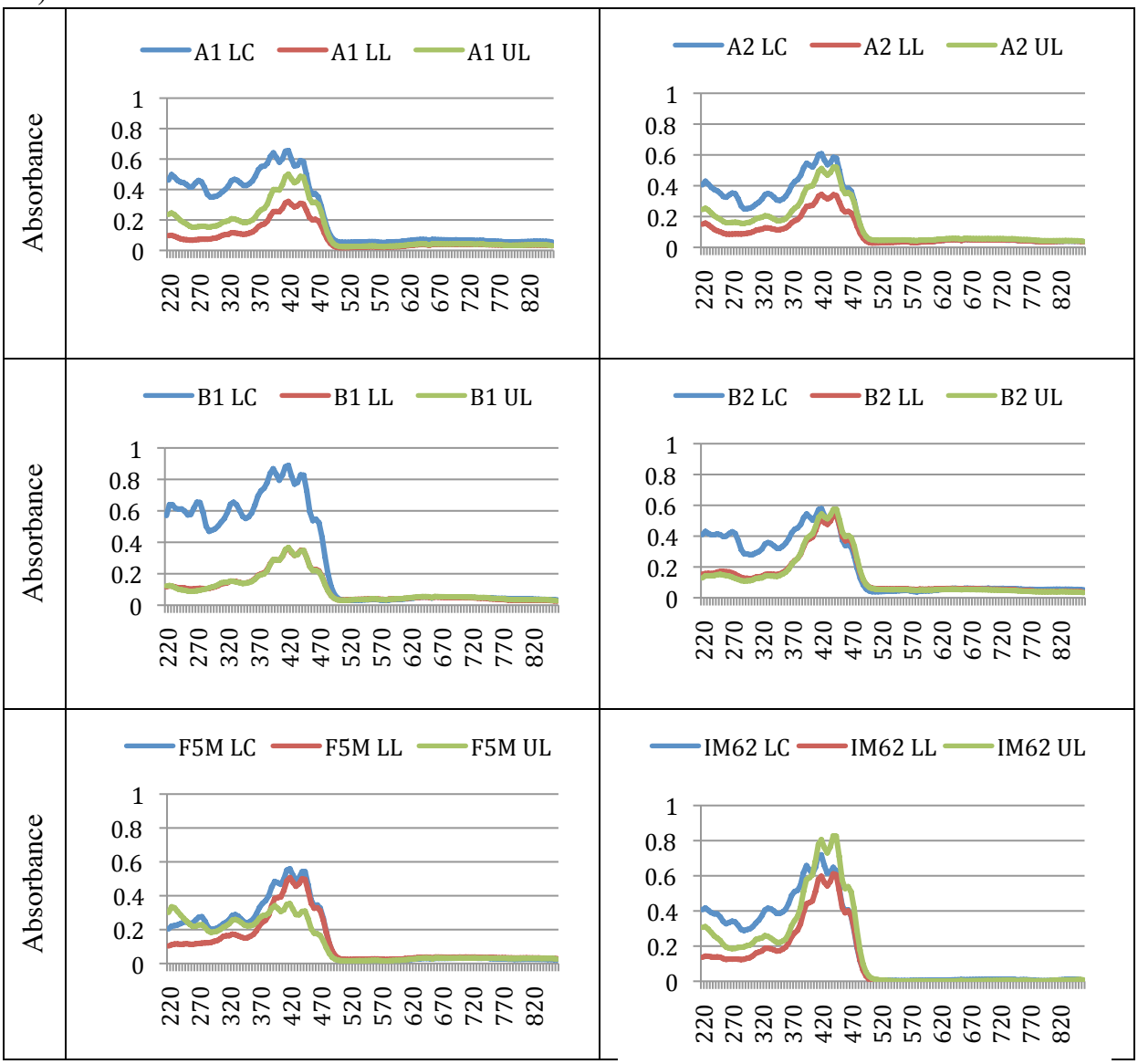
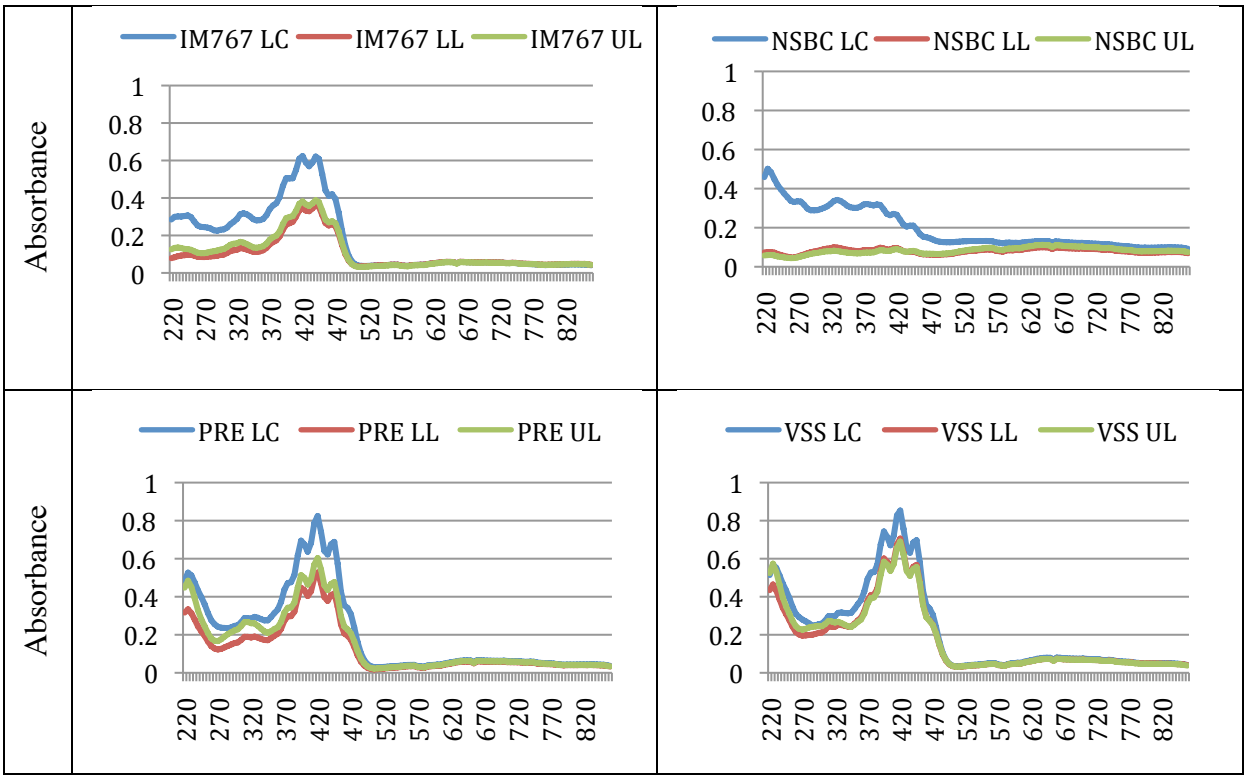


Figure 4–5. Absorbance spectra for extracted pigments from petal tissue. Panel A: Population averages at each of three extracted tissue locations (Figure 4–3). Panel B: Comparison among flowers within population A1. Individual A1–1 was documented by photography to have a normal UV pattern, while A1–4 and A1–6 flowers only reflected UV light. Wavelength (nm) is displayed on the x-axis, absorbance on the y-axis.

A)




Absorbance
IM767 LC
IM767 LL
IM767 UL
NSBC LC
NSBC LL
NSBC UL
PRE LC
PRE LL
PRE UL
VSS LC
VSS LL
VSS UL
0
0.2
0.4
0.6
0.8
1
220
270
320
370
420
470
520
570
620
670
720
770
820

B)

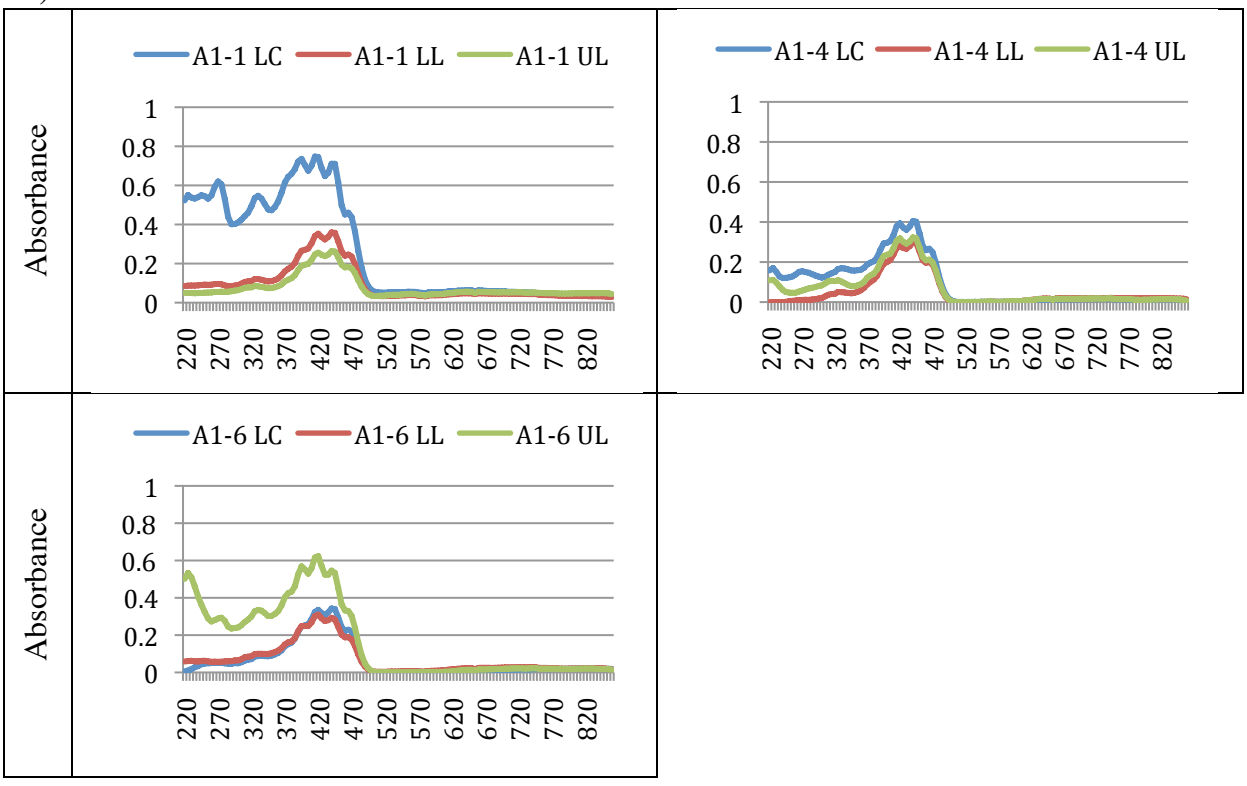
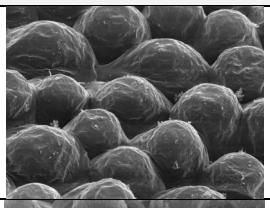
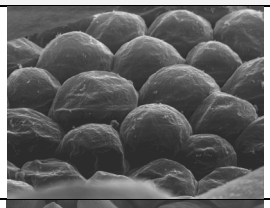
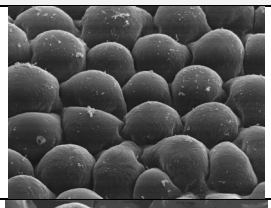
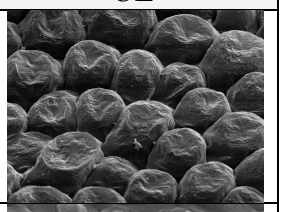
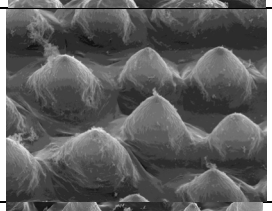
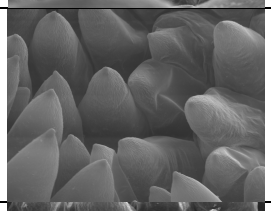
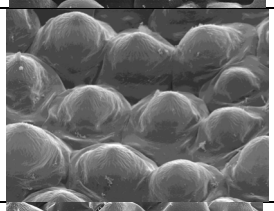
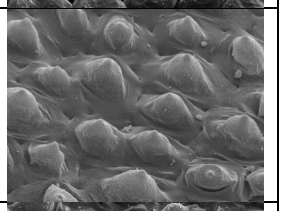
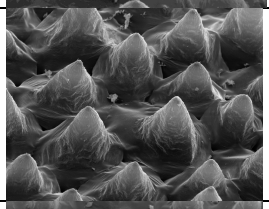
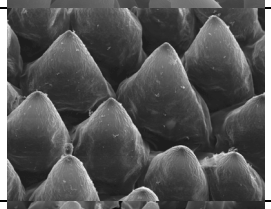
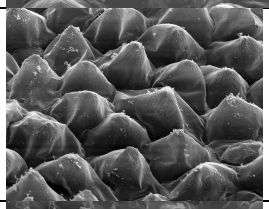
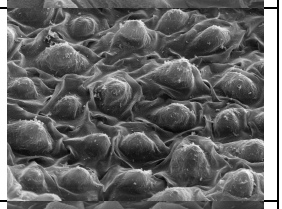
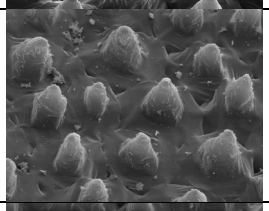
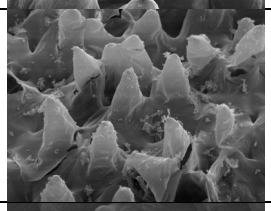
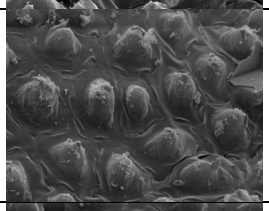
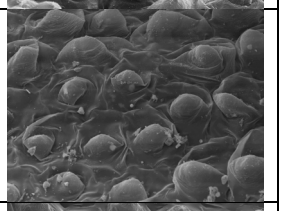
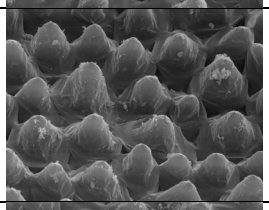
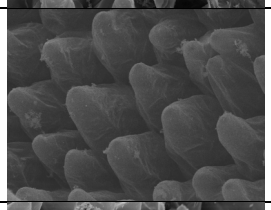
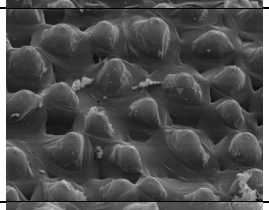
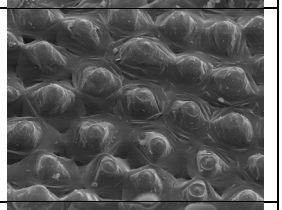
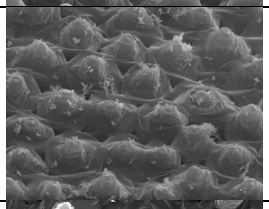
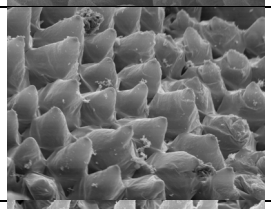
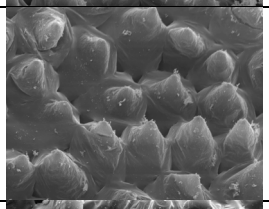
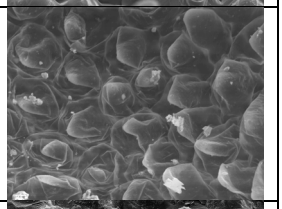
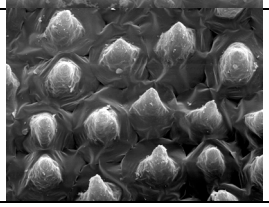
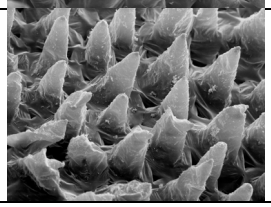
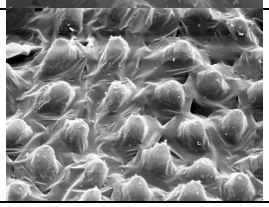
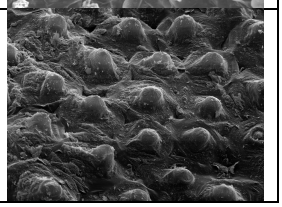
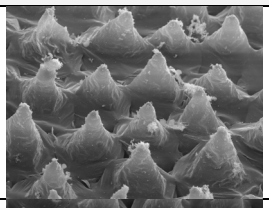
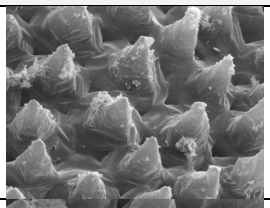
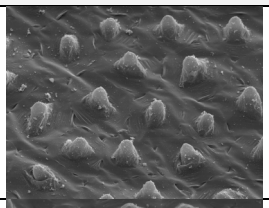
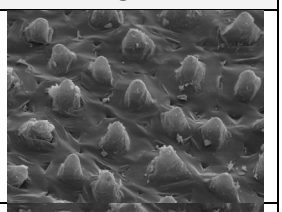
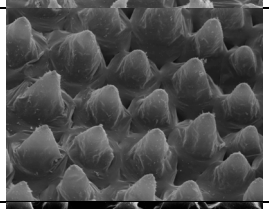
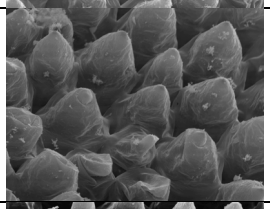
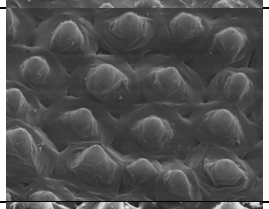
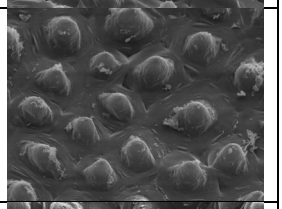
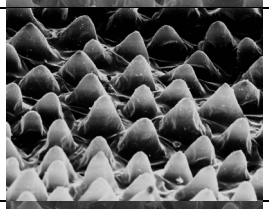
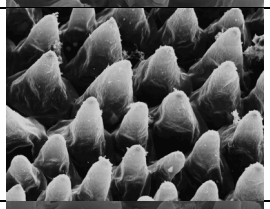
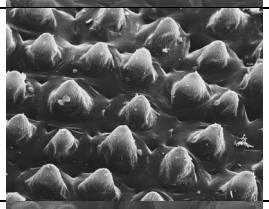
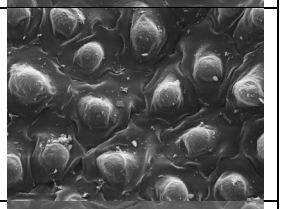
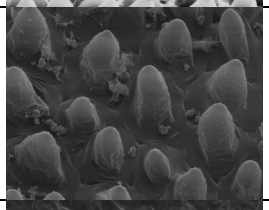
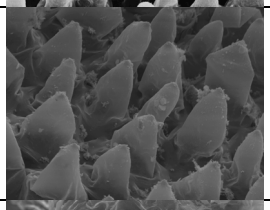
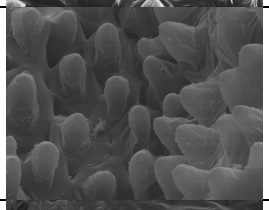
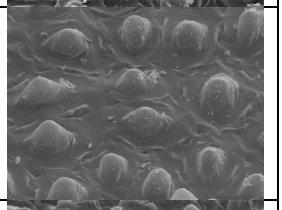
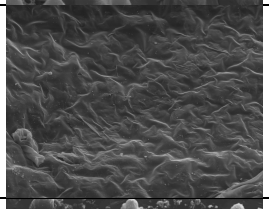
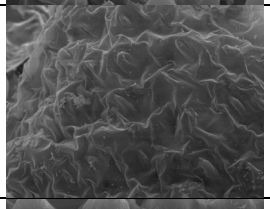
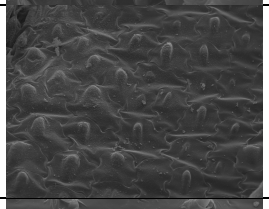
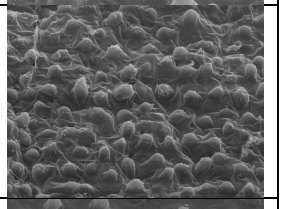
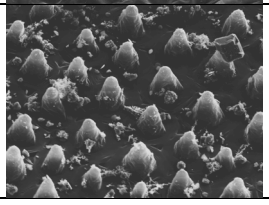
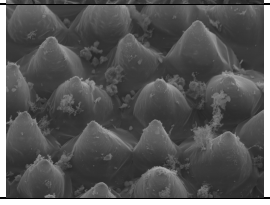
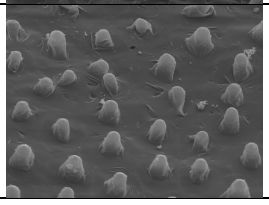
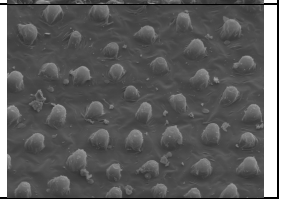

Absorbance
A1-1 LC
A1-1 LL
A1-1 UL
A1-4 LC
A1-4 LL
A1-4 UL
A1-6 LC
A1-6 LL
A1-6 UL
0
0.2
0.4
0.6
0.8
1
220
270
320
370
420
470
520
570
620
670
720
770
820

Figure 4–6. SEM images of *M. guttatus* and *M. nasutus* corolla tissue at four different locations (Figure 4–3). All images taken at a 45° angle, magnification 2.97 KX, EHT = 10 kV, working distance of 15 mm with an SE2 scanner. Width of each image is 105 μm. *denotes individual with no recorded UV pattern.

Population	LC	UC	LL	UL
PRE1				
PRE5				
VSS				
A1				
A1–6*				
A2				
B1				

Population	LC	UC	LL	UL
B2				
F5M				
IM 62				
IM767				
NSBC BCCER1				
NSBC BCCER2				

CONCLUSION

The overarching goal of my dissertation was to examine the mating system transition from outcrossing to selfing in flowering plants. More specifically, I asked which traits facilitate outcrossing via mutualisms with pollinators, and how those traits change in response to disruption of pollinator relationships. The ability of plant populations to rapidly adapt to environmental disturbances, including pollinator loss, is critical to their continued survival. Using the self-compatible wildflower, *Mimulus guttatus,* as a model, I investigated trait evolution shaped by either pollinator presence or absence.

In the first two chapters, I used experimental evolution to document the immediate effects of pollinator loss within predominately outcrossing *Mimulus guttatus* populations. I found evidence that pollinator loss does initiate rapid adaptation for selfing; I documented substantial trait evolution, corresponding with allele frequency change at multiple loci, in experimental populations after only five generations. Furthermore, observed patterns of phenotypic and genetic change favor a sequential model for the evolution of self-fertilization. Traits immediately essential to continued survival and reproduction evolved first (e.g. herkogamy and self-seed production) and were then followed by other traits associated with the selfing syndrome (e.g. flower shape). The genetic basis of mating system divergence appears to parallel this pattern. Genes with large effects (either deleterious or advantageous) experienced initial rapid purging or positive selection, respectively, while genes with smaller effects, likely corresponding to polygenic traits, accumulated allele frequency changes at a slower rate.

My third chapter investigated the relationship between specific *Mimulus* floral traits and the perceptive abilities of a bumblebee (*Bombus)* pollinator. I used bee behavioral trials to identify specific *Mimulus* floral traits attractive to bumblebees and determine how foraging patterns change as differences in preferred plant traits become more pronounced. Bees demonstrated clear preference for large flower size and discriminated against pale-colored flowers. Constancy, a measure of pollinator fidelity, was weak, but increased as divergent phenotypes became more distinct. The magnitude of pollinator preference demonstrated by *Bombus* indicates that pollinators are likely a strong selective force maintaining outcrossing *Mimulus* floral morphology. However, weak constancy among recently diverged population variants suggests *Bombus* is unlikely to initiate population divergence and ethological speciation in *Mimulus*.

The fourth chapter examined a cryptic pollination trait, floral ultraviolet (UV) patterning, which is often implicated as a nectar guide for attracting foraging pollinators. I found a striking UV pattern consisting of a lower corolla UV absorbing region surrounded by UV reflectance in twelve *Mimulus* populations. I observed UV pattern variation, in extent of petal coverage, within and among populations. UV patterns did not correspond with mating system; presence of a strong pattern in selfing *M. nasutus* suggests that UV patterning functions beyond pollinator attraction. The patterns appeared to be caused by variable concentrations of UV absorbing chemicals within the floral tissue and were not affected by the shape of corolla epidermal cells.

My research demonstrates that pollinator mutualisms play a key role in the formation and maintenance of mating systems in *Mimulus*. Bumblebees demonstrated clear preference for specific floral traits, suggesting that pollinators are a strong selective force for attractive traits in

naturally outcrossing populations. In contrast, the loss of pollinators created a strong selective force for self-fertilization traits. Plant traits that enhance selfing are often opposite of those attractive to pollinators, but the presence of UV patterning in selfing *M. nasutus* shows that attractive traits may be multi-faceted, functioning beyond solely pollinator attraction. Increasing anthropogenic environmental perturbations are predicted to generate novel selection pressures on plant populations, including continued disruption of plant-pollinator relationships. My results indicate that rapid adaptation and evolutionary rescue is possible for self-compatible populations experiencing sudden pollinator loss. However, the transition to a selfing mating system is not without cost as decreasing genetic diversity resulting from inbreeding may leave populations vulnerable to extinction if further environmental perturbations occur.

APPENDIX

Glutaraldehyde fixation protocol for SEM

Materials:

Glutaraldehyde (Electron Microscopy Science)

Sodium Phosphate Monobasic

Sodium Phosphate Dibasic

Solution recipes:

1M phosphate buffer:

Add 68.4 mL of 1M Na_2HPO_4 + 31.6 mL of NaH_2PO_4 to 900 mL dH_2O.

pH should be 7.2

Add 10 mL of 2% glutaraldehyde + 240 mL phosphate buffer = 250 mL total.

Fix whole flowers for 2+ hours in solution or overnight at 4°C, in 50 mL Falcon tubes.

Ethanol series:

50% EtOH for 1 hr. 30 min. at -20°C

70% EtOH for 1 hr. 30 min. at -20°C

85% EtOH for 1 hr. at 4°C

95% EtOH for 1 hr. at 4°C

95% EtOH overnight at 4°C

100% EtOH overnight at 4°C

100% EtOH for 1–2 hrs. at room temp.