

**UNCOVERING THE CONSEQUENCES OF CO-FLOWERING AND POLLINATOR
SHARING: EFFECTS OF LOCAL COMMUNITY CONTEXT ON POLLEN
TRANSFER DYNAMICS, FEMALE REPRODUCTIVE SUCCESS AND FLORAL
EVOLUTION IN *MIMULUS GUTTATUS***

by

Gerardo Arceo-Gómez

B.S., University of Yucatan, 2005

M.S., Institute of Ecology, 2008

Submitted to the Graduate Faculty of the
Kenneth P. Dietrich School of Arts and Sciences in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy, Biological Sciences

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH
KENNETH P. DIETRICH SCHOOL OF ARTS AND SCIENCES

This dissertation was presented

by

Gerardo Arceo-Gómez

It was defended on

February 26, 2014

and approved by

Dr. Susan Kalisz, Dept. of Biological Sciences, University of Pittsburgh

Dr. Randall J. Mitchell, Dept. of Biology, University of Akron

Dr. Brian Traw, Dept. of Biological Sciences, University of Pittsburgh

Dr. Stephen Tonsor, Dept. of Biological Sciences, University of Pittsburgh

Dissertation Advisor: Dr. Tia-Lynn Ashman, Dept. of Biological Sciences, University of Pittsburgh

Copyright © by Gerardo Arceo-Gómez

2014

**UNCOVERING THE CONSEQUENCES OF CO-FLOWERING AND POLLINATOR
SHARING: EFFECTS OF LOCAL COMMUNITY CONTEXT ON POLLEN
TRANSFER DYNAMICS, FEMALE REPRODUCTIVE SUCCESS AND FLORAL
EVOLUTION IN *MIMULUS GUTTATUS***

Gerardo Arceo-Gómez, PhD

University of Pittsburgh, 2014

While plant-pollinator interactions commonly take place within a larger community context, studies of plants and their pollinators have typically focused on pair-wise interactions. Co-flowering species within multi-species communities may influence pollinator foraging decisions and hence plant reproductive success and floral evolution. For instance, plants growing in highly diverse areas may be more pollen limited than plants in species-poor areas due to the high levels of pollinator competition and interspecific pollen transfer. As a result, stronger selection pressures can also be expected in highly diverse areas in order to increase the quantity and/or quality of pollen reaching conspecific stigmas. However, how plant community composition contributes to the severity of pollen limitation, what the potential underlying mechanisms are and how selection on floral traits changes with increasing community diversity is still unclear. In this study I use *Mimulus guttatus* as a model system to evaluate the effect of local co-flowering community context on quantity and quality aspects of pollen limitation, pollen transfer dynamics, heterospecific pollen effects and selection processes in high and low diversity areas. I show that the relative contribution of pollen quantity and quality limitation to overall pollen limitation of reproductive success depends on the co-flowering community context in which *M. guttatus* exists. I further uncover heterospecific pollen receipt as a potential mechanism underlying decreased reproductive success in highly diverse areas by showing that complex

interactions among multiple heterospecific pollen donors can exacerbate its effects and that heterospecific pollen receipt can have an even greater detrimental effect on self compared to outcross conspecific pollen. Finally, I show that co-flowering community context can be an important driver of selection that promotes floral trait differentiation among populations, in the case of *M. guttatus* in flower longevity. By combining observational, experimental, field and greenhouse approaches, this study extends our knowledge of the processes underlying insufficient pollination in natural communities, reveals new complexities in our understanding of heterospecific pollen effects and advances our understanding of the community properties shaping the evolutionary dynamics of constituent populations.

TABLE OF CONTENTS

| | |
|--|----|
| INTRODUCTION..... | 1 |
| 1.0 PATTERNS OF POLLEN QUANTITY AND QUALITY LIMITATION OF PRE-ZYGOTIC REPRODUCTION IN <i>MIMULUS GUTTATUS</i> VARY WITH CO- FLOWERING COMMUNITY CONTEXT..... | 5 |
| 1.1 INTRODUCTION | 5 |
| 1.2 MATERIALS AND METHODS..... | 10 |
| 1.2.1 Study system..... | 10 |
| 1.2.2 Patterns of pollen quantity and quality limitation in high- and low- diversity seeps..... | 11 |
| 1.3 RESULTS | 15 |
| 1.4 DISCUSSION..... | 16 |
| 1.5 CONCLUSIONS..... | 20 |
| 2.0 HETEROSPECIFIC POLLEN DEPOSITION: DOES DIVERSITY ALTER THE CONSEQUENCES? | 23 |
| 2.1 INTRODUCTION | 23 |
| 2.2 MATERIALS AND METHODS..... | 27 |
| 2.2.1 Study system..... | 27 |

| | | |
|-------|---|----|
| 2.2.2 | Experimental plant material..... | 28 |
| 2.2.3 | Pollination treatments | 29 |
| 2.2.4 | Components of female reproductive success..... | 30 |
| 2.2.5 | Data analyses..... | 31 |
| 2.3 | RESULTS | 32 |
| 2.3.1 | Overall pollen load composition and conspecific load size effects | 32 |
| 2.3.2 | Diversity and identity effects on stigma reopening and fertile seed production..... | 33 |
| 2.3.3 | Multi-species interactions on fertile seed production..... | 33 |
| 2.3.4 | Mechanisms: seed abortion and pollen tube growth interference | 34 |
| 2.4 | DISCUSSION..... | 35 |
| 2.4.1 | What are the effects of diversity in the heterospecific load? | 35 |
| 2.4.2 | What is the role of CP loss in <i>M. guttatus</i> reproductive success?..... | 36 |
| 2.4.3 | What are the mechanisms involved in fertilization failure? | 36 |
| 2.4.4 | Ecological and evolutionary implications in natural communities | 37 |
| 3.0 | CO-FLOWERING COMMUNITY CONTEXT INFLUENCES FEMALE FITNESS AND ALTERS THE ADAPTIVE VALUE OF FLOWER LONGEVITY IN <i>MIMULUS GUTTATUS</i> | 43 |
| 3.1 | INTRODUCTION | 43 |
| 3.2 | MATERIALS AND METHODS..... | 48 |
| 3.2.1 | Co-flowering community diversity..... | 48 |
| 3.2.2 | Pollinator visitation | 49 |
| 3.2.3 | Pollen receipt..... | 50 |

| | | |
|-------|--|----|
| 3.2.4 | Co-variation between floral traits and community context..... | 51 |
| 3.2.5 | Adaptive value of flower longevity | 51 |
| 3.2.6 | Pre-planned contrasts..... | 53 |
| 3.3 | RESULTS | 55 |
| 3.3.1 | Co-flowering community diversity..... | 55 |
| 3.3.2 | Pollinator visitation | 55 |
| 3.3.3 | Pollen receipt..... | 56 |
| 3.3.4 | Co-variation between floral traits and community context..... | 56 |
| 3.3.5 | Adaptive value of flower longevity | 57 |
| 3.4 | DISCUSSION..... | 58 |
| 3.4.1 | Co-flowering diversity effects on the pollination environment | 59 |
| 3.4.2 | Co-flowering diversity effects on the adaptive value of flower longevity . | 61 |
| 3.4.3 | Why flower longevity?..... | 63 |
| 3.4.4 | Species richness versus sampling and phylogenetic effects | 65 |
| 4.0 | HETEROSPECIFIC POLLEN RECEIPT AFFECTS SELF POLLEN MORE THAN OUTCROSS POLLEN IN <i>MIMULUS GUTTATUS</i> : IMPLICATIONS FOR MIXED-MATING PLANTS..... | 72 |
| 4.1 | INTRODUCTION | 72 |
| 4.2 | MATERIALS AND METHODS | 75 |
| 4.2.1 | Study species | 75 |
| 4.2.2 | Plant material..... | 76 |
| 4.2.3 | Hand-pollination treatments | 76 |
| 4.2.4 | Data collection..... | 77 |

| | | |
|--------------------|---|----|
| 4.2.5 | Data analyses..... | 78 |
| 4.3 | RESULTS | 79 |
| 4.4 | DISCUSSION..... | 81 |
| 4.4.1 | Heterospecific pollen effects on self and outcross conspecific pollen..... | 81 |
| 4.4.2 | Ecological and evolutionary implications for mixed mating plants in diverse communities..... | 83 |
| APPENDIX A | | 88 |
| BIBLIOGRAPHY | | 91 |

LIST OF TABLES

| | |
|---|----|
| Table 1. Description and location of the two high- and low-diversity seep pairs studied. Total co-flowering species richness, number of co-flowering species per plot, average <i>M. guttatus</i> flower density and mean visitation rate to <i>M. guttatus</i> flowers (Arceo-Gómez and Ashman 2014) are shown for each seep. | 21 |
| Table 2. Piecewise regression analyses of pollen tubes on pollen grains per flower. First (b1), second (b2) slopes, breakpoints (c) and their bootstrap confidence intervals (BCa) for each seep in two years. Average pollen loads per stigma and the frequency distribution of flowers below (I), within (II) and above (III) the confidence intervals of the breakpoint are also given with region with the most flowers for a particular seep/year combination is shown in bold. * indicates individual slopes that differ significantly from zero ($P < 0.01$). †Indicates significant ($P < 0.0001$) differences in the frequency distribution of data points among the three regions (I, II and III). | 21 |
| Table 3. Effect of the co-flowering context (seep) on the pollen grain-pollen tube relationship (b1) for the subset of flowers that fall below the breakpoint. Differences in b1 were tested for each high- and low-diversity seep-pair and year (see Table 1 for seep pairing) using ANCOVA. | 22 |

Table 4. GLM results for the proportion of open stigmas (χ^2 test) and number of fertile seeds (F test) (a). Planned linear contrasts to test for the effects of increasing donor diversity (b) and donor identity (c) on the proportion of open stigmas and the number of fertile seeds produced by *M. guttatus* following hand pollinations with different heterospecific pollen loads and conspecific load sizes. Flower size was used as a covariate for seed number. Significant effects are denoted as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ‘-’ factor absent from model. 38

Table 5. Mean proportion of open stigmas and least square mean (\pm SE) number of fertile seeds produced after hand pollinations with different HP treatments. Comparisons were made between pollinations with conspecific pollen alone (*M. guttatus*; G) and single-species within group C+1(*M. guttatus* +*H. exilis*; +H, *S. albens*; +S and *M. nudatus*; +N) and between the control (C) and C+1, C+2 and C+3 mixes. Means that do not share a letter are significantly different at $P < 0.05$. Differences between G and single-species within C+1 are indicated by upper case letters, whereas differences between C, C+1, C+2 and C+3 by lower case letters. 39

Table 6. Least square mean (\pm SE) number of aborted seeds and callose plugs at the bottom of the style after hand pollinations with different HP treatments. Comparisons were made between pollinations with *M. guttatus* (G) and single-species combinations within group C+1: *M. guttatus* +*H. exilis* (+H), *S. albens* (+S) and *M. nudatus* (+N). Different letters denote significant differences at $P < 0.05$ 39

Table 7. Co-flowering species richness and locations of the four sites used to assess the effects of the pollination environment on *M. guttatus* visitation rate, pollen transfer dynamics, reproductive success and flower longevity. For pre-planned contrasts these were grouped into two diversity categories (high and low). 67

| | |
|--|----|
| Table 8. (A) Two-way ANCOVA and (B) pre-planned contrast results for the effects of source diversity, flowering site diversity and their interaction on <i>M. guttatus</i> flower longevity, conspecific pollen deposition and seed production. (C) The effects of covariates, temporal cohort (early and late in the flowering season) and local <i>M. guttatus</i> (conspecific) flower density on each dependent variable..... | 67 |
| Table 9. (A) Results of mixed-model ANOVA for the effects of conspecific pollen type (CP type [self vs. outcross]), heterospecific pollen treatment (HP treatment [without HP, HP-CP simultaneous, HP prior to CP]) and their interaction on the proportion of the total CP grains on stigma that grow tubes that reach the base of the style (CP pollen tubes/total CP on stigma) and CP fertilization success (fertile seeds/total CP on stigma). (B) Pre-planned contrasts testing for differences between control and HP application treatments and (C) the effect of CP type within each HP treatment level. | 85 |
| Table 10 (Appendix Table A1) Site number, co-flowering species richness, GPS coordinates (location), mean flower size, stigma-anther distance, flower longevity and conspecific flower density of the 23 seep communities at the McLaughlin Natural Reserve in northern California, USA. Sites 1-4 correspond to the same sites described in Table 1 and Table A2. | 89 |
| Table 11 (Appendix Table A2) Complete list of co-flowering species recorded in all the 23 seep communities surveyed at the McLaughlin Natural Reserve in northern California, USA. ‡ denotes that a particular species is present at that site. | 90 |

LIST OF FIGURES

Figure 1. Relations of pollen tube number to pollen receipt during two years (A-B: 2010 and C-D: 2011) for high- (RHA and TPW; open circles and solid lines) and low- (RHL and TP8; closed circles and dotted lines) diversity seeps. All slopes (b_1) were significantly greater for high-diversity seeps ($P < 0.05$) except for TPW during 2010 (Table 3). See methods for detailed descriptions of seep pairing and analyses. Parameter estimates of each individual pollen grain-tube relationship are given in Table 2..... 22

Figure 2. Conceptual models for multi-species effects of heterospecific pollen deposition on plant reproductive success: a) effects of multispecies interactions and b) ‘selection effects’. One conspecific donor (C), and two heterospecific donors (H1, H2) are represented and their individual contributions to the total pollen load is reflected in the pie diagrams on the X-axis (total heterospecific load size is held constant and only the diversity is varied). a) Addition of a single heterospecific donor to a pure conspecific mix results in a decrease in plant reproductive success (1). Increasing the diversity of heterospecific donors (but keeping conspecific and heterospecific load sizes constant) results in a similar effect of reproductive success (2), indicating H1 and H2 act additively, or results in a decrease in plant reproductive success that cannot be explained by the simple average of their individual effects (3) indicating that H1 and

H2 have synergistic effects, or results in an increase in reproductive success (4), suggesting that H1 and H2 are acting antagonistically, perhaps because H1 inhibits H2 and when combined with H2, H1 has a indirect positive effect on reproductive success. b) Addition of a single heterospecific donor to a pure conspecific mix results in non-additive effects on plant reproductive success because one donor has a strong effect (5), representing a ‘selection effect’ when in combination, i.e., one donor dominates the response in combination. In both cases the average of the individual effects (H1 and H2) is expected to be different from the mean of the two species combined (dashed lines), but, the latter is not different from the strongest (H2; 5) of the individual effects..... 40

Figure 3. Differences in fertile seed production (least square means \pm SE) between the control (*M. guttatus*; G), the average (dotted line) of single-species effects and the two-species loads for (a) +H and +N versus +HN (b) +S and +N versus +SN, and (c) +H and +S versus +SH (*H. exilis*; +H, *M. nudatus*; +N, *S. albens*; +S). Different letters denote significant differences at $P < 0.05$ 42

Figure 4. Frequency histogram of the number of plant species co-flowering with *Mimulus guttatus* at seeps (N = 23) within the McLaughlin Natural Reserve in Northern California. 68

Figure 5. Mean (\pm SE) for A) insect visitation rate, B) conspecific pollen (CP) receipt on stigma after one day of open pollination, and C) proportion of heterospecific pollen (HP) on the stigmas of *M. guttatus* flowering at sites with high and low co-flowering co-flowering community diversity. Significance of pre-planned contrasts between high and low diversity denoted as: * $P < 0.05$, ** $P < 0.001$ 69

Figure 6. Correlation between *M. guttatus* flower longevity assessed under common greenhouse conditions and the in-situ number of co-flowering species at the site (($r = 0.52$, $P = 0.01$; $n = 23$).

..... 70

Figure 7. Mean (\pm SE) for A) flower longevity, B) conspecific pollen (CP) receipt on stigma at the end of flower life span, and C) seed number per fruit for *M. guttatus* from source populations with high and low co-flowering community diversity when flowering at high and low co-flowering diversity sites. Significance of pre-planned contrasts denoted as: $*P < 0.05$, $**P = 0.01$ 71

Figure 8. Mean (\pm SE) for the proportion of the total pollen grains on stigma that grow tubes and reach the base of the style (*M. guttatus* pollen tubes/total conspecific pollen [CP] on stigma) for (A) *M. guttatus* outcross and self pollen, (B) heterospecific pollen (HP) treatments: without HP (open bars), HP-CP applied simultaneously (dashed bars) and HP applied prior to CP (closed bars) and for (C) *M. guttatus* outcross and self pollen within each HP treatment. Different letters and * denote significant differences $P < 0.05$ 86

Figure 9. Mean (\pm SE) for CP fertilization success (fertile seeds/ total conspecific pollen [CP] on stigma) for (A) *M. guttatus* outcross and self pollen, (B) heterospecific pollen (HP) treatments: without HP (open bars), HP-CP applied simultaneously (dashed bars) and HP applied prior to CP (closed bars) and for (C) *M. guttatus* outcross and self pollen within each HP treatment. Different letters and * denote significant differences $P < 0.05$ 87

INTRODUCTION

Plants rarely grow solely with conspecifics; rather they typically co-occur, flower and interact with other plants species in a community (Olesen and Jordano 2002, Bascompte et al. 2003, Geber and Moeller 2006). The acknowledgement of this complexity has shifted the focus of research on plant-pollinator interactions in recent years (Larson and Barret 2000, Geber and Moeller 2006, Sargent and Ackerly 2008) from studies that emphasize simple pair-wise interactions to studies of more complex community-wide interactions (e.g., Olesen and Jordano 2002, Bascompte et al. 2003, Vamosi et al. 2006, Sargent and Ackerly, 2008, Alonso et al. 2013). Thus, interest in the extent to which co-flowering plants share pollinators (e.g., Waser et al. 1996, Olesen and Jordano 2002, Bascompte et al. 2003), compete for or facilitate pollination (e.g., Ghazoul 2006, Mitchell et al. 2009, Schuett and Vamosi 2010, Sargent et al. 2011), and influence each other's pollination sufficiency and reproductive success (e.g., Moeller and Geber 2005, Ghazoul, 2006, Vamosi et al. 2006, Alonso et al. 2013) is rapidly increasing. For instance, different plant species within communities can vary in the quantity, quality and type of rewards offered, so a pollinator may avoid species that offer insufficient or low quality reward or substitute one species for another with the same characteristics (Geber and Moeller 2006). Interspecific competition for pollinators can thus reduce reproductive success of a focal species when intermixed with simultaneously flowering species (e.g., Bell et al. 2005, Mitchell et al.

2009). On the other hand, multiple-species floral displays have also been hypothesized to facilitate pollination and thus increase reproductive success by attracting a greater number and/or diversity of pollinators (e.g., Ghazoul 2006). Recent meta-analyses, however, have revealed that reproductive success of plants growing in species-rich regions of the world are limited by the amount of pollen reaching stigmas to a greater degree than plants in species-poor regions (Vamosi et al. 2006, Alonso et al. 2010, Vamosi et al. 2013), suggesting perhaps that pollinator competition is more common. Pollen limitation of reproductive success, however, has been often considered only a quantitative problem (i.e., too few pollen grains reaching conspecific stigmas) even though it can also have a qualitative component if deposition of poor quality pollen results in a decrease in fruit or seed production (Ashman et al. 2004, Aizen and Harder 2007, Alonso et al. 2012). Thus, pollen limitation can be influenced not only by processes that influence the frequency and efficiency of pollinator visitation and hence pollen quantity but also by processes that reduce pollen viability and seed development and thus reduce the quality of pollen received (Ashman et al. 2004, Alonso et al. 2012). However, how the co-flowering community context influences the magnitude of these two components of pollen limitation (quantity and quality) and what the underlying mechanisms are is still unknown. The negative effects of pollen quality limitation, in particular, can be the result not only of reduced pollen viability (e.g., through inbreeding depression) but also of heterospecific pollen deposition which may interfere with performance and fertilization by conspecific pollen grains (reviewed in Morales and Traveset 2008). Unfortunately, our knowledge of heterospecific pollen effects is still in its infancy as many studies have overlooked the complexities associated with heterospecific pollen receipt in natural communities (Ashman and Arceo-Gomez 2013). For instance, we still do not know if the negative effects of heterospecific pollen deposition can be intensified when multiple donors co-

occur on the same stigma, as is usually the case (Montgomery and Rathcke 2012, Fang and Huang 2013, Ashman and Arceo-Gomez 2013). Furthermore, I still unclear if the effects of heterospecific pollen receipt are greater when combined with conspecific pollen loss, even though both heterospecific receipt and conspecific loss are two major components of interspecific pollen transfer (Morales and Traveset 2008, Mitchell et al. 2009). Finally, we do not know if the effects of heterospecific pollen receipt are the same for self and outcross conspecific pollen despite the fact that >46% of the animal-pollinated species studied to date display some degree of mixed mating (Vogler and Kalisz 2001, Goodwillie et al. 2005) and thus receive self and outcross conspecific pollen along with heterospecific pollen. Thus, in order to fully understand the ecological and evolutionary implications of pollinator sharing in natural communities we need to explore the consequences of interspecific pollen transfer under more realistic pollination contexts. Overall, very little is known about pollinator responses to the combined resources of complex multi-species communities and thus more studies are needed that evaluate how co-flowering community context affects plant-pollinator interactions and plant reproductive success.

The plant community context not only can shape pollinator behavior and influence plant reproductive success but it can also alter the course of evolution (Caruso 2000, Geber and Moeller 2006). However, questions of how multiple interacting species in a community alter the evolutionary trajectory of a focal species and whether variation in community composition leads to geographic mosaics of selection have received little attention (Thompson 1999). For instance, one of the main effects of inadequate pollination could be an increase in selection on plant mating system and floral traits to diminish the negative effects of pollen limitation (Caruso 2000, Ashman et al. 2004, Knight et al., 2005). Pollen limitation may decrease as plants evolve traits

that attract more specialized pollinators that deliver less heterospecific pollen, or reduce competition for pollinators (Vamosi et al. 2006). Thus, selection for floral traits may be particularly strong in pollen-limited populations, and pollen limitation may play an important role in the evolution of secondary sexual traits (Ashman and Morgan 2004, Knight et al. 2005). As pollinator responses may differ between multi-species plant communities and single-species populations we could expect differences in the strength of pollinator-mediated selection on floral traits along a gradient of species-richness (Geber and Moeller 2006). Thus, studies that are conducted on a single focal species across different levels of co-flowering community diversity are needed to identify how community context influences pollen transfer dynamics, pollen limitation and how spatial variation in plant community structure may influence the evolution of plant reproductive traits, all of which are largely unknown to this day (Geber and Moeller 2006).

Thus, to enhance our understanding of the ecological and evolutionary consequences of community-wide interactions in this study I evaluate the extent to which the co-flowering community context shapes the outcome of plant-pollinator interactions. In particular, I evaluate the effect of the local co-flowering community context on quantity and quality aspects of pollen limitation (chapter II), I assess heterospecific pollen effects under more realistic pollination scenarios (chapter III and V), and evaluate the effect of co-flowering diversity on visitation rate, pollen transfer dynamics and floral evolution (chapter IV).

1.0 PATTERNS OF POLLEN QUANTITY AND QUALITY LIMITATION OF PRE-ZYGOTIC REPRODUCTION IN *MIMULUS GUTTATUS* VARY WITH CO-FLOWERING COMMUNITY CONTEXT

Arceo-Gómez G. and Tia-Lynn Ashman. *Oikos*. In review.

1.1 INTRODUCTION

Pollen limitation of plant reproduction is widespread (Burd 1994, Larson and Barrett 2000, Knight et al. 2005) and can have important consequences for plant demography (e.g., Ehrlén and Eriksson 1995, Anderson et al. 2011), trait evolution (e.g., Totland 2001, Fishman and Willis 2008) and ecosystem function (Davis et al. 2004; also see Ashman et al. 2004). Empirical data on over 200 species in 34 angiosperm orders distributed across six continents (Vamosi et al. 2006) indicates more than 60% of populations display some degree of pollen limitation (Burd 1994, Ashman et al. 2004, Knight et al. 2005). However, the apparently high incidence of pollen limitation is based on pollen supplementation experiments (e.g., 1,013 studies in Vamosi et al. 2006) which can confound the effects of pollen quantity (hereafter, ‘Qt’) with those of pollen quality (hereafter, ‘Ql’) on reproductive success, low Ql pollen being considered here as low conspecific pollen viability and/or slow pollen tube growth rate as a consequence of genetic or

environmental effects (Ashman et al. 2004, Aizen and Harder 2007, Alonso et al. 2012). Thus, despite of the large body of data amassed on pollen limitation the relative importance of pollen Qt and Ql in limiting plant reproduction in natural populations is still poorly understood (but see Alonso et al. 2013).

Complete knowledge of the causes underlying pollen limitation is necessary to fully understand its potential ecological and evolutionary consequences. For instance, changes in pollinator visitation rate and efficiency typically influence the Qt of pollen received while processes that affect conspecific pollen viability and tube growth rate influence the Ql of pollen reaching stigmas (Wilcock and Neiland 2002), and thus different strategies may evolve to cope with insufficiencies in these two components of pollen limitation. On one hand, severe Qt limitation can select for floral traits that increase the amount of conspecific pollen receipt in individual flowers such as flower size (e.g., Caruso 2000, Totland 2001) and display size (e.g., Thompson 2001). On the other hand, under severe Ql limitation selection may favor traits that increase outcrossing and hence could enhance pollen Ql, such as increased herkogamy (e.g., Motten and Stone 2000) and flower longevity (Dudash and Ritland 1991). Differences in the relative importance of Qt and Ql limitation could then underlie the enormous variability observed in both, the target and intensity of selection in response to pollen limitation (e.g., Caruso 2000, Totland 2001, Ashman and Morgan 2004, Vanhoenacker et al. 2006, Fishman and Willis 2008). Thus, evaluating the extent to which pollen Qt and Ql limit plant reproduction is an important first step toward understanding the type of selection mediating floral and mating system evolution.

Furthermore, while the role of intrinsic factors such as reproductive (e.g., flower size, flower longevity, breeding system) and life history traits (e.g., growth form, number of

reproductive events) in influencing the magnitude of pollen limitation have been well documented (e.g., Larson and Barrett 2000, Knight et al. 2005, Vamosi et al. 2006, Alonso et al. 2012), much less is known about the effects of extrinsic factors (biotic and abiotic). This gap in the understanding of pollen limitation can be the result of extrinsic effects typically tested across broad taxonomic and spatial scales (Larson and Barrett 2000, Vamosi et al. 2006, Alonso et al. 2010) which encompass large intrinsic and environmental variability and thus lead to low explanatory value or inconclusive results (Larson and Barrett 2000, Vamosi et al. 2013). In this sense, replicated, population-level studies are ideal to specifically tease out the role of extrinsic ecological factors contributing to variation in pollen limitation within a single species (e.g., Gomez et al. 2010, Schuett and Vamosi 2010, Sargent et al. 2011). Furthermore, even though few within-species studies have evaluated how variation in extrinsic factors such as conspecific density (e.g., Kunin 1997, Knight 2003), pollinator diversity (e.g., Gomez et al. 2010, Albrecht et al. 2012) and phylogenetic structure of the co-flowering community (e.g., Schuett and Vamosi 2010, Sargent et al. 2011) influence overall pollen limitation, no study has addressed their effects on Q_t and Q_l limitation separately.

The increasing magnitude of pollen limitation with increasing plant species richness observed at the global scale (Vamosi et al 2006, Alonso et al. 2010, Vamosi et al. 2013) deserves further attention because both Q_t and Q_l aspects of pollen limitation could vary with co-flowering species richness but in different directions. Specifically, an increase in species diversity (i.e., the addition of co-flowering species to an area) can intensify interspecific competition for pollinators (e.g., Caruso 2000, Bell et al. 2005, Mitchell et al. 2009, Arceo-Gómez and Ashman 2014) which, in turn, could reduce the amount of conspecific pollen reaching stigmas, intensifying Q_t limitation. On the contrary, if facilitative plant-plant

interactions occur (e.g., Ghazoul 2006) plants at highly diverse sites would experience increased pollinator visitation and thus, less Qt limitation compared to plants at low diversity sites. On the other hand, a decrease in co-flowering diversity could increase conspecific density in some cases (Schuett and Vamosi 2010), which can reduce pollinator flight distances between plants. In genetically structured populations, this effect can reduce outcrossing rate (Herrera 1987, Lu 2000), and population genetic diversity (Hamrick and Godt 1996, Hughes et al. 2008), degrading pollen vigor and viability (Willis 1993, Carr and Dudash 1997). Such effects could manifest as increased Ql limitation. However, in the absence of genetic structure, an increase in conspecific density could lead to increased pollen delivery and reproductive success (e.g., Kunin 1997). On the other hand, species diversity and population genetic diversity may correlate positively for reasons not directly related to pollinator movements. For instance, more heterogeneous environments may impose stronger diversifying selection on both species and genes (Vellend and Geber 2005, Lankau and Strauss 2007). A positive association between species and genetic diversity could also create differences in pollen Ql, and manifest as stronger Ql limitation in low-diversity co-flowering communities than high ones. Thus, we can expect the contributions of Qt and Ql to pollen limitation to differ based on the co-flowering community context. Specifically, we predict that a plant species growing in a high-diversity/low-conspecific density co-flowering community receives limited high quality pollen and experiences stronger Qt than Ql limitation, whereas the same species growing in a low-diversity/high-conspecific density co-flowering community experiences stronger Ql than Qt limitation.

Recently proposed approaches that distinguish between Qt and Ql aspects of pollen limitation (Aizen and Harder 2007, Alonso et al. 2012) make it possible to evaluate our predictions. Specifically, the approach developed by Alonso et al. (2012) distinguishes between

Qt and Ql limitation of pre-zygotic phase (i.e., pollen tube success) of reproduction by characterizing the pollen grain-pollen tube relationship. This analysis framework quantifies overall pollen limitation of pre-zygotic reproduction as the percent of flowers in a population that do not receive sufficient or high enough quality pollen to reach an asymptote in the number of pollen tubes that enter the ovary. Furthermore, it allows evaluating differences in pollen Ql by identifying the Qt of pollen needed to saturate pollen tubes at the base of the style (Alonso et al. 2012). This approach is also valuable in that it avoids the confounding effects of post-pollination resource reallocation which can be a problem with the standard hand-pollination approach (Ashman et al. 2004, Knight et al. 2005, Alonso et al. 2012).

In this study we use the method of Alonso et al. (2012) to evaluate aspects of pollen Qt and Ql limitation in *Mimulus guttatus* (Phrymaceae) at the serpentine seeps in Northern California. This study system is ideal to evaluate the effects of co-flowering community context on Qt and Ql aspects of pollen limitation, because it grows in seeps that vary naturally in co-flowering diversity and *M. guttatus* density (Freestone and Inouye 2006, Arceo-Gómez G. unpublished data; Table 1). By comparing populations of *M. guttatus* within the same regional area we tested the effects of the local ecological context on both components of pollen limitation while controlling for plant characters (e.g., life history, etc.) and broad climatic differences. Specifically, we answer the following questions (seeps are referred to here by their differences in diversity for simplicity): Does the percent of flowers experiencing pre-zygotic pollen limitation differ between high- and low-diversity seeps? Is the quality of pollen received higher at high- compared to low-diversity seeps? Do *M. guttatus* flowers at high-diversity seeps receive a lower quantity of pollen than those at low-diversity seeps? Are the patterns of Qt and Ql limitation

consistent across years, suggesting consistency of the ecological conditions that affect Qt and Ql limitation (Price et al. 2005, Gomez et al. 2010)?

1.2 MATERIALS AND METHODS

1.2.1 Study system

We evaluate the effect of co-flowering community diversity on Qt and Ql aspects of pollen limitation using *M. guttatus*, the yellow monkey flower, as the focal species. *Mimulus guttatus* is an insect-pollinated, hermaphroditic, annual-to-perennial species distributed throughout western North America (Vickery, 1978). Although self-compatible (Willis 1993) *M. guttatus* is considered mainly an outcrossing species (Dudash and Ritland 1991) that is mostly visited by bees but it can also be visited by beetles, butterflies and flies among other insects (Arceo-Gómez and Ashman 2014). Its flowers can have more than 300 ovules (Carr and Dudash 1995, Arceo-Gómez and Ashman 2011) and in some cases produce up to 700 seeds (Arceo-Gómez pers. obs.). Pollen tubes reach the ovary typically 8h after pollination (Arceo-Gómez and Ashman 2011) and no differences in fertilization success has been observed between self and outcross conspecific pollen (Arceo-Gómez and Ashman in prep.).

This study of *M. guttatus* was conducted in the serpentine seep communities at the McLaughlin Natural Reserve (California, USA). Seep communities are discrete within a matrix of grassland communities and are spatially independent from each other (Harrison et al. 2000, Freestone and Harrison 2006). To evaluate whether Qt and Ql aspects of pre-zygotic pollen

limitation of *M. guttatus* vary with co-flowering community context, we selected four seeps that differed in number of co-flowering species per unit of area (Table 1). Number of co-flowering species at each site was recorded during the entire *M. guttatus* flowering season (Arceo-Gómez and Ashman 2014). Two pairs of geographically proximal seeps that differed in diversity (high–low: RHA-RHL and TPW-TP8; average within pair distance vs. between pairs: 1.2 km vs. 3 km). Pairing seeps in this manner controls for variation due to other biotic (e.g., pollinator community) and abiotic (e.g., resource availability) factors that may be spatially structured and influence pollination success. Within these seeps *M. guttatus* flower density is higher at low compared to the high co-flowering diversity seeps ($P < 0.01$; Arceo-Gómez G. unpublished data) and *M. guttatus* flowers at the former receive twice as many pollinator visits than at the latter (Arceo-Gómez and Ashman 2014; Table 1). Hereafter seeps are referred to by their differences in co-flowering diversity for convenience. *Mimulus guttatus* morphological features such as flower size, stigma-anther distance, and floral display size, however, do not differ between paired populations and delayed self-pollination is very low at all seeps (Arceo-Gómez and Ashman 2014) contrary to what has been reported for other populations (e.g., Dole 1990). In addition, no differences in fruit size have been observed between high and low diversity sites ($P = 0.1$, Arceo-Gomez G. unpublished data).

1.2.2 Patterns of pollen quantity and quality limitation in high- and low-diversity seeps

To evaluate how pollen Qt and Ql limits *M. guttatus* pre-zygotic reproductive success when flowering at high- versus low-diversity seeps we used the piecewise regression analysis described by Alonso et al. (2012). This analytical method assesses patterns of Qt and Ql

limitation by characterizing the relation between the number of pollen grains and tubes in un-manipulated wilted flowers (Alonso et al. 2012, Alonso et al. 2013). The dose-response (i.e., pollen grain-tubes) relation can be characterized by three main parameters. The percent of flowers below the lower confidence limit of the point where the pollen grain-tube relation changes, i.e., the breakpoint (region I; Alonso et al. 2012), indicates the extent to which flowers do not reach an asymptote in the number of pollen tubes that enter the ovary (overall pollen limitation). The rise of the first slope ‘ b_1 ’ and the break point ‘ c ’ (Toms and Lesperance 2003, Ryan and Porth 2007) are descriptors of pollen QI (Alonso et al. 2012, Alonso et al. 2013). For instance, a steeper b_1 and a lower c would reflect higher pollen QI because fewer pollen grains will be required to reach an asymptote (Alonso et al. 2012, Alonso et al. 2013). A second slope ‘ b_2 ’ reflects the degree to which pollen QI causes an increase in the number of pollen tubes even after an asymptote has been reached (Alonso et al. 2012). It is important to point out that even though we evaluate pollen limitation of the pre-zygotic stage, the number of pollen tubes at the base of the style is a good indicator of seed production in this species ($r = 0.6$, $P = 0.001$, $N = 168$; Arceo-Gomez G. unpublished data) and thus the results ought to be relevant to later stage as well.

To conduct this analysis we sampled wilted flowers (one per plant at least 12h after senescence) by randomly collecting 20 *M. guttatus* styles per seep per week for a total of five weeks (100 styles total per seep/year). With this scheme we sampled the pollination environment experienced by *M. guttatus* over the entire flowering season and achieved a large enough sample size to ensure proper fitting of the model and narrow confidence intervals of the estimated parameters (Alonso et al. 2012). Styles were collected in two consecutive years (2010 and 2011) to assess whether among site patterns of pollen Qt and QI limitation are consistent over time.

Styles were stored in vials with 70% ethanol until processed. Styles were stained with decolorized aniline blue and the number of conspecific pollen grains and tubes at the base of the style counted using a fluorescence microscope at 40x magnification (Kearns and Inouye 1993). Styles were firmly squeezed against the microscope slide to spread and separate pollen tubes for easier counting (for more details see Arceo-Gómez and Ashman 2011).

Dose-response pollen grain-tube number relationships were constructed for each seep [two high (RHA, TPW) and two low (TP8, RHL) diversity] and for each year independently. To do so we first tested the linearity of each relationship by assessing the significance of the nonlinear component using a generalized additive model (proc gam; SAS 2010) with a cubic smoothing spline, a Poisson error distribution and a log link function (Hastie and Tibshirani 1990, Alonso et al. 2012, Alonso et al. 2013). A significant spline component is an indicator of a better fit of a nonlinear relationship (Hastie and Tibshirani 1990). A piecewise regression was then fitted (proc nlin; SAS 2010) to each data set and $b1$, $b2$ and c values were estimated as well as the confidence intervals for c using non-parametric bootstrapping, ($n= 1000$) following Alonso et al. (2012). We then estimated the percent of flowers that fell below (region I), within (region II) and above (region III) the confidence intervals of c (Alonso et al. 2012). The percent of flowers below the breakpoint (region I) indicates the overall magnitude of pre-zygotic pollen limitation at the population level, the second region reflects the uncertainty in the location of the breakpoint while the third region indicates the percent of flowers for which pollen Q_t is no longer important or that have reached saturation in the number of pollen tubes that enter the ovary (Alonso et al. 2012). A χ^2 -test was used to compare the overall frequency of flowers within regions for each seep and year. We further tested whether the percent of pollen-limited flowers (region I) was affected by seep and/or year via an ANOVA (proc glm; SAS 2010).

Percentages were arc sine transformed in order to achieve normality. Seeps were chosen to represent different levels of community diversity and thus treated as a fixed effect, whereas year was treated as a random effect. Because all seeps differ in overall species richness they were considered as independent treatment levels of community diversity (e.g., Strauss and Murch 2004, Arceo-Gómez and Ashman 2014) and a pre-planned linear contrast (Rosenthal and Rosnow 1985) was conducted to evaluate whether percent of pollen-limited flowers differed between the two high- and low-diversity seeps (proc glm; SAS 2010). We then compared differences in $b1$ between high- and low-diversity paired seeps in each year (Table 1). The slopes ($b1$) were compared by splitting each data set at the breakpoint (c) and conduct ANCOVA (proc glm; SAS 2010) on pollen tubes using the subset of flowers that fall below c with seep as a fixed factor and pollen grains as a covariate (Alonso et al. 2012, 2013). A significant seep by pollen grain interaction indicates that $b1$, which is an indicator of pollen QI, differed between paired seeps. Furthermore, we used ANOVAs to test for the overall effect of seep and year (random effect) on $b1$ and evaluated differences between high- and low-diversity seeps via a preplanned contrast. Significant differences in $b1$ values between high- and low-diversity would also reflect differences in pollen QI. Finally, we evaluated whether total conspecific pollen receipt by *M. guttatus* stigmas differed between high- and low-diversity seeps and/or between years via a two-way ANOVA with seep (fixed) and year (random) as effects (proc glm; SAS 2010). The difference in the amount of conspecific pollen received by *M. guttatus* when growing at high- and low-diversity seeps was assessed via a preplanned contrast, as above. The number of conspecific pollen grains per stigma was square root transformed to achieve normality.

1.3 RESULTS

Mean conspecific pollen receipt varied significantly among seeps ($F_{3, 792} = 29.17$, $P < 0.0001$), but not between years ($F_{1, 68} = 0.60$, $P = 0.43$). The pre-planned contrast revealed that *M. guttatus* at high-diversity seeps received on average 29% less conspecific pollen (mean \pm SE: 325 ± 12.6) than at low-diversity seeps (456 ± 17.5 ; $F_{1, 792} = 31.79$, $P < 0.0001$).

For all seep-year combinations pollen receipt by most sampled flowers fell below (region I; 5 seep-year combinations) or within (region II; RHL- and TPW-2010) the break point, except for one (RHA-2010, region III; Table 2). The proportion of flowers differed significantly among ‘regions’ within all seeps during both years ($X^2 > 35$, $P < 0.001$, in all cases), except for RHA during 2010 (Table 2). However, the percent of pollen-limited flowers (region I) was not affected by seep ($F_{3, 7} = 1.23$, $P = 0.4$) or year ($F_{1, 7} = 0.93$, $P = 0.4$), nor did it differ between high- and low-diversity seeps ($F_{1, 7} = 0.67$, $P = 0.4$), suggesting that pollen-tube formation in most *M. guttatus* flowers at these sites is pollen limited, regardless of the co-flowering context.

For all seeps and years, pollen tube number varied non-linearly with pollen receipt (spline component, $P < 0.0001$ for all). Overall, piecewise analyses consistently revealed a tendency for larger *b1* and lower breakpoints at high-diversity compared to low-diversity seeps across both years (Table 2). Furthermore, comparisons between seep pairs revealed that *b1* was significantly higher at the high-diversity seep than the low-diversity one of each pair in each year (Table 3: Fig 1A, C, D), with the exception of TPW-TP8 in 2010 (Table 3; Fig 1B). When tested across all seeps, *b1* values were affected by seep ($F_{3, 7} = 10.24$, $P = 0.04$) but not by year ($F_{1, 7} = 1.41$, $P = 0.3$). Consistent with the above results, pre-planned contrast revealed that the overall *b1* values were significantly higher at high-diversity compared to low-diversity seeps (0.69 ± 0.06 and 0.46

± 0.01 ; $F_{1,7} = 22.12$, $P = 0.01$) suggesting better pollen QI at high-diversity seeps. The second slope (b_2) did not differ from zero in most seeps (all except for RHA; Table 2), indicating that addition of more or better quality pollen would not increase pollen tube formation.

1.4 DISCUSSION

Our study revealed that *M. guttatus* in serpentine seeps experience overall pollen limitation of pre-zygotic reproduction regardless of the co-flowering context. This is apparent from the high percent of flowers that do not receive enough quantity or high enough quality pollen to reach a ‘breakpoint’ (i.e., asymptote) in the number of pollen tubes that enter the ovary at either, high- or low-diversity seeps. Specifically, 50 to 61% of flowers fall below the breakpoint across years at high- and low-diversity seeps respectively (Table 2) and no difference was found among seeps indicating that at least half of the sampled flowers were limited by some aspect of pollen limitation. However, different components of pollen limitation at high- and low-diversity seeps underlie this overall pattern. In particular, Qt limitation is stronger at high-diversity seeps whereas QI is the most important limiting factor of pre-zygotic reproductive success at low-diversity seeps. This conclusion is supported by three lines of evidence. First, when tested across all seeps and years high-diversity seeps had significantly higher ‘ bI ’ estimates compared to low-diversity seeps. Second, ‘ bI ’ estimates were significantly steeper at high-diversity seeps in the majority (3 out of 4) of specific paired comparisons. Third, *M. guttatus* received 29% less conspecific pollen at high- compared to low-diversity seeps. Together, these results indicate

higher pollen QI (Alonso et al. 2012) at high-diversity seeps and suggest that *M. guttatus* growing in high diversity co-flowering communities are more limited by the amount rather than by the QI of pollen reaching the stigmas. This effect might be accentuated by the fact that the delayed self-pollination mechanism that many *M. guttatus* exhibit (Dole 1990) is not very effective or is completely absent in plants from all the studied seeps (Arceo-Gómez and Ashman 2014). Consistent with these results, previous studies have shown that *M. guttatus* receives 60% fewer pollinator visits at high- compared to low-diversity seeps (Arceo-Gómez and Ashman 2014; Table 1), suggesting high interspecific competition for pollinators may underlie the stronger pollen Qt limitation observed at these seeps. It is also possible, however, that lower conspecific densities at high-diversity seeps leads to lower pollinator recruitment and this results in a decrease in conspecific pollen transfer. Thus, future experiments that simultaneously manipulate conspecific density and co-flowering diversity would be ideal to dissect their individual effects on pollen Qt limitation.

On the other hand, the differences in pollen QI observed between high- and low-diversity seeps could have arisen from two non-mutually exclusive mechanisms. First, *M. guttatus* flower visitors may travel shorter distances between plants at the low-diversity seeps as *M. guttatus* flower density is at least 25% higher at these seeps than high-diversity ones (Table 1). This change in pollinator foraging behavior at low-diversity seeps could then lead to an increase in biparental inbreeding which can have negative effects on aspects of pollen QI, such as pollen viability (e.g., Willis 1993, Carr and Dudash 1997). Second, higher pollen QI at high diversity seeps may result from a correlation between species and genetic diversity which can be driven by the parallel effects of local environmental heterogeneity on both levels of diversity or by the direct effects of one level of diversity on another (Vellend and Geber 2005, Lankau and Strauss

2007, Hughes et al. 2008). For instance, an increase in the number of competitors in an area may impose stronger and more varied selection pressures on genotypes within a population and increase genetic diversity (Vellend and Geber 2005, Lankau and Strauss 2007). Evaluating whether these mechanisms are responsible for the differences in pollen QI observed is necessary to fully understand the causes of pollen QI limitation seen here.

It is important to acknowledge that pollen QI can also be affected by other factors that may differ between seeps. For instance, pollen viability has also been shown to be influenced by environmental conditions such as the amount of solar exposure (Galen and Stanton 2003), water stress (Gay et al. 1987), temperature (Ledesma and Sugiyama 2005) and ultraviolet-B radiation (Demchik and Day 1996). However, in our study plants from high-diversity seeps (with high QI pollen in our study) do not seem to experience better resource and environmental conditions compared to plants at low-diversity sites (with lower pollen QI; Arceo-Gómez and Ashman 2014). It is important to point out, however, that we cannot rule out the possibility of environmental factors influencing our results and experiments that simultaneously manipulate the co-flowering context and environmental conditions are needed to tease apart their effects on pollen quality. It is also unlikely that differences in heterospecific pollen receipt between plants growing at high and low diversity sites are the cause of differences in pollen QI limitation in this study because *M. guttatus* at high diversity sites receives larger and more diverse heterospecific pollen loads (Arceo-Gómez and Ashman 2014) but conspecific pollen quality is the highest here. If heterospecific pollen was the cause of pollen QI differences we would have expected the opposite pattern whereby high diversity sites would have the lowest pollen quality as greater heterospecific pollen receipt would have further reduced pollen germination and tube growth rate at these sites (Arceo-Gómez and Ashman 2011). Finally, other studies have shown that

communities of closely related individuals tend to experience higher levels of pollen limitation (Schuett and Vamosi 2010). However, phylogenetic relatedness was unlikely to have influenced our results as *M. nudatus* the closest relative of *M. guttatus* exists at all four of our study sites (Arceo-Gómez and Ashman 2014).

Overall, our results support the prediction that *M. guttatus* at high-diversity seeps receives low amounts of high QI pollen (i.e., stronger Qt limitation), whereas at low-diversity seeps plants receive more pollen of inferior QI (i.e., stronger QI limitation). And, these patterns are consistent across years. This suggests that local ecological factors, such as *M. guttatus* population size and the frequency and species composition of its flower visiting fauna (Price et al. 2005, Vanhoenacker et al. 2006, Gomez et al. 2010) which are thought to induce changes in pollen Qt and QI limitation, did not differ appreciably during the studied years.

Meta-analytical approaches have suggested that the magnitude of pollen limitation can be greater for plants in highly diverse areas (e.g., Vamosi et al. 2006, Alonso et al. 2010). Our study gives new empirical support to this idea by showing that plants in high-diversity seeps receive less conspecific pollen compared to plants at low-diversity seeps, and that pre-zygotic reproductive success is mostly limited by pollen Qt at these sites. However, plants at low-diversity seeps were also pollen limited, although for the species studied here by low QI, rather than by low amounts of pollen. This apparent discrepancy between our results and previous findings (e.g., Vamosi et al. 2006) may be due to the different components of reproductive success being evaluated (fruit-set vs. pollen tube formation). However, pollen supplementation studies (as the ones evaluated in Vamosi et al. 2006) often confound Qt and QI limitation (Ashman et al. 2004, Aizen and Harder 2007, Alonso et al. 2012) and no response to pollen supplementation is expected when reproductive success is mostly limited by pollen QI (Aizen

and Harder 2007). Thus, previous studies (e.g., Vamosi et al. 2006, Alonso et al. 2010) may have underestimated the overall magnitude of pollen limitation in low-diversity communities by overlooking the effect of Ql limitation. Few studies have evaluated the importance of pollen Qt and Ql in determining plant reproductive success (Vaughton and Ramsey 2010, Alonso et al. 2012, Alonso et al. 2013). However, while these have evaluated their contribution to pollen limitation in a single population (Vaughton and Ramsey 2010, Alonso et al. 2012) our study is the first to show how the relative importance of these two components can vary with aspects of the community context.

1.5 CONCLUSIONS

Here we show that receipt of inadequate pollen Ql as well as Qt can contribute to variation in pollen limitation of pre-zygotic reproductive success, adding to recent studies (Aizen and Harder 2007, Alonso et al. 2013) and expanding on them by revealing variation with extrinsic factors. Thus, we emphasize the need to differentiate these two aspects if we are to fully understand the causes and consequences of plant reproductive failure in natural populations. Such a pursuit is becoming increasingly important as disturbances such as invasive species (Vila et al. 2009), climate change (Hegland et al. 2009) and habitat fragmentation (Aguilar et al. 2006) change the communities e.g., by altering population size and the number of co-flowering species within communities in ways that may affect both Qt and Ql aspects of pollen limitation.

Table 1. Description and location of the two high- and low-diversity seep pairs studied. Total co-flowering species richness, number of co-flowering species per plot, average *M. guttatus* flower density and mean visitation rate to *M. guttatus* flowers (Arceo-Gómez and Ashman 2014) are shown for each seep.

| Pair | Diversity category | Seep name | Total co-flowering species richness | Mean \pm SE number of co-flowering species in 2m ² | Mean \pm SE <i>M. guttatus</i> flower density in 2m ² | Mean \pm SE visits to <i>M. guttatus</i> flowers per hour | Location (GPS) |
|------|--------------------|-----------|-------------------------------------|---|--|---|------------------------------|
| 1 | High | RHA | 19 | 4.9 \pm 0.24 | 15 \pm 2.1 | 0.18 \pm 0.06 | 38°51'29.45"N 122°24'33.49"W |
| 1 | Low | RHL | 9 | 1.8 \pm 0.22 | 130 \pm 25.2 | 0.54 \pm 0.18 | 38°51'13.38"N 122°24'21.43"W |
| 2 | High | TPW | 17 | 2.5 \pm 0.23 | 66 \pm 11.4 | 0.3 \pm 0.06 | 38°51'56.62"N 122°27'02.30"W |
| 2 | Low | TP8 | 8 | 1.7 \pm 0.16 | 79 \pm 9.4 | 0.6 \pm 0.24 | 38°51'30.91"N 122°25'55.88"W |

Table 2. Piecewise regression analyses of pollen tubes on pollen grains per flower. First (b1), second (b2) slopes, breakpoints (c) and their bootstrap confidence intervals (BCa) for each seep in two years. Average pollen loads per stigma and the frequency distribution of flowers below (I), within (II) and above (III) the confidence intervals of the breakpoint are also given with region with the most flowers for a particular seep/year combination is shown in bold. * indicates individual slopes that differ significantly from zero ($P < 0.01$). [†]Indicates significant ($P < 0.0001$) differences in the frequency distribution of data points among the three regions (I, II and III).

| Pair | Seep | Year | Diversity | b1 | b2 | c (BCa) | Mean \pm SE pollen load | Flower distribution (%) | | |
|------|------|------|-----------|-------|-------|-----------------|---------------------------|-------------------------|-----------|-----------------|
| | | | | | | | | I | II | III |
| 1 | RHA | 2010 | High | 0.88* | 0.32* | 382 (295-461) | 438 \pm 23 | 29 | 27 | 44 |
| 1 | RHL | 2010 | Low | 0.46* | 0.07 | 813 (155-1402) | 466 \pm 39 | 31 | 66 | 3 [†] |
| 1 | RHA | 2011 | High | 0.7* | 0.23* | 501 (369-705) | 379 \pm 22 | 55 | 38 | 7 [†] |
| 1 | RHL | 2011 | Low | 0.48* | 0.11 | 661 (486-847) | 476 \pm 39 | 62 | 21 | 17 [†] |
| 2 | TPW | 2010 | High | 0.58* | 0.18 | 484 (122-524) | 185 \pm 16 | 47 | 49 | 4 [†] |
| 2 | TP8 | 2010 | Low | 0.5* | -0.45 | 1208 (835-1277) | 491 \pm 32 | 89 | 7 | 4 [†] |
| 2 | TPW | 2011 | High | 0.6* | 0.19 | 553 (328-710) | 263 \pm 25 | 69 | 25 | 6 [†] |
| 2 | TP8 | 2011 | Low | 0.41* | -0.23 | 1011 (426-1011) | 389 \pm 30 | 62 | 35 | 3 [†] |

Table 3. Effect of the co-flowering context (seep) on the pollen grain-pollen tube relationship (b1) for the subset of flowers that fall below the breakpoint. Differences in b1 were tested for each high- and low-diversity seep-pair and year (see Table 1 for seep pairing) using ANCOVA.

| Seep pair | Year | DF | F | P |
|-----------|------|--------|-------|----------|
| RHA-RHL | 2010 | 1, 118 | 23.57 | < 0.001 |
| TPW-TP8 | 2010 | 1, 85 | 0.04 | 0.84 |
| RHA-RHL | 2011 | 1, 147 | 12.74 | 0.0005 |
| TPW-TP8 | 2011 | 1, 173 | 19.28 | < 0.0001 |

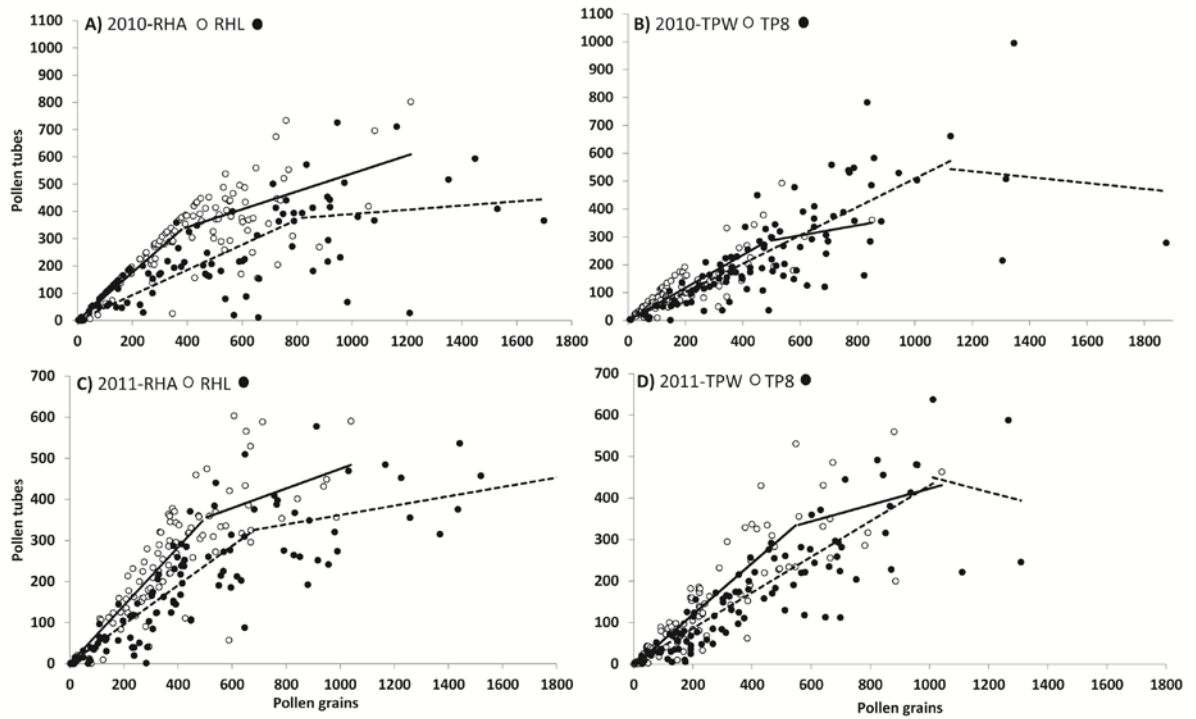


Figure 1. Relations of pollen tube number to pollen receipt during two years (A-B: 2010 and C-D: 2011) for high- (RHA and TPW; open circles and solid lines) and low- (RHL and TP8; closed circles and dotted lines) diversity seeps. All slopes (b1) were significantly greater for high-diversity seeps ($P < 0.05$) except for TPW during 2010 (Table 3). See methods for detailed descriptions of seep pairing and analyses. Parameter estimates of each individual pollen grain-tube relationship are given in Table 2.

2.0 HETEROSPECIFIC POLLEN DEPOSITION: DOES DIVERSITY ALTER THE CONSEQUENCES?

Arceo-Gómez G. and Tia-Lynn Ashman. 2011. *New Phytologist* 192:738-746

2.1 INTRODUCTION

Flowering plants often share pollinators (Waser *et al.*, 1996; Bascompte *et al.*, 2003; Lazaro *et al.*, 2009; Mitchell *et al.*, 2009). Indeed, generalized pollination systems, where many plants are visited by diverse pollinator assemblages (Oelsen & Jordano, 2002; Bascompte *et al.*, 2003; Petanidou *et al.*, 2008), are more common than specialized systems where exclusive plant-pollinator relationships exist (Herrera, 1988; Waser *et al.*, 1996; Lazaro *et al.*, 2009). Pollinator sharing can lead to interspecific pollen transfer that may decrease plant reproductive success (Feinsinger *et al.*, 1988, Feinsinger & Tiebout, 1991; Bell *et al.*, 2005; Morales & Traveset, 2008; Mitchell *et al.*, 2009), and may underlie the high levels of pollen limitation of seed set for plants in very diverse communities (Vamosi *et al.*, 2006; Alonso *et al.*, 2010). Thus, to fully understand the ecological and evolutionary implications of pollinator sharing in natural communities we need to explore the consequences of interspecific pollen transfer.

Interspecific pollen transfer can reduce seed production through two different mechanisms, conspecific pollen (hereafter, CP) loss and heterospecific pollen (hereafter HP) deposition (Wilcock & Neiland, 2002; Morales & Traveset, 2008; Mitchell *et al.*, 2009). Pollinator movements between plant species can lead to loss of CP to heterospecific flowers and thus reduce the amount of pollen that reaches conspecific stigmas (Feinsinger & Tiebout, 1991; Larson *et al.*, 2006; Flanagan *et al.*, 2009; Mitchell *et al.*, 2009). In turn, mixed pollen loads on pollinators (e.g., >8 different plant species; Bartomeus *et al.*, 2008) leads to stigmas receiving diverse communities of HP (Feinsinger *et al.*, 1986; Neiland and Wilcock, 1999) and sometimes in substantial amounts relative to CP load size (e.g., 70%; Bartomeus *et al.*, 2008).

Reduction in CP deposition directly affects fertilization opportunities (Waser & Fugate, 1986; Galen & Gregory, 1989; Caruso & Alfaro, 2000; Brown & Mitchell, 2001; Moragues & Traveset, 2005), whereas HP receipt can reduce female reproductive success through a number of indirect mechanisms. Heterospecific pollen can interfere with CP adhesion and germination (Galen & Gregory, 1989), as well as with pollen tube growth, ovule fertilization and seed development (Wilcock & Neiland, 2002; Morales & Traveset, 2008). For example, allelopathic effects of pollen can inhibit both pollen tube growth and ovule development (Thomson *et al.*, 1981), and pollen of closely related species can preemptively fertilize ovules that then fail to develop (Harder *et al.*, 1992). The variable results obtained from single heterospecific donor experiments, i.e., some show detrimental effects (Brown & Mitchell, 2001; Larson *et al.*, 2006; Moragues & Traveset, 2005), while others do not (Kohn & Waser, 1985; Caruso & Alfaro, 2000), may reflect differences in the severity or the type of mechanism involved but we do not yet know because most studies only record seed production (Table 3 in Morales & Traveset,

2008) and do not uncover the underlying mechanism (but see Thomson *et al.*, 1981; Galen & Gregory, 1989; Harder *et al.*, 1992).

While numerous studies have assessed the effects of a single HP donor on reproductive success (reviewed in Morales & Traveset, 2008), only one has assessed the effects of two (Flanagan *et al.*, 2010), and none have assessed the effects of three or more HP species. This represents a significant gap in our understanding of the consequences of HP deposition as the number of heterospecific donors on a single stigma can be large (e.g., 9 species; Neiland and Wilcock, 1999), and these may interact. For instance, while the addition of a single heterospecific donor (H1) to a CP load (C) may result in a decrease in reproductive success (Fig. 1a-1), increasing the diversity of heterospecific donors (here keeping total heterospecific load constant and assuming linear responses) may have diverse outcomes. Three outcomes are possible with two heterospecific donors (H1 and H2). First, the two pollen donors are similarly detrimental and act additively such that the effect of their combination is simply the average of their independent effects (Fig. 1a-2). Second, the two donors interact synergistically such that their combined effect is more detrimental than the average of their independent effects (Fig. 1a-3), e.g., one facilitates the interference mechanism of the other. And third, the two donors interact antagonistically such that their combined effect is less detrimental than the average of their independent effects (Fig. 1a-4), e.g., one may inhibit the other and thus have an indirect positive effect on focal plant reproductive success. It is worth noting that non-linear relationships between the size of the HP load and the magnitude of the effect have been observed (Thomson *et al.*, 1981), and these could be particularly important when one heterospecific donor has a strong effect and thus dominates the process (i.e. “selection effect”; Loreau & Hector 2001). Interactions identified as synergistic or antagonistic under assumptions of linearity and

additivity, instead could reflect the effect of the heterospecific donor with a dominant effect (Fig. 1b). One can test for a ‘selection effect’ once additivity is rejected by comparing the combined effect to the singular effect of the strongest, if the combined effect is stronger (Fig 1b-5), of the interacting species (see also Loreau & Hector 2001 for advanced statistical methods for more complex communities). When the pollen community on the stigma becomes more diverse one also needs to consider ‘sampling (lottery or chance) effects’ where communities with more species also have a greater probability of containing a species with a strong effect (e.g., Loreau & Hector 2001; Leps *et al.*, 2001). Thus, while our additive model is the most basic, it combined with these additional considerations provides a conceptual foundation for understanding how multi-species pollen loads may determine the shape of the response to increasing heterospecific donor diversity.

Finally, the negative impact of HP deposition may be exacerbated when combined with CP loss. Since CP loss likely reduces the amount of CP reaching a stigma, the relative amount of HP will increase with increasing conspecific loss. If the level of interference by HP depends on its relative abundance on a style then there may be an interaction between conspecific loss and heterospecific deposition. However, to our knowledge, the interactive effects on seed production have not yet been determined in any system.

To address these issues, we sought to understand the effects of multi-species HP loads on *Mimulus guttatus* female reproductive success. This species exists in the highly diverse serpentine seep communities in northern California (Freestone & Inouye, 2006) where HP deposition is common (Arceo-Gómez, unpublished). It also has a ‘touch sensitive’ stigma that provides a rapid indicator of ovule fertilization success (Fetscher & Kohn, 1999). Thus, we simulated the effect of increasing HP diversity and increasing CP loss on *M. guttatus* stigmas to

address the following specific questions: (1) Does HP deposition and/or reduced CP deposition affect the probability that a stigma reopens, or the production of fertile seeds? (2) Does the outcome of HP receipt depend on the diversity of donors, species identity, or CP load size? (3) Are the effects of multi-species heterospecific loads on fertile seed production additive, or is there evidence for synergistic, antagonistic or other types of effects? (4) Are ovule usurpation (and subsequent seed abortion) or pollen tube growth interference the mechanisms responsible for reduced fertilization?

2.2 MATERIALS AND METHODS

2.2.1 Study system

Focal species: *Mimulus guttatus*, the yellow monkeyflower (Phrymaceae) was both the pollen recipient and CP donor. It is an insect-pollinated, self-compatible, hermaphroditic, annual-to-perennial species distributed throughout western North America (Vickery, 1978). Its flowers have a bilobed stigma that closes ~2 s after being touched and reopens in response to low ovule fertilization (Fetscher & Kohn, 1999). Thus, reopening probability is an early indicator of insufficient ovule fertilization as 95% of pollen tubes reach the ovary in 8 hrs (Arceo-Gómez, unpublished). Flowers are herkogamous, last 5-6 days and only autonomously self-pollinate as corollas abscise (Dole, 1990; Arathi & Kelly, 2004, Arceo-Gómez, pers., obs.). *Mimulus guttatus* pollen grains are spheroidal and ~30 μm in diameter. *Mimulus guttatus* fruits produce numerous seeds (377 ± 136 ; Arceo-Gómez, unpublished).

Community members: We selected three species as HP donors (*Helianthus exilis* [Asteraceae], *Stachys albens* [Lamiaceae] and *Mimulus nudatus* [Phrymaceae]) that intermingle with *M. guttatus* in high densities (Freestone & Inouye, 2006; Arceo-Gómez, pers., obs.). All four species, including *M. guttatus*, co-flower during the summer (Gardner & Macnair, 2000; Harrison *et al.*, 2000; Arceo-Gómez, pers., obs.), and are mainly bee-pollinated (Robertson *et al.*, 1999; Wolf *et al.*, 1999; Gardner & Macnair, 2000; Arceo-Gómez, pers., obs.). Pollinator sharing and HP transfer between *M. guttatus* and these species has been recorded (Gardner & Macnair, 2000; Arceo-Gómez, pers., obs.).

The serpentine sunflower *Helianthus exilis* is an annual forb endemic to serpentine outcrops in northern California. It produces many yellow flower heads and its pollen grains are spheroidal, ~25 μm in diameter, and have a highly ornamented surface. *Stachys albens* is a perennial herb endemic to California. It produces white flowers in spikes, and its pollen is ~20 μm , spheroidal and unornamented. *Mimulus nudatus*, is an annual species endemic to serpentine seeps in northern California. It has small yellow flowers, and large (~32 μm), spheroidal pollen grains.

2.2.2 Experimental plant material

Focal species: *Mimulus guttatus* seeds (obtained from David Carr, University of Virginia) were sown in trays of Sunshine™ germination mix in the greenhouse (14hday/10h night; 26/21°C) at the University of Pittsburgh. Forty-five 3-week old seedlings were individually transplanted into eight cm^2 pots containing Fafard #4 soil, and received six beads of 13:13:13 N-P-K Nutricote™ fertilizer.

HP donors: *Helianthus exilis* seeds and *Stachys albens* plants were purchased from native plant nurseries (www.diggingdog.com, www.hedgerowfarms.com, respectively) and *M. nudatus* seeds were obtained from the wild by Paul Aigner (McLaughlin Natural Reserve, UC Davis). All plants were grown to flowering in the greenhouse at the University of Pittsburgh.

2.2.3 Pollination treatments

Pollen load composition: To test the effects of HP on components of female reproductive success, we created eight different pollen mixes with *M. guttatus* and the three HP donors as follows: *M. guttatus* plus one HP species (3 mixes), *M. guttatus* plus two species (3 mixes), *M. guttatus* plus three species (1 mix) and *M. guttatus* alone (control). We prepared pollen mixes prior to pollination using a ‘recipe’ based on the mean number of pollen grains/anther for each species. We created the mixes with the desired donor proportions keeping the total heterospecific load constant across all treatments (~ 200 grains). Thus, our heterospecific donor diversity was not confounded with total heterospecific load size. Pollen mixes were made using pollen from recently open flowers one or two days in advance.

CP load size: To assess the role of CP loss and its interaction with HP deposition on female reproductive success, we simulated conspecific loss by creating two CP load sizes based on a high (2:1) and low (1:1) pollen-ovule ratio (for ~ 400 ovules per *M. guttatus* ovary), so the final low (~ 700 total pollen grains) and high (~ 1200 total pollen grains) treatments consisted of ~72 and 84% of CP and the fully-crossed experiment had 16 pollen mixes. We confirmed the effectiveness of our pollinations on 5 randomly selected stigmas per treatment. We achieved the

two conspecific load sizes (mean \pm SD; high: 1047 ± 399 and low: 490 ± 240 *M. guttatus* pollen grains), and the constant total heterospecific load (224 ± 101).

Hand pollinations: Each of the 45 *M. guttatus* plants received all 16 pollination treatments in random order. High pollen loads were applied with a paint brush and low loads with a tooth pick. We collected styles one day after hand pollination and prior to autonomous self-pollination.

2.2.4 Components of female reproductive success

Stigma reopening: To determine whether the rate of ovule fertilization was affected by pollen load size or composition, we recorded whether the stigma was open or closed 24hrs after hand pollination, and collected and fixed styles in 70% ethanol.

Seed production: The number of fertile (large and swollen) and aborted (small and shriveled) seeds per ripe fruit was counted with the aid of a dissecting microscope for 37 plants ($N = 592$ fruits). Size (i.e., corolla length x width) of each flower was measured (± 0.1 mm) for use as a covariate.

Pollen tubes: To assess whether HP deposition leads to fewer pollen tubes penetrating ovules we scored callose plugs at the base of the style for a subset of the plants ($N = 7$). Callose plug number is a good proxy for pollen tube number in *M. guttatus* ($r = 0.78$ $P = 0.001$; C. Kohler & G. Arceo-Gómez, unpublished) and other species (Weller & Ornduff, 1989; Snow & Spira, 1991). We prepared styles following Dafni (1992) and counted callose plugs in a 0.21mm^2 basal area of the style with the aid of a fluorescence microscope. We scored callose plugs for *M.*

guttatus alone and for each heterospecific single donor in the low treatment so that we could assess whether interference was species specific.

2.2.5 Data analyses

Stigma reopening: In order to evaluate the effects of HP deposition on the probability of stigma reopening we used a linear model for categorical data (proc catmod; SAS, 2010) with pollen load composition, CP load size and their interaction as fixed factors. We scored stigmas as ‘0’ (closed) or ‘1’ (open). We performed *A-priori* linear contrasts to test for the effect of increasing HP diversity. We compared the control i.e., *M. guttatus* alone (hereafter, C), to the mean of *M. guttatus* plus one species (hereafter, group C+1), the mean of *M. guttatus* plus two other species (hereafter, group C+2) and the mean of *M. guttatus* plus all three species (hereafter, group C+3). In addition, we also performed linear contrasts to compare each single-species combination within the C+1 group (i.e., *Mimulus guttatus* +*Helianthus*, +*M. nudatus*, +*Stachys*, hereafter, +H, +N, +S) to *M. guttatus* (G) alone to test for a species-specific effect on the proportion of stigmas that reopen.

Seed production: To evaluate the effects of HP deposition on the number of fertile or aborted seeds we performed ANCOVAs (proc glimmix; SAS, 2010). We used a mixed model where plant was treated as a random factor, pollen load composition, conspecific load size and load composition*conspecific load size interaction were fixed factors and flower size was used as a covariate to account for differences in ovule number among flowers. Planned linear contrasts were made as described above to test for an effect of increasing diversity in the HP load on fertile seed number and to test for species-specific effects on fertile and aborted seed numbers.

We also performed contrasts to test for additive, synergistic or antagonistic interactions of two-species loads on fertile seed number. We compared the mean of the single-species effects to their combined effects (e.g., mean of +N and +H versus the +NH mix). A significant difference in the combined relative to the mean of the individual effects would suggest a deviation from additive effects (see Fig. 1a). Finally, to test for a “selection effect” (Loreau & Hector 2001) we compared the effect of the two-species mix to the strongest of the single-species effects, when the test of nonadditivity implied a synergistic effect.

Pollen tubes: To test for the interference mechanism of HP we performed an ANCOVA (proc glimmix; SAS, 2010) on the number of callose plugs at the bottom of the style. In this model, plant was a random factor, pollen load composition was a fixed factor and flower size was a covariate. Planned linear contrasts were performed to test for differences between each combination within group C+1 and the control (G). All continuous variables were normally distributed.

2.3 RESULTS

2.3.1 Overall pollen load composition and conspecific load size effects

Pollen load composition and conspecific load size significantly affected the probability of stigma reopening and fertile seed production (Table 1a). Stigmas had a higher probability of reopening after deposition of low (mean \pm SE; 0.26 ± 0.02) compared to high (0.17 ± 0.01) CP loads. Flowers produced more fertile seeds when receiving the high (least square mean \pm SE; $322 \pm$

9.39) versus the low (237 ± 8.39) CP load treatment. There was no interaction between load size and composition for both response variables ($P > 0.05$) so the interaction term was removed from the models.

2.3.2 Diversity and identity effects on stigma reopening and fertile seed production

The probability of stigma reopening varied with the number and identity of heterospecific donors (Table 2). But *A-priori* linear contrasts revealed stigmas only reopened significantly more often after C+2 relative to the control (Table 1b, 2). Single-species comparisons (G versus those within C+1) revealed that only *H. exilis* had a significant positive effect on stigma reopening (Table 1c, 2).

Significantly more fertile seeds were produced following pollination with pure *M. guttatus* pollen (C) than any of the other groups (C+1, C+2 and C+3; Table 1b, 2). Moreover, *A-priori* linear contrasts revealed flowers in the C+2 treatment produced significantly fewer seeds than those in C+1, but additional reduction was not seen in C+3 (Table 1b, 2). All species reduced fertile seed production, but differences in magnitude were evident: *H. exilis* had the strongest effect, *M. nudatus* an intermediate effect and *S. albens* the weakest effect (Table 1c, 2).

2.3.3 Multi-species interactions on fertile seed production

The outcomes of mixtures of HP on fertile seed production were diverse. The means individual effects of *H. exilis* and *M. nudatus* as well as that of *H. exilis* and *S. albens* were equivalent to their effects in mixture (mean of +H, +N vs. +HN; $F = 1.36$, $df = 1$, $P = 0.24$; mean of +H, +S vs.

+HS; $F = 2.37$, $df = 1$, $P = 0.12$) suggesting the absence of an interaction for these pairs (Fig. 2a,c). However, the mean of the individual effects of *S. albens* and *M. nudatus* was significantly weaker than their mixture ($F = 8.43$, $df = 1$, $P = 0.003$) (Fig. 2b). Moreover, this combined effect was significantly greater than the effect of strongest single-species effect (i.e., *M. nudatus*; $F = 4.03$, $df = 1$, $P = 0.045$), rejecting the hypothesis of a ‘selection effect’ (Fig. 1b-5). Taken together these results suggest a synergistic interaction between *S. albens* and *M. nudatus* on *M. guttatus* seed production.

2.3.4 Mechanisms: seed abortion and pollen tube growth interference

Pollen load composition ($F_{7, 534} = 37.69$, $P < 0.0001$; Table 3) but not conspecific load size ($F_{1, 534} = 1.94$, $P = 0.16$) nor their interaction ($F_{7, 534} = 1.24$, $P = 0.27$), significantly affected aborted seed number. Of the single HP donors in the C+1 group, only the presence of *M. nudatus* significantly increased the number of aborted seeds compared to G ($F = 117.5$, $df = 1$, $P < 0.0001$; Table 3). Moreover, the number of aborted seeds increased in direct proportion to the representation of *M. nudatus* in the HP load (data not shown).

Composition significantly affected the number callose plugs, and thus pollen tubes, at the base of the style ($F_{3, 17} = 3.48$, $P = 0.03$). Although all heterospecific treatments tended to reduce callose plugs, only the presence of *H. exilis* pollen significantly reduced (>50%) callose plugs number relative to *M. guttatus* alone ($F = 8.96$, $df = 1$, $P = 0.008$; Table 3).

2.4 DISCUSSION

HP deposition significantly reduced *M. guttatus* female reproductive success. The effect was independent of the CP load size, but its magnitude depended on the diversity and identity of the HP contributors. The underlying mechanisms were species-specific and responses to multi-species loads were suggestive of post-pollination interactions between HP donors. We discuss these results and their ecological and evolutionary implications in natural communities below.

2.4.1 What are the effects of diversity in the heterospecific load?

Although the negative effects of HP increased with increasing donor diversity (Table 2), the magnitude of the response to mixed loads depended on the species involved (i.e., when combined effects were compared to single-species effects) potentially reflecting post-pollination interactions among donors. For example, when *S. albens* was combined with *M. nudatus* a greater than average effect was observed (Fig. 2b) and this effect was stronger than the effect of *M. nudatus* alone suggesting the absence of a “selection effect” (Loreau & Hector 2001), and indicative of a synergistic effect (Fig. 1a-3). In contrast, the detrimental effects of the combinations of *H. exilis*+*N. nudatus* and *H. exilis*+*S. albens* were consistent with additive linear effects (Fig. 2a,c; Fig. 1a- 2). Interestingly, the effects of C+3 were not worse than C+2, perhaps reflecting the existence of a ‘lottery’ effect. Our results combined with others (reviewed in Morales & Traveset, 2008) support the notion that HP effects can be species-specific but extend these by demonstrating that they can also vary when the number of donors increases beyond one (also see Flanagan *et al.*, 2010), suggesting that the potential outcomes from natural

‘communities’ of HP grains on stigmas may be quite diverse. This occurrence opens the possibility for selection to act to reduce negative interactions between community members over the course of evolution (Sargent *et al.*, 2011).

2.4.2 What is the role of CP loss in *M. guttatus* reproductive success?

We found a dramatic decrease in seed production following a simulated 50% loss of CP, but no interaction with HP deposition suggesting that these factors affect female function independently. Such an interaction may only occur when HP physically displaces CP on stigmas. Although conspecific loss can be high in nature (e.g., >75%; Murcia & Feinsinger, 1996), the magnitude is not known for *M. guttatus*, and whether conspecific load size varies with species-richness is not well known for any species (but see Schuett & Vamosi, 2010).

2.4.3 What are the mechanisms involved in fertilization failure?

Our results suggest HP donors reduced *M. guttatus* fertile seed production by different mechanisms. Closely related *M. nudatus* (Gardner & Macnair, 2000) pollen germinated and fertilized *M. guttatus* ovules (i.e., lowering the proportion of stigmas that reopened), but because a strong postzygotic barrier exists (Gardner & Macnair, 2000) those seeds were aborted (Table 3). Similar usurpation of ovules was observed in other pairs of closely related species (Harder *et al.*, 1992; Fishman & Wyatt, 1999), suggesting a positive association between this mechanism and relatedness of the species involved. Distantly related *H. exilis* pollen, however, interfered with ovule fertilization at an earlier stage by preventing *M. guttatus* pollen tubes from reaching

the bottom of the style. *Helianthus exilis* pollen is large, spiny and germinated on *M. guttatus* stigmas so interference could be the result of physical interactions. Alternatively, pollen allelopathy has been documented several species in the family Asteraceae (Sukhada & Jayachandra, 1980; Murphy & Aarssen, 1995), so chemical interference by *H. exilis* is also possible. Even though *S. albens* did not significantly reduce callose plug number (Table 3) an interference mechanism that manifests more severely when in combination with other HP donors (Fig 2b) is hypothesized.

2.4.4 Ecological and evolutionary implications in natural communities

Our results join others that find the severity of the effects of interspecific pollen transfer on plant reproductive success depend more on the identity and relatedness of the species present (Schuett & Vamosi, 2010; Sargent *et al.*, 2011) than to species richness itself (but see Vamosi *et al.*, 2006; Alonso *et al.*, 2010). Recent studies have shown that the presence of closely related species in a community can enhance quantity aspects of pollination by facilitating visitation by specialist pollinators (Sargent *et al.*, 2011). However, our results and those of others (Harder *et al.*, 1992; Fishman & Wyatt, 1999; Schuett & Vamosi, 2010) show that close relatives can also have detrimental effects on quantity (e.g., reducing the number of viable seeds) and potentially quality (e.g., hybridization) aspects of pollen limitation. Nevertheless, our results also show that even distantly related taxa, by themselves or through interactions with others, can have detrimental effects and these too may underlie the patterns of increased pollen limitation with regional species richness (Vamosi *et al.*, 2006; Alonso *et al.*, 2010). We are not yet in the position to

differentiate between the impact of species versus phylogenetic diversity on plant reproductive success, so studies that consider their influence on both aspects (i.e., quantity and quality) of pollen limitation are needed. Furthermore, native heterospecific donors in stable communities with a long history of interactions may have a weaker effect on a focal species than those exerted by novel heterospecific donors in a community (i.e., invasive species; Schuett & Vamosi, 2010; Sargent *et al.*, 2011).

Table 4. GLM results for the proportion of open stigmas (χ^2 test) and number of fertile seeds (F test) (a). Planned linear contrasts to test for the effects of increasing donor diversity (b) and donor identity (c) on the proportion of open stigmas and the number of fertile seeds produced by *M. guttatus* following hand pollinations with different heterospecific pollen loads and conspecific load sizes. Flower size was used as a covariate for seed number. Significant effects are denoted as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ‘-’ factor absent from model.

| Contrast | <i>Stigma reopening</i> | | <i>Fertile seeds</i> | | |
|---|-------------------------|-----------|----------------------|---------|----------|
| | df | χ^2 | df | SS | F value |
| (a) Source of variation | | | | | |
| Plant | - | - | 36 | 2756102 | 4.77*** |
| Sp composition | 7 | 18.86** | 7 | 654897 | 5.83*** |
| Pollen load | 1 | 150.33*** | 1 | 1198720 | 74.72** |
| Flower size | - | - | 1 | 1479027 | 92.19*** |
| Error | 7 | | 534 | 8567102 | |
| (b) Composition: donor diversity | | | | | |
| C vs C+1 | 1 | 3.45 | 1 | 305517 | 19.04*** |
| C vs C+2 | 1 | 4.15** | 1 | 638784 | 39.82*** |
| C vs C+3 | 1 | 0.47 | 1 | 284183 | 17.71*** |
| C+1 vs C+2 | 1 | 0.06 | 1 | 125827 | 7.84** |
| C+2 vs C+3 | 1 | 1.36 | 1 | 23478 | 1.46 |

| (c) Composition: donor identity | | | | | |
|---------------------------------|---|--------|---|--------|----------|
| G vs H | 1 | 8.69** | 1 | 104188 | 6.49** |
| G vs S | 1 | 1.99 | 1 | 64598 | 4.03* |
| G vs N | 1 | 0.03 | 1 | 201399 | 12.55*** |

Table 5. Mean proportion of open stigmas and least square mean (\pm SE) number of fertile seeds produced after hand pollinations with different HP treatments. Comparisons were made between pollinations with conspecific pollen alone (*M. guttatus*; G) and single-species within group C+1(*M. guttatus* +*H. exilis*; +H, *S. albens*; +S and *M. nudatus*; +N) and between the control (C) and C+1, C+2 and C+3 mixes. Means that do not share a letter are significantly different at $P < 0.05$. Differences between G and single-species within C+1 are indicated by upper case letters, whereas differences between C, C+1, C+2 and C+3 by lower case letters.

| Response variable | G(C) | C+1 | | | C+1 | C+2 | C+3 |
|----------------------------|----------------------------|-----------------------|-------------------------|--------------------------|-------------------------|-------------------------|--------------------------|
| | | +H | +S | +N | | | |
| Proportion of open stigmas | 0.17 ^{a(A)} | 0.37 ^B | 0.26 ^A | 0.19 ^A | 0.26 ^{ab} | 0.27 ^b | 0.22 ^{ab} |
| Number of fertile seeds | 360 (20.2) ^{a(A)} | 262 (18) ^C | 307 (18.9) ^B | 286 (16.7) ^{BC} | 285 (10.4) ^b | 251 (10.6) ^c | 272 (15.9) ^{bc} |

Table 6. Least square mean (\pm SE) number of aborted seeds and callose plugs at the bottom of the style after hand pollinations with different HP treatments. Comparisons were made between pollinations with *M. guttatus* (G) and single-species combinations within group C+1: *M. guttatus* +*H. exilis* (+H), *S. albens* (+S) and *M. nudatus* (+N). Different letters denote significant differences at $P < 0.05$.

| Response variable | G | +H | +S | +N |
|-------------------------|-------------------------|-------------------------|------------------------|-------------------------|
| Number of aborted seeds | 23.9 (3.5) ^a | 22.3 (4.9) ^a | 19.2(3.2) ^a | 85.4 (6.3) ^b |

| | | | | |
|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Number of callose plugs | 336 (62.7) ^a | 153 (33.8) ^b | 260 (59.4) ^a | 308 (48.1) ^a |
|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|

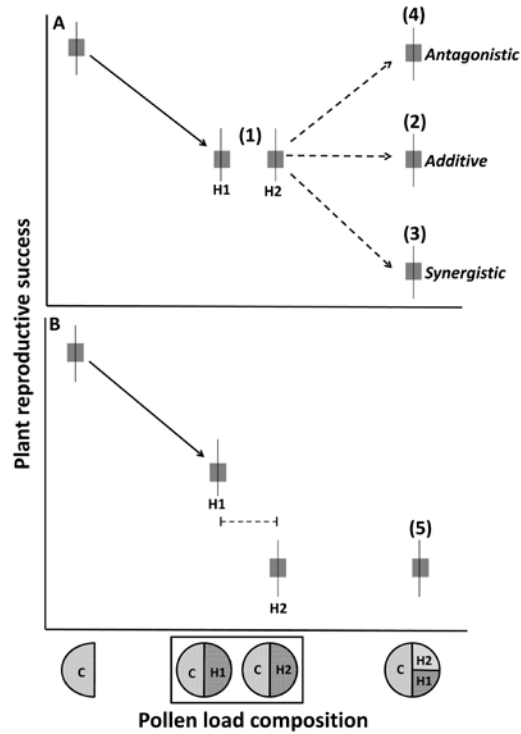


Figure 2. Conceptual models for multi-species effects of heterospecific pollen deposition on plant reproductive success: a) effects of multispecies interactions and b) ‘selection effects’. One conspecific donor (C), and two heterospecific donors (H1, H2) are represented and their individual contributions to the total pollen load is reflected in the pie diagrams on the X-axis (total heterospecific load size is held constant and only the diversity is varied). a) Addition of a single heterospecific donor to a pure conspecific mix results in a decrease in plant reproductive success (1). Increasing the diversity of heterospecific donors (but keeping conspecific and heterospecific load sizes constant) results in a similar effect of reproductive success (2), indicating H1 and H2 act additively, or results in a decrease in plant reproductive success that cannot be explained by the simple average of their individual effects (3)

indicating that H1 and H2 have synergistic effects, or results in an increase in reproductive success (4), suggesting that H1 and H2 are acting antagonistically, perhaps because H1 inhibits H2 and when combined with H2, H1 has an indirect positive effect on reproductive success. b) Addition of a single heterospecific donor to a pure conspecific mix results in non-additive effects on plant reproductive success because one donor has a strong effect (5), representing a 'selection effect' when in combination, i.e., one donor dominates the response in combination. In both cases the average of the individual effects (H1 and H2) is expected to be different from the mean of the two species combined (dashed lines), but, the latter is not different from the strongest (H2; 5) of the individual effects.

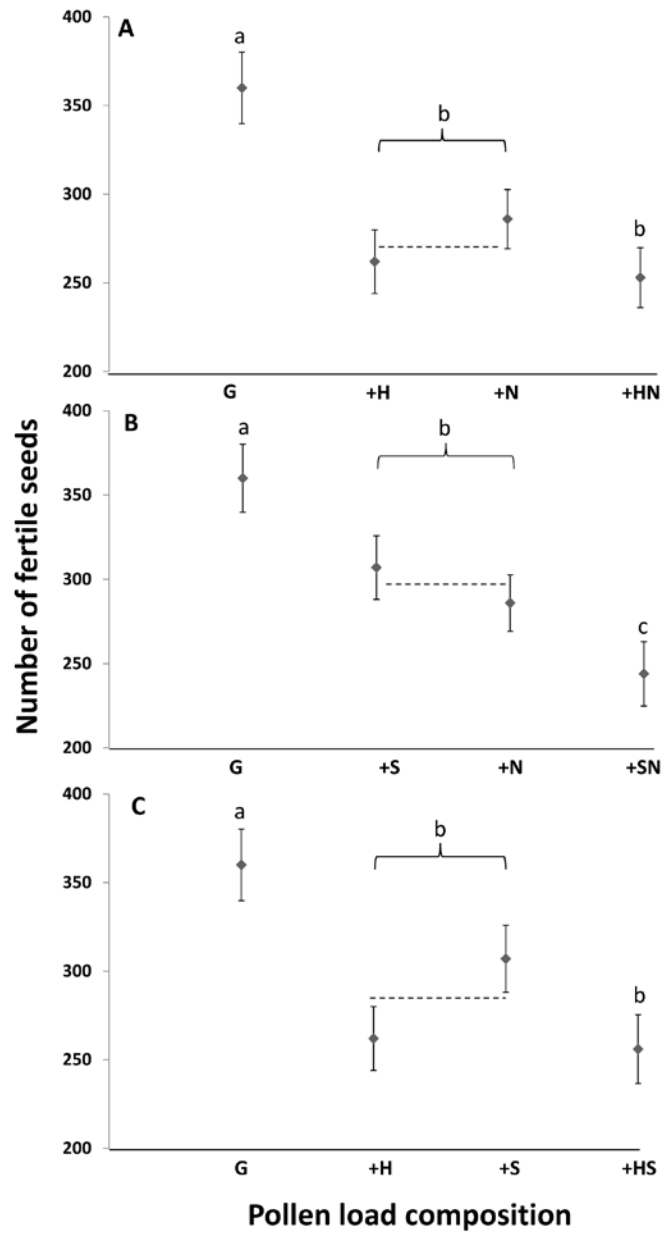


Figure 3. Differences in fertile seed production (least square means \pm SE) between the control (*M. guttatus*; G), the average (dotted line) of single-species effects and the two-species loads for (a) +H and +N versus +HN (b) +S and +N versus +SN, and (c) +H and +S versus +HS (*H. exilis*; +H, *M. nudatus*; +N, *S. albens*; +S). Different letters denote significant differences at $P < 0.05$.

3.0 CO-FLOWERING COMMUNITY CONTEXT INFLUENCES FEMALE FITNESS AND ALTERS THE ADAPTIVE VALUE OF FLOWER LONGEVITY IN *MIMULUS* *GUTTATUS*

Arceo-Gómez G. and Tia-Lynn Ashman. 2014. *The American Naturalist*. 183:E50-E63

3.1 INTRODUCTION

Phenotypic traits are often shaped by interactions among multiple species within a community as opposed to selection imposed by a single community member (Iwao and Rausher 1997; Strauss and Irwin 2004; Strauss et al. 2005). Thus, the selective regime experienced by a particular species may change depending on the community in which it occurs (i.e., diffuse selection; Janzen 1980; Gould 1988; Iwao and Rausher 1997; Stinchcombe and Rausher 2001; Strauss et al. 2005). Even though this process has received considerable theoretical attention, few studies have experimentally evaluated changes in the trait-fitness relationship as a result of changes in community composition (Strauss et al. 2005; Johnson and Stinchcombe 2007). This is surprising given the importance of understanding how community context can shape microevolutionary processes within populations (Thompson 1999; Strauss and Irwin 2004), as well as uncovering the underlying mechanisms. A major goal for ecologists and evolutionary biologists is to

incorporate the complexity of multispecies interactions into our understanding of the ecological and evolutionary dynamics of natural populations. The complexity of natural systems cannot be fully understood by only examining subsets of natural communities (e.g., species pairs) and there is a need for studies that evaluate the effects of multi-species interactions (Inouye and Stinchcombe 2001; Strauss et al. 2005).

In particular, studies of the interactions between plants and their pollinators have focused on pair-wise interactions (Geber and Moeller 2006; Mitchell et al. 2009; Sargent et al. 2011) even though plant species commonly occur within complex plant-pollinator communities (Olesen and Jordano 2002; Bascompte et al. 2003). Therefore, while we have a good understanding of how floral traits influence pollinator attraction, pollen receipt and seed production for many species (e.g., Jones and Reithel 2001; Armbruster et al. 2005; Nattero et al. 2010; Leonard et al. 2011), or how species pairs affect each other's success (e.g., Brown et al. 2002; Feldman et al. 2004; Bell et al. 2005; Flanagan et al. 2009), our understanding of how attributes of the whole co-flowering community affect the pollinator visitation and reproduction of plants or whether changes in these attributes lead to divergent selective pressures is limited (but see Vamosi et al. 2006; Schuett and Vamosi 2010; Sargent et al. 2011). Variation in co-flowering communities can result from differences in species composition, species number, or abundance per species (Leps et al. 2001; Loreau and Hector 2001). Given the complexity of diverse, naturally occurring, co-flowering communities, an important first step is to evaluate whether variation in species richness has an effect on pollination success. Once this has been established, then one can ask whether effects are due to overall richness or individual species effects (i.e., changes in composition or abundances).

Co-flowering taxa can influence reproduction of a focal species at both the pre- and post-pollination stages. At the pre-pollination phase, several possibilities exist. First, the presence of co-flowering species can increase per capita visitation rate by increasing the floral display and/or diversity of floral rewards in a patch (Potts et al. 2003; Moeller 2004; Ghazoul 2006; Lazaro et al. 2009). Such facilitative interactions could result in greater conspecific pollen (hereafter, CP) receipt and seed production by individuals inhabiting diverse co-flowering communities relative to those in species-poor communities (Ghazoul 2006). Second, co-flowering species may compete for pollinators, and thus experience reduced per-capita visitation, CP receipt, and seed production (reviewed in Mitchell et al. 2009) in high diversity patches/communities compared to those in less diverse communities. Third, while some pollinators may discriminate among co-flowering species, others may move freely between flowers of different species. This lack of pollinator constancy could lead to CP loss and/or heterospecific pollen (hereafter, HP) receipt (reviewed in Morales and Traveset 2008; Ashman and Arceo-Gómez 2013). Thus, for plants with generalized pollination systems (Olesen and Jordano 2002; Bascompte et al. 2003), the amount and diversity of HP received is predicted to be higher when they exist in species-rich communities than in species-poor ones. Yet, it is not clear how plant traits or community attributes influence HP receipt in natural communities (but see McLernon 1996; Montgomery and Rathcke 2012).

Co-flowering plants may also influence reproduction via post-pollination processes, and these may reinforce negative pre-pollination interactions (competition) or reduce the benefits of positive pre-pollination ones (facilitation). Specifically, HP receipt can interfere with CP adhesion, germination, pollen tube growth and/or ovule fertilization and seed production (Morales and Traveset 2008; Arceo-Gómez and Ashman 2011). A high proportion or a high

diversity of HP can exacerbate interference, and in some cases, accentuate reproductive failure (Arceo-Gómez and Ashman 2011). Thus, plants growing in highly diverse co-flowering communities may face challenges to reproduction at both the pre- and post-pollination phases (Vamosi et al. 2006). Yet, no study has compared these key components of reproduction for a focal species under naturally varying co-flowering species richness (but see Schuett and Vamosi 2010).

Moreover, if the pollination environment is more challenging in diverse relative to depauperate co-flowering communities (Vamosi et al. 2006), then selection to maximize CP receipt and reduce HP receipt should be stronger in the former than in the latter. Thus, we can envision three evolutionary trajectories for plant traits in diverse communities: (1) Plant traits could evolve in a manner that reduces competition for, and sharing of, pollinators, i.e., via divergence in flowering time, in the placement of pollen on pollinator's bodies (e.g., Mosquin 1971; Waser 1978; Aizen and Vazquez 2006) or in the primary pollinator (i.e., specialization, Fleming and Holland 1998; Sargent and Otto 2006). (2) Plant traits could evolve in a way that reduces dependence on pollinators and increases reproductive assurance via autonomous selfing (Dole 1992; Fishman and Wyatt 1999; Morgan and Wilson 2005; Grossenbacher and Whittall 2011). For instance, reduction in stigma-anther distance (e.g., Fenster and Ritland 1994; Fishman and Willis 2008), loss of protandry (e.g., Holtsford and Ellstrand 1992), smaller flowers (e.g., Elle and Carney 2003), and shorter flower lifetimes (Primack 1985) all lead to greater autonomous selfing. (3) Plant traits could evolve to increase competitive ability via enhanced attractiveness to pollinators (Caruso 2000; Totland 2001). Large and/or long-lived floral displays receive more pollinator visits and lead to greater CP receipt (e.g., Johnston 1991; Conner et al. 1996). Such displays can be achieved by production of more or larger flowers (e.g., Totland

2001) and/or increased individual flower lifetimes (Ashman and Schoen 1994; Marshall et al. 2010). Thus, for a wild species living in a gradient of community diversity, we would expect that 1) populations will be differentiated in one or more of these key traits, such that trait means vary with diversity (e.g., Holtsford and Ellstrand 1992; Bradshaw et al. 1998; Sandring and Agren 2009), and/or 2) fitness value of one of these key traits depends on the co-flowering community (i.e., diffuse selection; Iwao and Rausher 1997; Stinchcombe and Rausher 2001; Strauss et al. 2005).

Mimulus guttatus (Phrymaceae), the yellow monkey flower, is a good species to evaluate how variation in co-flowering community diversity affects pollination, reproductive success and floral trait evolution because it is a self-compatible, hermaphroditic, insect-pollinated species that shows variation in traits thought to be important in variable pollination environments, i.e., corolla size, stigma-anther distance (Fenster and Ritland 1994; Kelly and Arathi 2003), flower longevity (Arathi et al. 2002) and autonomous self-pollination (Dole 1990; Arathi and Kelly 2004). Its flowers also have the ability to respond to variation in pollination via a stigma that closes after being touched but reopens when ovule fertilization is low (Fetscher and Kohn 1999; Arceo-Gómez and Ashman 2011). Moreover, *M. guttatus* grows within the highly variable communities of serpentine seeps (Freestone and Harrison 2006; Freestone and Inouye 2006). Environmental heterogeneity and spatial discontinuity lead to high variation among seeps in overall species richness (Freestone and Inouye 2006) providing replicate communities within which to answer the following questions: 1) Is there variation in co-flowering species richness among seep communities? 2) Does pollinator visitation rate to *M. guttatus* flowers differ between high and low diversity co-flowering communities? 3) Is *M. guttatus* CP receipt lower and HP receipt higher in high compared to low diversity co-flowering communities? 4) Does variation in

M. guttatus flower size, longevity and/or stigma-anther distance correlate with seep species richness? 5) Does the adaptive value of the floral trait most strongly correlated with species richness (flower longevity) differ between high versus low diversity co-flowering communities?

3.2 MATERIALS AND METHODS

3.2.1 Co-flowering community diversity

We identified 23 seeps with *M. guttatus* at the McLaughlin Natural Reserve in California, USA (Table A1, available online). The seeps are discrete, separated by 2.5 ± 1.6 km (range: 0.4-5.7) on average, and surrounded by a matrix of grassland species. They provide independent replication of co-flowering community context because they are spatially independent from each other (Harrison et al. 2000; Freestone and Harrison 2006). While a positive association between total plant diversity and soil moisture/resource availability has been found in seeps at this location (Freestone and Harrison 2006), the community richness of the co-flowering insect-pollinated herbaceous species (between June –July) we studied did not covary in this manner. High diversity sites tended to be more dry and less productive and *M. guttatus* at these sites were 15% shorter ($P = 0.001$), but did not differ from species-poor sites in other features that often reflect resource status (e.g., flower size, floral display and/or flowering time [$P > 0.05$ in all cases; Arceo-Gómez unpublished data]). However, we cannot rule out the possibility of other confounds of diversity.

To determine co-flowering species richness at each seep we recorded every insect-pollinated herbaceous plant in flower during *M. guttatus*'s flowering season (June/July in 2010). In addition, we recorded *M. guttatus* flower density in five 1 x 1m² plots/site in a subset of seeps ($n = 12$). We selected four sites that were separated by at least 1km for further study, two with lower and two with higher co-flowering diversity (Table 1, sites 1-4 in A1).

3.2.2 Pollinator visitation

Given that *M. guttatus* is visited by a variety of pollinators (small, medium and large bees, bumblebees, honey bees, lepidopterans, coleopterans, bee flies and other flies; Thorp et al. 1983; Macnair et al. 1989) that also visit at least 15 other species within the seeps (Meindl et al. unpublished manuscript) we evaluated whether visitation rate to *M. guttatus* differs for plants at high versus low diversity co-flowering communities. We recorded insect visitation at the four sites that varied in co-flowering species richness (Table 1). At peak flowering during the summer of 2011 we conducted pollinator observations between 0800 and 1400 h (when pollinators were most active; Arceo-Gómez pers. obs.) in three minute censuses at five randomly located 1 x 2m² plots/site. Censuses were conducted twice per plot/day for four days for a total of 8 h of observation across all sites. Within each plot, we recorded the number of *M. guttatus* flowers and visits they received. We estimated the average visitation rate (visits/flower/min) for each plot/day and constructed a model using repeated-measures ANOVA with site, day and their interaction as main factors. Plot was the repeated subject with an unstructured covariance matrix, which better fit the data (proc mixed; SAS 2010). Pre-planned contrasts were conducted to specifically test whether visitation rate differed between high and low diversity sites (see 'Pre-

planned contrasts' below). Visitation rates were arcsine (square root + 0.001) transformed prior to analysis to meet the assumption of normality.

3.2.3 Pollen receipt

To evaluate patterns of pollinator-mediated CP and HP receipt in high versus low diversity co-flowering communities we haphazardly selected 18-20 plants of approximately the same size at each of the four sites (Table 1), marked one bud per plant ($n = 77$) and recorded open flower display size on the same day in the summer of 2011. Because *M. guttatus* flowers are capable of delayed self-fertilization (Dole 1990; Arathi and Kelly 2004) we collected styles after one day of exposure to pollinators and thus, avoided autonomous self-pollen receipt. Stigmas were processed following Arceo-Gómez and Ashman (2011) and CP and HP were enumerated with the aid of a fluorescence microscope. We constructed a linear model using site as a random factor and flower display as a covariate (proc glm; SAS 2010). To determine whether amount of CP and/or proportion HP (HP/total pollen load) received varied with co-flowering diversity we used pre-planned contrasts (see 'Pre-planned contrasts' below). We analyzed proportion of HP on stigmas to take into account variation in HP relative to that in the CP load, as the combination can be most influential in determining seed production (e.g., Thomson et al. 1981). Conspecific pollen and proportion HP were square root and arcsine square root transformed respectively to meet the assumption of normality.

3.2.4 Co-variation between floral traits and community context

To assess whether flower size, longevity and/or stigma-anther distance were correlated with co-flowering species richness, we collected one seed from 15 plants in each of the 23 *M. guttatus* seeps (Table A1). In 2010, one seedling/plant/site was sown in 8-cm² pots containing Fafard #4 soil and grown under common conditions in the greenhouse (day/night: 14h/10h and 26/21°C) at the University of Pittsburgh. Plants were arranged in 15 spatial ‘blocks’ (each containing one plant/site) on greenhouse benches. We measured flower size (corolla length x width) and stigma-anther distance with digital calipers (± 0.1 mm) and scored flower longevity by recording the day of anthesis and senescence (wilted corolla) on the first flower of each plant. We tested for a correlation between population means of traits assessed under common conditions and *in-situ* co-flowering species richness of the source population, and for correlations among the floral traits. We also tested for a correlation between average conspecific flower density/m² and species richness or floral trait means for the subset of the sites ($n = 12$; proc corr; SAS 2010). We consider significant covariation of floral traits with co-flowering richness to suggest a pattern of genetic differentiation (in the broad sense) for these traits consistent with variation in an agent of selection (e.g., Stinchcombe et al. 2004).

3.2.5 Adaptive value of flower longevity

Because population-mean flower longevity was the only trait significantly correlated with co-flowering species richness (see results below) we focused our tests of adaptive value on this trait at the high and low diversity sites. Specifically, we conducted a reciprocal ‘transplant’

experiment with potted plants in the field in 2011 to evaluate 1) whether plants collected from high and low diversity sites were differentiated for flower longevity under field conditions (i.e., to verify greenhouse results in the field) and 2) whether the adaptive value of longevity (over the entire flower life span) depended on co-flowering community context. We randomly collected 32 *M. guttatus* seedlings from each of the two high and two low diversity sites (128 total), transplanted each seedling into a 8-cm² pot containing Fafard #4 soil, and grew them to flowering at a common location. To simulate natural conditions, but keep soil resources homogeneous across all plants (and ‘transplant’ sites), we fertilized seedlings with four beads of 13:13:13 N-P-K Nutricote™ and bottom-watered them for the duration of the experiment. Just before flowering we created 32 arrays of four evenly-sized plants, one from each source location. At each site we placed eight arrays (divided into two temporal cohorts: five ‘early’ and three ‘late’) during peak flowering. We scored conspecific flower density in a 1m radius circle around each array two times during flowering. On each plant we scored flower longevity of one flower. When its corolla wilted we collected its style, and later its fruit. We scored CP load (at the end of flower life span) and seed production following Arceo-Gómez and Ashman (2011). Plant death and herbivory reduced sample sizes slightly ($n = 125, 120, 116$ for flower longevity, CP load, and seed production respectively). For all response variables (i.e., flower longevity, CP load and seed production) we constructed a mixed model using population of origin, ‘transplant’ site and their interaction as random factors (proc glm; SAS 2010). We included cohort and conspecific density rather than array in the overall models to avoid over-specifying the models but to account for sources of pollination microsite variation. However, the results are the same when array is used instead (data not shown). To specifically test for differences between high and low diversity co-flowering communities as well as their interaction we used pre-planned contrasts (see ‘Pre-

planned contrasts' below). Conspecific pollen load (square root) and seed number (square root [seed number + 0.5]) were transformed to achieve normality. Finally, to isolate and confirm the role of flower longevity in *M. guttatus* on seed production from the contributions of flowering sites and sources, we reran the model described above with flower longevity as a covariate.

3.2.6 Pre-planned contrasts

We used pre-planned contrasts to evaluate the effects of high and low co-flowering community diversity on the pollination environment and to evaluate whether response to pollination environment depended on plant source in a manner indicative of an adaptive value to flower longevity. Pre-planned contrasts are a powerful means of asking focused questions and testing *a priori* predictions and/or patterns within interactions (i.e., 'interaction contrasts') (Rosnow and Rosenthal 1985; 1989; Wahlsten 1991; Rosnow and Rosenthal 1995; Abelson and Prentice 1997; Rosenthal et al. 2000; Marini 2003) and are recommended over omnibus tests across all levels of a factor (here, all sites which were unique in species richness/composition) or interactions (Rosnow and Rosenthal 1989; Wahlsten 1991; Myers and Well 1995; Rosenthal et al. 2000; see also Galen 1985; Molfsky and Fisher 1993; Juenger and Bergelson 1997; Negishi and Richardson 2003; Strauss and Murch 2004). In each pre-planned contrast a specific pair of means is compared using an *F*-test (1 *df*) constructed with the full MSE as the denominator of the *F*-ratio (i.e., using the full sample for estimating the error variation) and thus have high power (Rosnow and Rosenthal 1985; 1995; Abelson and Prentice 1997; Rosenthal et al. 2000). Because pre-planned contrasts are guided by strong conceptual reasons they can be performed instead of the more general omnibus *F*-tests of ANOVA and the significance of omnibus tests is

not a prerequisite for conducting the contrasts (Steele and Torrie 1980; Rosnow and Rosenthal 1989; Lentner and Bishop 1993; Rosnow and Rosenthal 1995; Abelson and Prentice 1997; Rosenthal et al. 2000; Marini 2003). Thus, we present the results of the full models and the pre-planned contrasts, but not omnibus tests for the following analyses. First, we conducted pre-planned contrasts to test for differences in pollinator visitation, CP receipt and proportion of HP deposited on stigmas between high and low diversity co-flowering communities (i.e., two low versus two high diversity sites; Table 1). We specifically wanted to know if pollinator visitation and CP receipt decreased and HP receipt increased at high compared to low diversity sites. Second, in our reciprocal transplant experiment, we not only conducted pre-planned contrasts between the two high versus the two low diversity sources (i.e., plant origin) and the two high versus the two low diversity flowering sites (i.e., pollination environment) but we also evaluated whether response to pollination environment depended on plant source (analogous to G x E) so we conducted pre-planned ‘interaction contrasts’ for all response variables. We were interested in determining whether flowers from high diversity sources have longer longevities and higher reproductive success at high but not low diversity sites, and whether this site-dependence was absent for flowers from less diverse sources. Such an outcome would suggest that the adaptive value of flower longevity was flowering context-specific and conferred only in high diversity communities. Back transformed means \pm SE are presented unless otherwise indicated (all data is available in the Dryad Digital Repository: doi:10.5061/dryad.653k7; Arceo- Gómez and Ashman 2013).

3.3 RESULTS

3.3.1 Co-flowering community diversity

The co-flowering, insect-pollinated, herbaceous species richness of 23 seeps varied from one to 21 (Fig. 1; Table A1). Naturally occurring *M. guttatus* at low diversity sites coexist with half as many species and these represent a subset of the dominant species present at high diversity sites (Table A2, available online). Thus, the high diversity sites contain additional less frequent species. *Mimulus guttatus* density also varied across sites (mean \pm SE: 67 ± 12.2 , range: 35-178 flowers/m², $n = 12$) but species richness was not correlated with *M. guttatus* floral density ($r = -0.29$, $P = 0.3$). Species richness of the four study sites (Table 1) reflected the mode and high extreme of this spectrum.

3.3.2 Pollinator visitation

We recorded a total of 284 insect visits across all sites. Ninety percent of visitors were bees, primarily bumble bees and medium size bees (e.g., *Lassioglossum* sp.). Beetles (6%) and lepidopterans (2%) were the next most frequent visitors. Only 2% of visits were made by insects not typically considered to be pollinators (e.g., orthopterans). The pre-planned linear contrast revealed that visitation rate was 60% higher at low relative to high diversity sites ($F_{1, 17} = 5.52$, $P = 0.03$; Fig. 2A). The overall model showed that neither day ($F_{3, 17} = 0.67$, $P = 0.5$) nor the site-day interaction had an effect ($F_{7, 17} = 1.31$, $P = 0.3$).

3.3.3 Pollen receipt

The overall models for amount of CP ($F_{4, 72} = 2.45$, $P = 0.05$) and HP ($F_{4, 72} = 4.13$, $P < 0.01$) received by *M. guttatus* in one day were significant. However, pre-planned contrasts did not detect differences in the number of CP grains received at high versus low diversity sites ($F_{1, 72} = 0.52$, $P = 0.4$; Fig. 2B). In contrast, the proportion of total pollen that was HP was four times higher (0.16 ± 0.04 vs. 0.04 ± 0.01) for flowers at high compared to low diversity sites ($F_{1, 72} = 13.02$, $P < 0.001$; Fig. 2C). Floral display size did not affect CP ($F_{1, 72} = 1.49$, $P = 0.2$) or HP receipt ($F_{1, 72} = 1.54$, $P = 0.2$).

3.3.4 Co-variation between floral traits and community context

When grown under common conditions, *M. guttatus* from the 23 populations (Table A1) varied in flower size (mean \pm SE: 424.3 ± 6.7 , range: 82.1 - 743.4 mm²), longevity (5.3 ± 0.08 , range: 1 - 12 days) and in stigma-anther distance (2.7 ± 0.05 , range: 0 - 7.5 mm). In addition, we found a positive correlation between population mean flower longevity and co-flowering species richness ($r = 0.52$, $P = 0.01$; Fig. 3). However, there was no significant correlation of species richness with flower size ($r = -0.03$, $P > 0.05$) or with stigma-anther distance ($r = -0.04$, $P > 0.05$), nor between the latter two traits and flower longevity ($r = -0.08$, $r = 0.07$, $P > 0.05$ respectively, $n = 23$). None of the floral traits was correlated with *M. guttatus* floral density ($P > 0.2$ in all cases).

3.3.5 Adaptive value of flower longevity

The overall model for flower longevity was significant (Table 2A) and the pre-planned contrast revealed that *M. guttatus* from populations with high co-flowering diversity had significantly longer flower longevity in the field (3 ± 0.11 days) than plants from less diverse sources (2.5 ± 0.12 ; Table 2B). This result is consistent with population genetic differentiation under common greenhouse conditions (see results above). Furthermore, potted plants had similar flower longevity when exposed to high versus low diversity pollination environments, i.e., flowering sites (2.9 ± 0.12 vs. 2.6 ± 0.12 days; Table 2B). However, interaction contrasts (Table 2B) revealed that at high diversity flowering sites *M. guttatus* from high diversity sources had flowers that lived 17% longer (3.1 ± 0.14 vs. 2.7 ± 0.18) than those from less diverse sources, but they did not differ in flower lifetime (2.6 ± 0.18 vs. 2.4 ± 0.15) when exposed to pollination environments of the low diversity sites (Fig. 4A). Other aspects of the pollination context also had significant effects on flower longevity under field conditions (flower longevity decreased with increasing conspecific density and increased later in the flowering season [cohort]; Table 2C).

The overall model for the amount of CP on *M. guttatus* stigmas at the end of flower life was significant (Table 2A), but pre-planned contrasts did not detect differences in CP receipt between high (201.6 ± 23.9) and low (210.8 ± 20.8) diversity sites, nor between high (217.5 ± 15) and low (195.4 ± 23.6) diversity sources (Table 2B). However, interaction contrasts revealed that when flowering at high diversity sites, *M. guttatus* from high diversity sources received 38% more CP than those from less diverse sources, but the same was not true at low diversity sites—here, no significant difference between high and low diversity sources was found (Table 2B; Fig

4B). Conspecific flower density had no effect on the amount of CP received, although cohort did (Table 2C).

The model for the number of seeds per fruit was significant (Table 2A) and *M. guttatus* from high diversity sources produced 41% more seeds/fruit (57.7 ± 7.7) than those from low diversity sources (33.6 ± 6.0 ; Table 2B). However, seed/fruit did not differ between *M. guttatus* flowering at high (50.4 ± 8.8) versus low (36 ± 5.0) diversity sites (Table 2B). Furthermore, interaction contrasts revealed that when flowering at high diversity sites, *M. guttatus* from high diversity sources produce 59% more seeds/fruit (81.3 ± 5 vs. 40.9 ± 8.8) than those from low diversity sources, but these did not differ (33.6 ± 11.2 vs. 32.4 ± 7.2) when flowering at low diversity sites (Table 2B; Fig. 4C). Similar to CP receipt, conspecific flower density had no effect on the number of seeds produced but cohort did (Table 2C).

Finally, flower longevity had a significant effect on seed production when it was added as a covariate in the ANOVA model ($F_{1, 98} = 5.61$, $P = 0.01$) suggesting that flower longevity directly explains variation in *M. guttatus* reproductive success, at least partially, because the main effect of source was also still significant ($F_{3, 98} = 4.28$, $P = 0.03$).

3.4 DISCUSSION

The importance of multispecies interactions in driving evolution has been debated over the past few decades (Strauss and Irwin 2004; Johnson and Stinchcombe 2007). In spite of the considerable theoretical attention given to the concept of diffuse selection (Janzen 1980; Iwao and Rausher 1997; Stinchcombe and Rausher 2001; Strauss et al. 2005) only a few studies have

measured both traits and fitness in communities of varying composition, and could thereby assess how community context alters the selective regime of a focal species (Strauss et al. 2005; Johnson and Stinchcombe 2007). Here, we confirm the power of reciprocal transplant experiments for studies of diffuse selection (Johnson and Stinchcombe 2007) and demonstrate that the community context changes the relationship between flower longevity and fitness. This result lends credence to the idea that diffuse selection can be mediated by the co-flowering community. Other studies have also shown evidence of diffuse selection, but mostly by manipulating the presence or absence of species in factorial designs (e.g., Pilson 1996; Juenger and Bergelson 1998; Stinchcombe and Rausher 2001). Thus, these studies have only manipulated a few members of the community and therefore could not account for the overall dynamics of species within a community. To our knowledge, this is one of the first studies to show evidence of community-mediated selection, as well as, to uncover the potential underlying mechanisms responsible for the observed changes in fitness. We discuss these mechanisms as well as the evidence for community-mediated selection in more detail below.

3.4.1 Co-flowering diversity effects on the pollination environment

Visitation rate to *M. guttatus* flowers was reduced by more than half in high compared to low diversity sites, consistent with the prediction of stronger competition, rather than facilitation, for pollinators with increasing co-flowering species richness. Thus, our results support the hypothesis that the increase in pollinator recruitment to an area, as a result of increased floral resources, may be offset by a decrease in per capita visitation rate (Schuett and Vamosi 2010). Pollinator sharing among co-flowering species at the studied sites is high. In particular, *M.*

guttatus at high diversity sites shares pollinators with more than 15 other plant species (Meindl et al. unpublished manuscript), which increases the potential for pollinator competition. Pollinators typically adjust their foraging to the floral resources available (Pyke 1978). Thus, at high diversity sites other equally and/or more rewarding species must be present that draw shared pollinators away from *M. guttatus* and decrease per capita visitation compared to low diversity sites. In fact, recent results show that pollen quantity is more limiting for *M. guttatus* reproduction at high compared to low diversity sites (Arceo-Gómez and Ashman unpublished manuscript). It is worth noting, however, that differences in overall pollinator abundance between these communities could also influence pollinator visitation rates.

The proportion of HP received was four times higher for plants flowering at high diversity sites compared to those at low diversity sites, a result consistent with the prediction of high interspecific pollen transfer in diverse co-flowering communities. Interspecific pollen transfer can reinforce competition among co-flowering species by reducing pollen quality (Bell et al. 2005; Mitchell et al. 2009), as well as by reducing conspecific ovule fertilization, even in small amounts (e.g., Thomson et al. 1981). Our recent work (Arceo-Gómez and Ashman 2011) showed that multiple species of HP can act synergistically to further reduce seed production in *M. guttatus* compared to when these pollen species occur alone on a stigma. Thus, at high diversity sites the HP received may be even more detrimental, as it is more diverse than at low diversity sites (number of species per stigma: 2.6 ± 0.2 vs. 1.6 ± 0.1 ; Arceo-Gómez and Ashman, unpublished data). So, even though we did not find an effect of co-flowering diversity on pollinator-mediated CP receipt (i.e., after one day of open pollination), the high proportion and diversity of HP received at high diversity sites may reduce ovule fertilization and reinforce the effects of pre-pollination competition. Although studies have documented HP receipt in natural

communities (McLernon et al. 1996; Montgomery and Rathcke 2012), more studies are needed to connect these to attributes of the co-flowering community (e.g., Feinsinger et al. 1986), especially if we are to determine whether HP receipt is the cause of pollen limitation in highly diverse areas (Vamosi et al. 2006).

3.4.2 Co-flowering diversity effects on the adaptive value of flower longevity

Species facing strong competition in stable communities are expected to evolve mechanisms to mitigate its effects in order to co-exist (Strauss et al. 2006; Sargent et al. 2011). Here, we found population differentiation in ‘maximum’ flower longevity (i.e., without pollination and under common garden conditions; Ashman and Schoen 1996). A positive correlation between population-mean flower longevity and *in situ* co-flowering species richness suggests that this trait may have evolved in response to stronger competition for pollination in co-flowering species-rich seeps. It is important to note, however, that maternal effects could also contribute to the population variation in flower longevity that we observed in the greenhouse as a full decomposition of genetic effects was not conducted. Although our preliminary data suggest that co-flowering diversity does not covary with resource conditions in the direction previously observed (Freestone and Harrison 2006; see methods above), high diversity was associated with drier seeps indicating that plants at high and low diversity seeps may be exposed to different abiotic conditions. Thus, we cannot rule out the possibility that flower longevity might be influenced by other unmeasured environmental factors that covary positively with co-flowering species richness. With respect to biotic interactions, however, interspecific competition may be more likely than limited mate availability as the selective force because there was no correlation

between flower longevity and conspecific plant density. Consistent with this conclusion, plants from high diversity sources had longer flower lifetimes than plants from low diversity sources regardless of the pollination environment, providing evidence for population (seep) differentiation in ‘realized’ flower longevity (under variable pollination and climate environments; Ashman and Schoen 1996). Moreover, longer flower lifetimes led to higher female fitness only at high diversity sites demonstrating the context-specific adaptive value of this trait. It is important to acknowledge, however, that we did not formally test for local adaptation (‘local vs. foreign’; Kawecki and Ebert 2004). Instead, because we had an *a priori* hypothesis of the potential agent of selection, we use a ‘parallel local adaptation’ approach (*sensu* Kawecki and Ebert 2004) wherein we studied replicate populations within a defined habitat type (i.e., high and low diversity). This design allowed us to exclude the possibility that differentiation may arise due to random differences among populations. However, as the number of destination and source populations is increased the hypothesis must be statistically reformulated, now as tests for specific forms of destination-site x source-site interactions (Kawecki and Ebert 2004). This differs with standard local adaptation studies where the local genotype is expected to do better than foreign genotypes at their home destination (assessed via a significant destination x source interaction). In our case, we expected one significant (i.e., difference between sources at high diversity seeps) and one non-significant result (i.e., no difference between sources at low diversity seeps). Thus, we acknowledge that low statistical power could also contribute to this specific combination of outcomes. The consistency in the pattern of the results across all response variables (longevity, CP deposition and seeds per fruit), however, makes this unlikely. While statistical contrasts have been used in tests of adaptation under particular circumstances (e.g., Joshi et al. 2001), testing for significant destination x source

interactions is nevertheless considered the standard for demonstrating adaptation (e.g., Byars et al. 2007, Gonzalo-Turpin and Hazard 2009).

3.4.3 Why flower longevity?

Mimulus guttatus exhibits variation in several characteristics (e.g., flower size, stigma-anther distance) that are predicted to confer a reproductive advantage in pollination-limited environments, yet only flower longevity varied with species richness (Fig. 1). Why might this be? The answer may reside in the fact that *M. guttatus* has a touch sensitive stigma. It closes in response to CP receipt but reopens if ovule fertilization is incomplete (Fetscher and Kohn 1999). This ability imposes a constraint on flower life time-- the lag time between closing and reopening- and thus additional time that a flower must remain alive to obtain more pollen. Since the stigma closes in response to HP as well as CP and the lag time in reopening is similar (Arceo-Gómez, unpublished data), flowers that receive high HP loads must remain viable for longer than those that receive pure CP loads. Evolution of extended flower longevity may be facilitated by two additional factors 1) the low cost of flower maintenance and 2) high potential fitness per flower (Ashman and Schoen 1994, 1997). *Mimulus guttatus* flowers do not produce nectar at the seeps used in this study (Arceo-Gómez pers. obs.), but do produce many seeds (Arceo-Gómez and Ashman 2011) and thus require high CP loads to fertilize all ovules. Interestingly, differences in CP receipt at the end of flower life occurred even though *M. guttatus* is capable of delayed self-fertilization suggesting that this mechanism may be ineffective in the populations we studied, putting a premium on pollinator-mediated pollen transfer.

It is possible that traits not measured in this study, but correlated with flower longevity, may be the real targets of selection and thus be responsible for the patterns we observed. For instance, *M. guttatus* height differed between high and low diversity seeps (see methods above) and this trait can influence plant visitation rate and fertility in other systems (e.g., Levin and Kerster 1973; Hainsworth et al. 1984). However, in our study plant floral display, which is correlated with plant size ($r = 0.2$, $P < 0.001$), was not correlated with flower longevity ($r = 0.15$, $P = 0.1$), and analysis of display size does not mirror that of floral longevity or fitness (Arceo-Gómez and Ashman unpublished). Furthermore, our results on *M. guttatus* are consistent with theoretical expectations for the evolution of flower longevity. Flower longevity is predicted to increase when the potential for fitness gain over time exceeds the costs associated with flower maintenance (i.e., slow fitness accrual) such as nectar production and respiration by floral structures (Ashman and Schoen 1997). If we assume that costs are identical at each site, then when pollinator competition and/or HP interference is high, as it occurs in high diversity sites, fitness accrual is expected to be slower and the potential gain during subsequent visits may exceed the costs of flower maintenance. Thus, extended flower longevities in *M. guttatus* are expected to be adaptive. Conversely, when visitation is high and/or HP interference is low, as occurs at low diversity sites, shorter flower life spans should be favored as the costs of flower maintenance may exceed potential fitness gain (Ashman and Schoen 1994, 1997). Overall, our results are consistent with the idea that differences in the co-flowering community context lead to changes in the adaptive value of *M. guttatus* flower longevity and influence its evolution. Experimental manipulations of diversity and floral longevity are needed, however, to confirm the target of selection (Conner 2003) in this system. Moreover, which trait-- floral lifetime, or others, e.g., flower size (Caruso 2000), flowering time (Waser 1978)—is likely to reflect the ‘path of

least resistance' to selection imposed by diverse communities in other systems remains to be seen.

3.4.4 Species richness versus sampling and phylogenetic effects

Diversity effects on ecological processes (e.g., pollination) are often questioned because species rich-communities have a greater probability of containing particular species that may be responsible for the overall effects (sampling or selection effect; Loreau 2000; Leps et al. 2001; Loreau and Hector 2001; Loreau et al. 2001; Hector et al. 2002; Fargione and Tilman 2005; Cardinale et al. 2007). Thus, effects may be driven by changes in species composition rather than diversity (species richness) and therefore species-specific effects need to be distinguished from those of species complementarity (i.e., when effects are driven by processes that involve multiple species; Loreau 2000; Loreau et al. 2001; Cardinale et al. 2007). In particular, in our study, higher competition at high diversity sites may be driven by the presence of one or a few species that are absent from low diversity sites (changes in species composition) rather than by an increase in overall species richness. Our use of natural variation, unfortunately does not allow us to formally differentiate between sampling and complementarity effects (e.g., comparisons of mix species array vs. arrays of *M. guttatus* plus each individual competitor; Tillman et al. 2001; Hector et al. 2002; Cardinale et al. 2007), and given the complexity of our communities, whole community manipulations would be daunting. However, sampling effects are unlikely to be responsible for the patterns we observed because the importance of a given species for pollinators appears to be determined by its relative abundance and not by species-specific characteristics (i.e., flower size, shape, amount of rewards and floral display; Meindl et al.

unpublished manuscript), suggesting that species identity '*per se*' plays a minor role. Other studies have shown that species complementarity effects can be highly important as these can exceed those of the most productive (Tilman et al. 2001, Cardinale et al. 2007) and most detrimental species (Arceo-Gómez and Ashman 2011). In addition, the strength of pollinator competition can also vary with phylogenetic relatedness of co-flowering species rather than with species richness alone (Schuett and Vamosi 2010). This is because closely related species often share similar flower traits and attract the same pollinators (Bell et al. 2005; Schuett and Vamosi 2010). While we have not tested this formally, this is unlikely to be responsible for the differences seen as the closest relative of *M. guttatus* (i.e., *M. nudatus*), which is very similar in its floral color, shape (Ritland and Ritland 1989) and pollinator community (Gardner and McNair 2000; Meindl et al. unpublished manuscript), occurs in all four of the focal sites (high and low diversity; Table A2). Nevertheless, we have shown that co-flowering species richness correlates with altered plant-pollinator interactions, plant fitness components and may drive floral evolution. However, experimental manipulation of the community will be necessary to confirm diversity as the agent of selection, as well as distinguish between species-specific and species complementarity effects (e.g., Loreau and Hector 2001; Leps et al. 2001; Cardinale et al. 2007).

Table 7. Co-flowering species richness and locations of the four sites used to assess the effects of the pollination environment on *M. guttatus* visitation rate, pollen transfer dynamics, reproductive success and flower longevity. For pre-planned contrasts these were grouped into two diversity categories (high and low).

| Site | Species richness | Diversity category | Location |
|------|------------------|--------------------|------------------------------|
| 1 | 17 | High | 38°51'29.45"N 122°24'33.49"W |
| 2 | 19 | High | 38°51'56.62"N 122°27'02.30"W |
| 3 | 9 | Low | 38°51'13.38"N 122°24'21.43"W |
| 4 | 8 | Low | 38°51'30.91"N 122°25'55.88"W |

Table 8. (A) Two-way ANCOVA and (B) pre-planned contrast results for the effects of source diversity, flowering site diversity and their interaction on *M. guttatus* flower longevity, conspecific pollen deposition and seed production. (C) The effects of covariates, temporal cohort (early and late in the flowering season) and local *M. guttatus* (conspecific) flower density on each dependent variable.

| | Flower longevity | | | Conspecific pollen | | | Seed number | | |
|--|------------------|-------|---------------|--------------------|--------|---------------|-------------|--------|---------------|
| (A) Overall model | df | SS | F value | Df | SS | F value | df | SS | F value |
| Model | 17 | 25.18 | 1.75* | 17 | 1077.9 | 1.78* | 17 | 574.6 | 2.23** |
| Error | 108 | 91.6 | | 103 | 3663.9 | | 99 | 1502.8 | |
| (B) Pre-planned contrasts | | | | | | | | | |
| Source diversity | | | | | | | | | |
| High vs low | 1 | 5.08 | 5.99** | 1 | 18.8 | 0.5 | 1 | 66.8 | 4.4* |
| Flowering Site diversity | | | | | | | | | |
| High vs low | 1 | 0.01 | 0.02 | 1 | 21.7 | 0.6 | 1 | 43.6 | 2.8 |
| Source diversity at high diversity sites | | | | | | | | | |
| High vs low | 1 | 4 | 4.71* | 1 | 160.7 | 4.5* | 1 | 90.8 | 5.9** |
| Source diversity at low diversity sites | | | | | | | | | |
| High vs low | 1 | 1.43 | 1.69 | 1 | 44.1 | 1.2 | 1 | 5 | 0.3 |
| (C) Additional sources of variation | | | | | | | | | |
| Cohort | 1 | 5.5 | 6.5** | 1 | 264.2 | 7.43** | 1 | 65.2 | 4.3* |
| Conspecific density | 1 | 4.3 | 5.1* | 1 | 26.1 | 0.73 | 1 | 16.7 | 1.1 |



Figure 4. Frequency histogram of the number of plant species co-flowering with *Mimulus guttatus* at seeps (N = 23) within the McLaughlin Natural Reserve in Northern California.

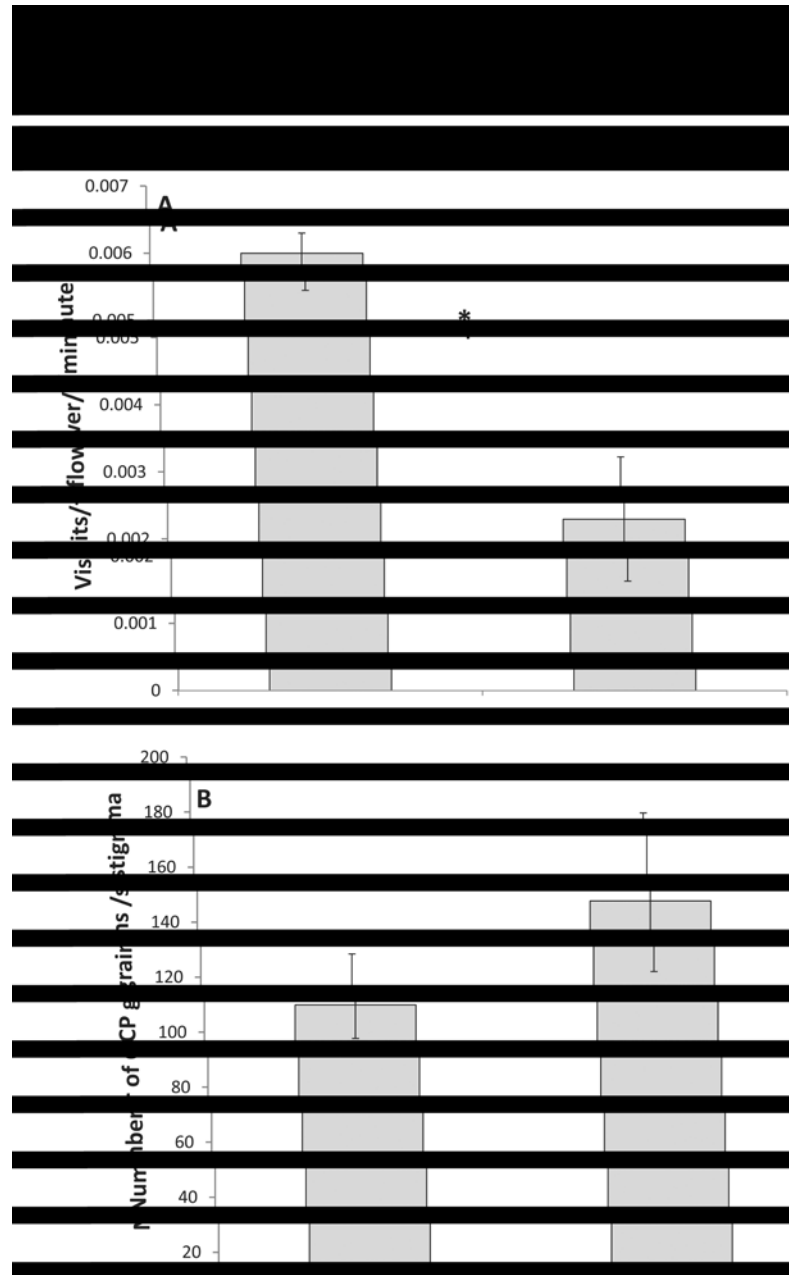


Figure 5. Mean (\pm SE) for A) insect visitation rate, B) conspecific pollen (CP) receipt on stigma after one day of open pollination, and C) proportion of heterospecific pollen (HP) on the stigmas of *M. guttatus* flowering at sites with high and low co-flowering co-flowering community diversity. Significance of pre-planned contrasts between high and low diversity denoted as: * $P < 0.05$, ** $P < 0.001$

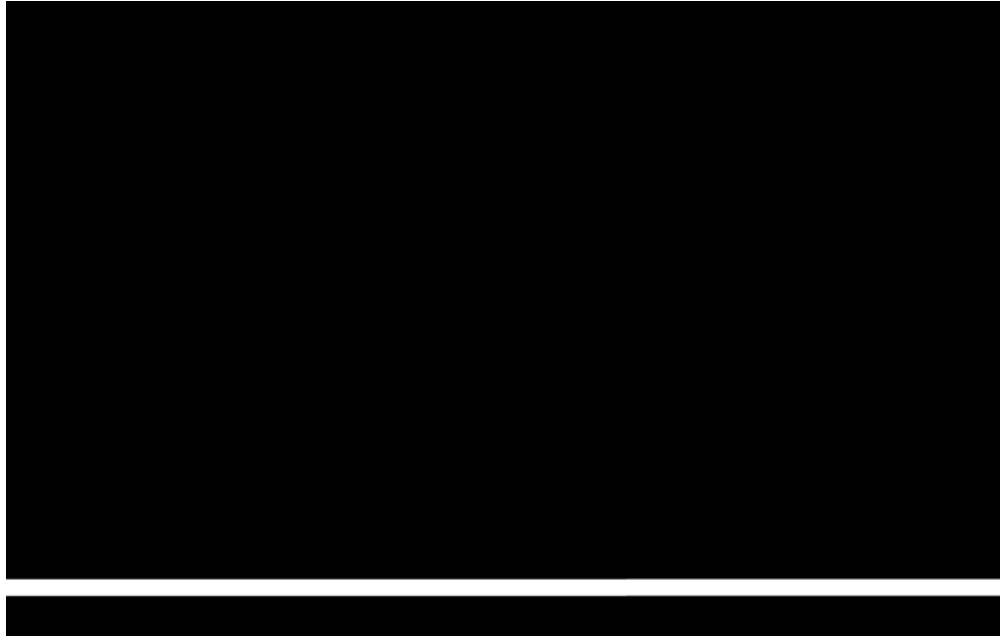


Figure 6. Correlation between *M. guttatus* flower longevity assessed under common greenhouse conditions and the in-situ number of co-flowering species at the site (($r = 0.52$, $P = 0.01$; $n = 23$).

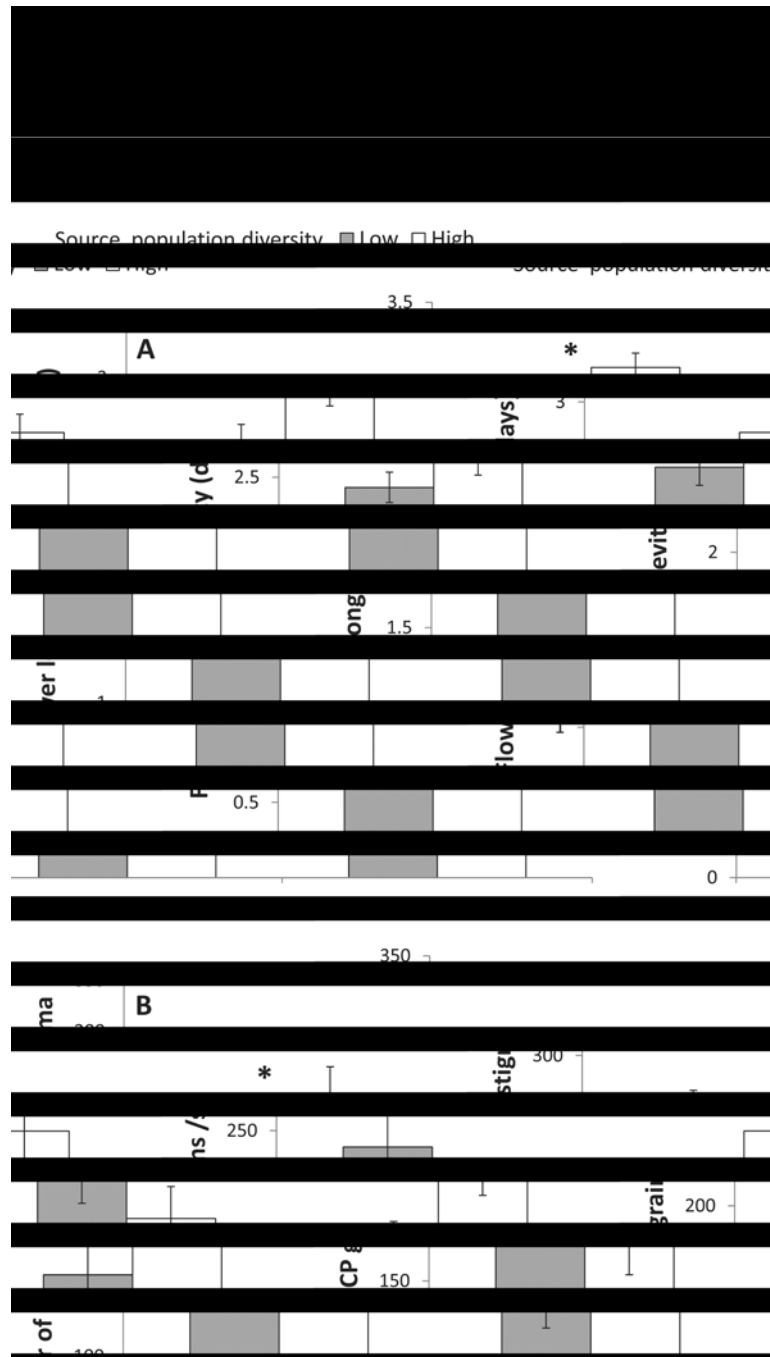


Figure 7. Mean (\pm SE) for A) flower longevity, B) conspecific pollen (CP) receipt on stigma at the end of flower life span, and C) seed number per fruit for *M. guttatus* from source populations with high and low co-flowering community diversity when flowering at high and low co-flowering diversity sites. Significance of pre-planned contrasts denoted as: * $P < 0.05$, ** $P = 0.01$.

4.0 HETEROSPECIFIC POLLEN RECEIPT AFFECTS SELF POLLEN MORE THAN OUTCROSS POLLEN IN *MIMULUS GUTTATUS*: IMPLICATIONS FOR MIXED- MATING PLANTS

Arceo-Gómez G. and Tia-Lynn Ashman. *Ecology*. In review.

4.1 INTRODUCTION

Plants do not grow in isolation but typically co-occur and flower with other plant species in a community. Thus, studies that seek to understand the ecological and evolutionary processes that govern the existence of plant populations in nature must consider the complexity of the ecological context in which they occur. Community-wide studies of plant-pollinator interactions (e.g., Olesen and Jordano, 2002, Bascompte et al. 2003, Petanidou et al. 2008, Lazaro et al. 2009, Fang and Huang 2013) have demonstrated the multitude of direct and indirect interactions that exist, and in doing so revealed the limits of our understanding of the costs/benefits pollinator sharing (e.g., Sargent and Ackerly 2008, Mitchell et al. 2009). One gap, in particular, is the consequence of interspecific pollen transfer (e.g., Morales and Traveset 2008, Ashman and Arceo-Gómez 2013, Fang and Huang 2013). Until recently evidence for interspecific pollen transfer (donation or receipt) was scattered and focused on amount rather than diversity

(reviewed in Morales and Traveset 2008) and thus its importance for plant reproductive success and floral evolution was debated (Morales and Traveset 2008, Mitchell et al. 2009, Muchhala et al. 2010). However, a recent synthesis (Ashman and Arceo-Gómez 2013) and detailed community-wide surveys (Fang and Huang 2013, Arceo-Gómez et al. in prep), show that the incidence of heterospecific pollen (hereafter, HP) receipt, in particular, can be substantial, occurring in >50% of flowers, intense for some species, constituting >60% of the total load (Ashman and Arceo-Gómez 2013) and involve multiple HP donors (>7, Fang and Huang 2013). This work has revitalized inquiry regarding the ecological impact of such transfer in natural communities.

Another reason the importance of HP receipt to ecological processes has been debated is because negative effects of HP receipt can sometimes be minimal, even under heavy deposition (e.g., Campbell and Motten 1985, Kwak and Jennersten 1991). However, this may be because the effects of HP can depend on the time it arrives relative to conspecific pollen (hereafter, CP) (e.g., Waser and Fugate 1986, Caruso and Alfaro 2000) and on the mechanism by which HP interferes with CP (Reviewed in Morales and Traveset 2008). Nevertheless, a recent survey has revealed that variation across species exists even when CP-HP timing and deposition rate are held constant suggesting species- or trait-specific modifiers play roles (Ashman and Arceo-Gómez 2013). Furthermore, acknowledging the diversity of HP on natural styles (e.g., Montgomery and Rathcke 2012, Fang and Huang 2013, Arceo-Gómez and Ashman 2014, Arceo-Gómez et al. in prep), other recent studies have shown that pollen from more than one heterospecific species can interact in complex ways and lead to greater than additive effects (Arceo-Gómez and Ashman 2011, Flanagan et al. 2011). Thus, in the last few years the features of the HP donors that determine the fitness consequence of HP receipt are being clarified but

much less attention has been paid to the role of the HP recipient (Ashman and Arceo-Gómez 2013). In particular, the source of the conspecific pollen (outcross or self) that interacts with the HP has been ignored entirely, despite the fact that both types of pollen are often involved in seed production (i.e., mixed mating).

Over half of the animal-pollinated species studied to date have mixed mating systems (Vogler and Kalisz 2001, Goodwillie et al. 2005) and thus to understand the effects of HP receipt in natural populations the effects of HP on both self and outcross CP must be evaluated. This is particularly important because there is good reason to believe that the effects may differ with CP source. First, numerous studies have demonstrated that self pollen is slower to germinate and grow tubes than outcross pollen (e.g., Weller and Ornduff 1977, Bowman 1987, Aizen et al. 1990, Cruzan and Barrett 1993, Kruszewski and Galloway 2006) and since both of these processes are commonly affected by the presence of HP (e.g., Sukhada and Jayachandra 1980, Murphy and Aarssen 1995, Morales and Traveset 2008, Arceo-Gómez and Ashman 2011), HP may interfere more with self than outcross CP pollen. Second, many mixed-mating species exhibit delayed selfing mechanisms in which self pollen is deposited after xenogamous outcross pollen (e.g., Dole 1990, Lloyd and Schoen 1992, Kalisz et al. 1999, Etcheverry et al. 2003, Sun et al. 2005, Qu et al. 2007), and evidence suggests that when HP is received prior to CP it is most detrimental to CP germination and tube growth (reviewed in Morales and Traveset 2008). Considering these factors, we predict that 1) HP will have greater effects on self than outcross CP, and 2) HP will have a greater effect when it arrives prior to CP than when HP and CP are deposited simultaneously. Because these predictions have not been tested in any species, the full potential for effects of HP receipt on plant fitness have not been explored. In this study, we address this gap by answering the following questions: 1) Is the effect of HP receipt on pollen

tube growth and seed production greater when interacting with self compared to outcross CP? 2)
Is the magnitude of the effect of HP receipt larger when HP is deposited prior to CP than when it is deposited simultaneously with CP?

4.2 MATERIALS AND METHODS

4.2.1 Study species

We tested the effects of HP on self and outcross CP success using *Mimulus guttatus* (Phrymaceae) as the pollen recipient (and CP donor) and *Helianthus exilis* (Asteraceae) as the HP donor. Both species are hermaphroditic annuals that co-occur in the highly diverse serpentine seep communities in northern California (Harrison et al. 2000, Freestone and Inouye, 2006, Arceo-Gómez and Ashman 2011) where their flowering times overlap, they share primarily bee pollinators (Arceo-Gómez and Ashman 2011), and pollen transfer between them occurs (Arceo-Gómez G., unpublished data). When *H. exilis* pollen constitutes 20% of the pollen load it can reduce *M. guttatus* outcross pollen tube growth and seed production by more than 25% (Arceo-Gómez and Ashman 2011). These interactions may be mediated by allelopathic compounds because although *H. exilis* pollen grains germinate on *M. guttatus* stigmas their tube growth is minimal, usually only a few μm (Arceo-Gómez and Ashman 2011; Arceo-Gómez pers. obs.).

Mimulus guttatus is self-compatible and has a mixed mating system (Dudash and Carr 1998). Flowers are herkogamous, but self-pollination can occur geitonogamously or via delayed selfing as the corolla abscises (Dole 1990), usually three days after anthesis (Arceo-Gómez and

Ashman 2014). Its flowers have a bilobed stigma that closes ~2 s after being touched and reopens within ~ 2 h in response to low ovule fertilization (Fetscher and Kohn, 1999; Arceo-Gómez, pers. obs.). It typically produces more than 300 seeds per flower (Arceo-Gómez and Ashman 2011). *Helianthus exilis* is self-incompatible (Harrison et al. 2000) with highly ornamented pollen grains that are slightly smaller than *M. guttatus* pollen grains (Arceo-Gómez and Ashman 2011).

4.2.2 Plant material

We planted 30 *M. guttatus* seeds each from a different maternal plant from population ‘1’ (in Arceo-Gómez and Ashman 2014) at the McLaughlin Natural Reserve in northern California. Twenty-eight of these plants were designated pollen recipients and functioned as self pollen donors, and the remaining two functioned as outcross pollen donors. We planted five *H. exilis* seeds acquired from a native plant nursery (www.diggingdog.com) to serve as HP donors (as in Arceo-Gómez and Ashman 2011). All seeds were sown into eight cm² pots containing Fafard #4 soil with six beads of 13:13:13 N-P-K Nutricote™ fertilizer and grown to flowering in the greenhouse (14hday/10h night; 26/21°C) at the University of Pittsburgh.

4.2.3 Hand-pollination treatments

To test the effect of HP on self and outcross CP tube growth and fertilization success, as well as the dependence on the timing of HP arrival on the stigma, we applied six different hand-pollination treatments to each *M. guttatus* recipient as follows: 1) *M. guttatus* outcross pollen

only (mix of two donors); 2) *M. guttatus* self pollen only; 3) mix of *M. guttatus* outcross pollen and *H. exilis* pollen applied simultaneously; 4) mix of *M. guttatus* self pollen and *H. exilis* pollen applied simultaneously; 5) *H. exilis* pollen applied ~6 hours prior to *M. guttatus* outcross pollen; and 6) *H. exilis* pollen applied ~6 hours prior to *M. guttatus* self pollen. The six treatments were randomly applied to flowers on each recipient ($N = 168$). The CP-HP mixes contained 20% HP which reflects the average level of HP receipt in nature across species (Ashman and Arceo-Gómez 2013) and these were created based on the mean number of pollen grains/anther for each species following Arceo-Gómez and Ashman (2011). Hand-pollination treatments were applied with a tooth pick which has proved effective in previous experiments (Arceo-Gómez and Ashman 2011). All *M. guttatus* styles were collected one day after hand-pollination, after enough time for fertilization (8 h) but before autonomous self-pollination could occur, and fixed in 70% ethanol (Arceo-Gómez and Ashman 2011). Fruits were collected at maturity and seeds were counted with the aid of a dissecting microscope following Arceo-Gómez and Ashman (2011).

4.2.4 Data collection

We assessed whether *H. exilis* interferes with *M. guttatus* self and outcross pollen tube growth by evaluating differences in the proportion of CP pollen grains on the stigma that grew tubes that reached the base of the style. For this styles were softened and stained with aniline blue and the number of pollen grains on the stigma and tubes at the base of the style counted with the aid of a fluorescence microscope (Dafni 1992, Arceo-Gómez and Ashman 2011). This proportional index (*M. guttatus* pollen tubes/total CP on stigma) takes into account variation in the number of pollen

tubes that reach the ovary due to differences in the CP load applied during hand-pollinations. The number of *H. exilis* pollen grains on stigmas was counted to confirm the effectiveness of our hand-pollination treatments. We achieved the desired HP load composition (mean % HP load \pm SE: 19 % \pm 0.01, $N = 112$) and this did not vary with application timing (simultaneously vs. prior) or mix type (HP-self vs. HP-outcross) ($P > 0.05$ for both, data not shown). In addition, we evaluated differences in CP fertilization success (fertile seeds/total CP on stigma) among the six hand-pollination treatments. This relative measure of reproductive success also takes into account variation due to differences in the amount of CP deposited on stigmas. Nine fruits did not reach maturity and thus CP fertilization success was estimated for a total of 159 fruits.

4.2.5 Data analyses

To evaluate the effects of *H. exilis* pollen on *M. guttatus* pollen tube growth and fertilization success we performed mixed models (proc mixed; SAS 2010) with CP type (self vs. outcross), HP treatment (control [without HP], HP-CP simultaneously and HP prior to CP) and their interaction as fixed factors. We accounted for variation among recipients by including plant as a random factor in the models but did not test its significance. When HP treatment had an overall significant effect we conducted pre-planned linear contrasts (Rosenthal and Rosnow 1985, Abelson and Prentice 1997) to test specific hypotheses regarding the presence and arrival time of HP receipt (e.g., Strauss and Murch 2004, Arceo-Gómez and Ashman 2011, 2014). Specifically, we wanted to know if HP reduced pollen tube growth and/or fertilization success when applied either simultaneously and/or prior to CP and thus we compared each of those two treatment levels to the control (without HP). If HP receipt caused a decrease in either response

variable under both deposition scenarios then we will proceed to compare between the HP treatments to determine whether prior HP arrival results in greater fitness reduction than simultaneous HP arrival. We further constructed pre-planned linear contrasts to test specific hypotheses regarding the HP treatment by CP type interaction (Rosenthal and Rosnow 1985, Abelson and Prentice 1997, Arceo-Gómez and Ashman 2014), but only when the main effect of the interaction was significant. Specifically, we wanted to know if *M. guttatus* self and outcross pollen tube growth and fertilization success differed when *H. exilis* pollen was present but not when it was absent. This result would indicate a differential effect of *H. exilis* pollen on *M. guttatus* self and outcross pollen. We were also interested in determining whether differences between self and outcross CP in both response variables were only observed when *H. exilis* pollen was applied prior to *M. guttatus* pollen but not when it was applied simultaneously. This result would indicate that the timing of HP arrival is important in determining its effect on self and outcross CP success. Thus, linear contrasts were constructed to test for the effect of CP type (self vs. outcross) on pollen tube growth and fertilization success within each of the three HP treatments. Both response variables were square root transformed in order to meet assumptions of normality of residuals.

4.3 RESULTS

Overall, CP type (self vs. outcross) only had a marginally significant effect on the proportion of pollen tubes at the base of the style (Table 1a; Fig. 1a). Heterospecific pollen treatment, however, had a significant effect (Table 1a) and pre-planned linear contrasts revealed that the

proportion of pollen tubes was significantly reduced (by 17%) when *H. exilis* was present compared to the control, but this was only the case when *H. exilis* was applied simultaneously and not prior to *M. guttatus* pollen --where a slight increase was observed (Table 1b; Fig. 1b). Furthermore, the HP treatment by CP type interaction was significant (Table 1a) and pre-planned contrasts revealed that *M. guttatus* self pollen tube growth was 32% lower than outcross pollen when they were applied simultaneously with *H. exilis* pollen, but only minor non-significant differences were observed when HP was absent (10%) or applied prior (3%) to *M. guttatus* pollen (Table 1c; Fig. 1c).

In addition, CP type had an overall significant effect on CP fertilization success (Table 1a) with *M. guttatus* self pollen being 14% less effective at fertilizing seeds than outcross pollen across all HP treatments (Fig 2a). Heterospecific pollen treatment did not have an overall effect on CP fertilization success (< 8% difference among all HP treatments; Table 1a; Fig. 1b), rather it varied with CP type (Table 1a; HP treatment by CP type interaction). Pre-planned contrasts revealed that *M. guttatus* self pollen was 39% less effective at fertilizing seeds than outcross pollen when *H. exilis* was applied simultaneously with *M. guttatus* pollen, but no (<1%) difference was observed when HP was absent or applied prior to *M. guttatus* pollen (Table 1c; Fig. 2c).

4.4 DISCUSSION

4.4.1 Heterospecific pollen effects on self and outcross conspecific pollen

Our results indicate that for self-compatible plants which display mixed mating (Goodwillie et al. 2005) the fitness consequences of HP receipt can be more detrimental than previously thought. To our knowledge, all previous studies have only evaluated HP effects on outcross CP (reviewed in Morales and Traveset 2008; but see Neiland and Wilcock 1999) and, as shown here, the detrimental effects of HP receipt can be even greater when self pollen is involved (32-39% further reduction of CP tube growth and fertilization success when HP deposition is simultaneous with CP). Thus, the full consequences of HP receipt in natural communities may be severely underestimated. For instance, in 13 of the 20 studies reviewed in Ashman and Arceo-Gómez (2013) where costs of HP receipt were evaluated, a self-compatible species was used as the pollen recipient and CP donor, and, to our knowledge, none of these studies evaluated HP effects on self pollen success. In the case of *M. guttatus*, negative effects of *H. exilis* pollen on outcross CP fertilization success have been previously observed (Arceo-Gómez and Ashman 2011), however, the present study suggests that the detrimental effect experienced by *M. guttatus* in natural communities could be much greater it typically receives a mix of self and outcross pollen. For instance, *M. guttatus* outcrosses 60 to 80% of the time (Dudash and Carr 1998), and if we assume this also reflects natural levels of self and outcross pollen receipt and HP is received at mean levels, then the effect of HP receipt would be a 40-32% decrease in seed production instead of the 25% estimated when complete outcrossing was assumed (Arceo-Gómez and Ashman 2011). However, one needs to acknowledge that the differential effects of

HP receipt on self and outcross CP could also alter the identity of the seeds produced. For instance, when HP is deposited simultaneously with self and outcross CP its greater detrimental effect on self pollen would lead to higher outcrossing rate than would be expected in the absence of HP. Such an outcome could lead to correlations between realized mating system and intensity of HP deposition among individuals or populations. Studies are needed to assess the deposition rates of self and outcross CP and HP, as well as selfing rates, in natural populations that vary in HP deposition to quantify the effects directly.

Interestingly, we did not observe any effect of *H. exilis* on *M. guttatus* tube growth or fertilization success when it was applied 6 h prior to CP suggesting that HP effects are dependent upon arrival time, but not in the direction we predicted. We expected a more detrimental effect when HP was applied prior to CP mainly due to stigma clogging (e.g., Caruso and Alfaro 2000) or reduced stigma receptivity due to closure of stigma lobes (e.g., Waser and Fugate 1986) or allelopathic effects (e.g., Murphy and Aarssen 1995). To our knowledge, this is the first study to show negative effects only when HP is applied simultaneously and not when is applied prior to CP. This result suggests that *H. exilis* pollen interference may result from a mechanism other than physical displacement of CP grains on the stigma since physical displacement would have occurred regardless of time of HP arrival. Thus, our results corroborate previous findings that have suggested allelopathic effects of *H. exilis* pollen on *M. guttatus* pollen germination and tube growth (Arceo-Gómez and Ashman 2011). Pollen allelopathy is one of the strongest mechanisms of HP interference (Morales and Traveset 2008) and has been observed in other species within the Asteraceae (Murphy 2000). Our results could indicate that pollen allelopathy only lasts for a limited time after which the allelopathic compounds volatilize or degrade (Fisher, 1980, Zhu and Mallik 1994). Although volatilization of allelopathic compounds in vegetative tissues is common

(e.g., Fisher, 1980, Zhu and Mallik 1994) its implications for pollen allelopathy have not been assessed but could be important. For instance, if allelopathic compounds of HP grains volatilize then selection for delayed self-pollination may be favored when a species interacts often with another species with allelopathic pollen. Future studies of the fitness consequences of HP receipt need to consider not only the effect of HP on self and outcross CP but also the pattern of HP and CP arrival to the stigma. Such work will provide a fuller understanding of the role of HP receipt in diverse communities (Ashman and Arceo-Gómez 2013).

4.4.2 Ecological and evolutionary implications for mixed mating plants in diverse communities

The potential increase in outcrossing in mixed-mating plants as a result of differential effects of HP receipt on self and outcross CP could also have important ecological and evolutionary consequences for natural populations. For instance, higher outcrossing, due to greater HP receipt in diverse communities (e.g., Fang and Huang 2013, Arceo-Gómez and Ashman 2014), could increase population genetic diversity and thus influence the rate of evolutionary change within populations (Hughes et al. 2008). Furthermore, increased genetic diversity can influence community level processes by generating and maintaining species diversity (Vellend and Geber 2005, Vellend 2006, Hughes et al. 2008). Specifically, genetic diversity and species diversity have been hypothesized to covary in space due to parallel processes that may affect both levels of diversity or because of direct effects of one level of diversity on the other (Vellend and Geber 2005). Our results provide the tantalizing possibility that HP effects contribute to such a correlation in flowering plant communities since high diversity communities have higher levels

of interspecific pollen transfer (e.g., Arceo-Gómez and Ashman 2014) and self-compatible species may exhibit higher outcrossing rates in these compared to when they flower alone or in less diverse communities. Studies are needed that evaluate the role of pollinator-sharing, patterns of HP receipt, outcrossing rates and levels of genetic diversity in natural populations to test this idea. In conclusion, our results not only add to the existing understanding of the effects of HP receipt by revealing differential effects on self and outcross CP but also suggest that HP receipt could have ecological and evolutionary implications that have not been previously acknowledged.

Table 9. (A) Results of mixed-model ANOVA for the effects of conspecific pollen type (CP type [self vs. outcross]), heterospecific pollen treatment (HP treatment [without HP, HP-CP simultaneous, HP prior to CP]) and their interaction on the proportion of the total CP grains on stigma that grow tubes that reach the base of the style (CP pollen tubes/total CP on stigma) and CP fertilization success (fertile seeds/total CP on stigma). (B) Pre-planned contrasts testing for differences between control and HP application treatments and (C) the effect of CP type within each HP treatment level.

| | CP tubes/ total CP on stigma | | Fertile seeds/ total CP on stigma | |
|-----------------------------------|---------------------------------|------------------|--------------------------------------|----------------|
| | <i>DF</i> | <i>F</i> value | <i>DF</i> | <i>F</i> value |
| (A) Source of variation | | | | |
| CP type | 1 | 3.2 [†] | 1 | 4.1* |
| HP treatment | 2 | 7.2** | 2 | 0.6 |
| CP type*HP treatment | 2 | 3.01* | 2 | 6.2** |
| (B) HP treatment contrasts | | | | |
| Control vs. HP-CP simultaneous | 1 | 4.56* | 1 | - |
| Control vs. HP prior to CP | 1 | 2.74 | 1 | - |
| (C) Interaction contrasts | | | | |
| Control (without HP) | | | | |
| self vs. outcross | 1 | 0.15 | 1 | 0.33 |
| HP-CP simultaneous | | | | |
| self vs. outcross | 1 | 8.97** | 1 | 16.38** |
| HP prior to CP | | | | |
| self vs. outcross | 1 | 0.08 | 1 | 0.001 |

Note: bold face *F* values indicates significant differences, [†]*P* = 0.07, **P* = < 0.05, ***P* = < 0.01, - = not tested

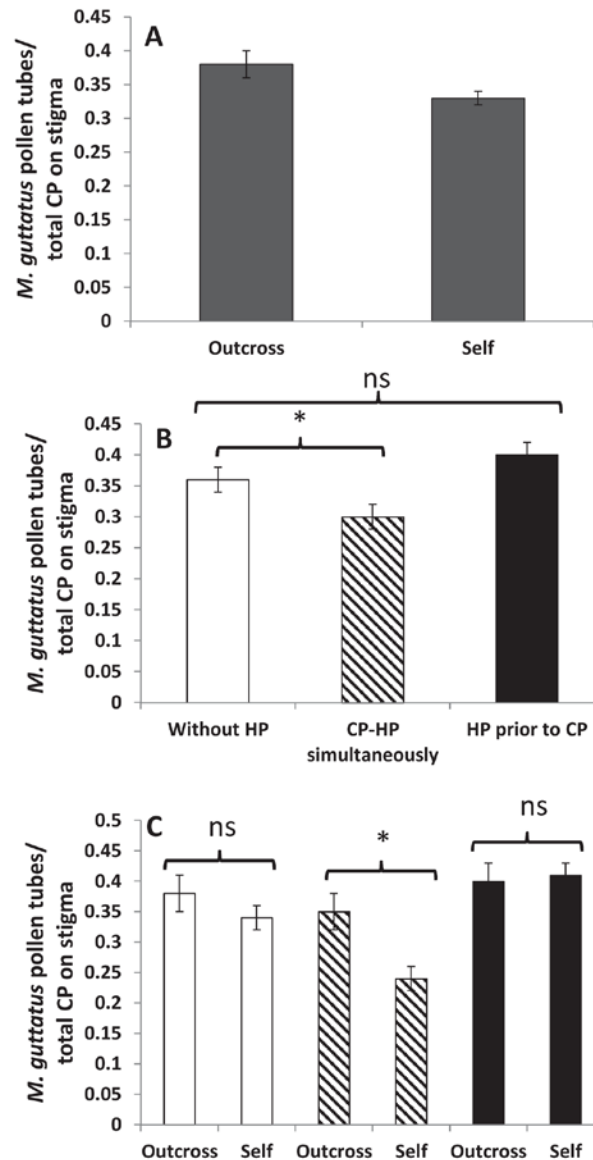


Figure 8. Mean (\pm SE) for the proportion of the total pollen grains on stigma that grow tubes and reach the base of the style (*M. guttatus* pollen tubes/total conspecific pollen [CP] on stigma) for (A) *M. guttatus* outcross and self pollen, (B) heterospecific pollen (HP) treatments: without HP (open bars), HP-CP applied simultaneously (dashed bars) and HP applied prior to CP (closed bars) and for (C) *M. guttatus* outcross and self pollen within each HP treatment. Different letters and * denote significant differences $P < 0.05$.

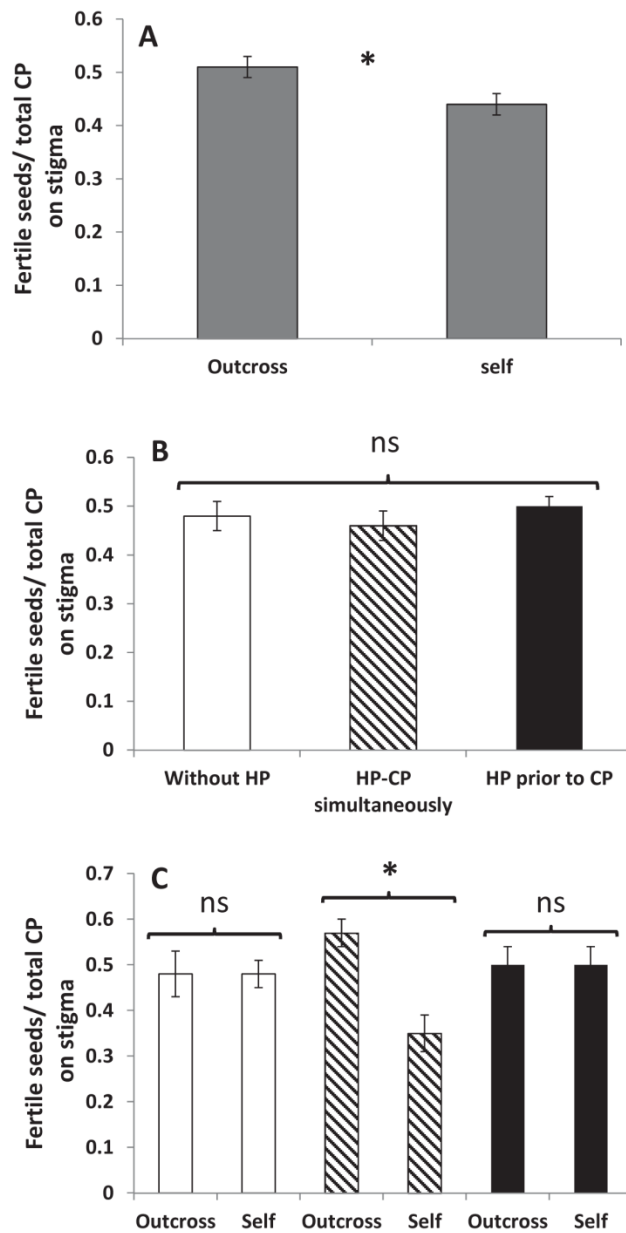


Figure 9. Mean (\pm SE) for CP fertilization success (fertile seeds/ total conspecific pollen [CP] on stigma) for (A) *M. guttatus* outcross and self pollen, (B) heterospecific pollen (HP) treatments: without HP (open bars), HP-CP applied simultaneously (dashed bars) and HP applied prior to CP (closed bars) and for (C) *M. guttatus* outcross and self pollen within each HP treatment. Different letters and * denote significant differences $P < 0.05$.

APPENDIX A

DESCRIPTION OF SEEP COMMUNITIES

Table 10 (Appendix Table A1) Site number, co-flowering species richness, GPS coordinates (location), mean flower size, stigma-anther distance, flower longevity and conspecific flower density of the 23 seep communities at the McLaughlin Natural Reserve in northern California, USA. Sites 1-4 correspond to the same sites described in Table 1 and Table A2.

| Site | Location | | Co-flowering species richness | Flower size (length x width mm) | Stigma-anther distance (mm) | Flower longevity (days) | Conspecific floral density (flowers x m ²) |
|------|----------------|-----------------|-------------------------------|---------------------------------|-----------------------------|-------------------------|--|
| 1 | 38°51'29.45"N | 122°24'33.49"W | 17 | 370.1 | 2.89 | 5.71 | 24.5 |
| 2 | 38°51'56.62"N | 122°27'02.30"W | 19 | 479.9 | 2.81 | 6 | 88.75 |
| 3 | 38°51'13.38"N | 122°24'21.43"W | 9 | 414.5 | 3.1 | 5.06 | 178.75 |
| 4 | 38°51'30.91"N | 122°25'55.88"W | 8 | 474 | 2.84 | 5.07 | 92.5 |
| 5 | 38° 51' 41.4"N | 122° 23' 54.8"W | 21 | 433.8 | 2.89 | 5.75 | 16.25 |
| 6 | 38° 51' 43.6"N | 122° 24' 56.6"W | 2 | 457.7 | 3.15 | 4.25 | . |
| 7 | 38° 51' 34.8"N | 122° 25' 05.8"W | 4 | 326.9 | 2.72 | 5 | . |
| 8 | 38° 51' 38.1"N | 122° 25' 09.7"W | 2 | 459.7 | 2.9 | 5.26 | 152.75 |
| 9 | 38° 52' 02.5"N | 122° 24' 08.2"W | 10 | 462.3 | 2.77 | 6.46 | 35 |
| 10 | 38° 52' 05.6"N | 122° 24' 09.3"W | 3 | 363.1 | 2.42 | 4.9 | . |
| 11 | 38° 52' 07.7"N | 122° 24' 06.7"W | 8 | 444.8 | 2.68 | 5.4 | 20.75 |
| 12 | 38° 52' 11.8"N | 122° 24' 05.8"W | 1 | 431.3 | 2.67 | 5.26 | . |
| 13 | 38° 51' 48.6"N | 122° 25' 05.8"W | 5 | 379.3 | 2.26 | 4.8 | . |
| 14 | 38° 51' 20.7"N | 122° 24' 09.5"W | 4 | 459.2 | 2.66 | 5.43 | . |
| 15 | 38° 50' 52.7"N | 122° 23' 27.3"W | 8 | 506.8 | 2.5 | 5.35 | . |
| 16 | 38° 51' 24.6"N | 122° 24' 28.5"W | 16 | 328.2 | 2.45 | 5.87 | 71 |
| 17 | 38° 51' 28.8"N | 122° 24' 33.8"W | 10 | 401.1 | 2.38 | 5.56 | 77.5 |
| 18 | 38° 51' 27.3"N | 122° 24' 31.1"W | 6 | 384.7 | 2.52 | 4.5 | 33.25 |
| 19 | 38° 51' 36.9"N | 122° 24' 42.1"W | 5 | 438.4 | 3.11 | 6.13 | 22.75 |
| 20 | 38° 51' 50.7"N | 122° 25' 39.3"W | 7 | 460.8 | 2.8 | 4.46 | . |
| 21 | 38° 51' 27.0"N | 122° 24' 56.5"W | 6 | 385.5 | 2.7 | 5.78 | . |
| 22 | 38° 51' 39.1"N | 122° 25' 54.6"W | 8 | 475.4 | 2.88 | 5.6 | . |
| 23 | 38° 51' 32.7"N | 122° 25' 53.2"W | 8 | 419.6 | 3.11 | 5.42 | . |

Table 11 (Appendix Table A2) Complete list of co-flowering species recorded in all the 23 seep communities surveyed at the McLaughlin Natural Reserve in northern California, USA. ‡ denotes that a particular species is present at that site.

| Family | Species | Site | | | | | | | | | | | | | | | | | | | | | | |
|------------------|-------------------------------------|------|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
| Liliaceae | <i>Allium amplexans</i> | ‡ | ‡ | ‡ | | ‡ | | | | | | | | ‡ | | ‡ | ‡ | ‡ | | | ‡ | ‡ | | ‡ |
| Primulaceae | <i>Anagallis arvensis</i> | | ‡ | | | ‡ | | | | | | | | | | | | | | | | | | |
| Scrophulariaceae | <i>Antirrhinum cornutum</i> | ‡ | ‡ | | | ‡ | | | | | | | | | | | ‡ | | | | | ‡ | ‡ | ‡ |
| Apiaceae | <i>Angelica tomentosa</i> | ‡ | ‡ | | | ‡ | | | | | | | | | | | ‡ | | | | | | | |
| Fabaceae | <i>Astragalus clevelandii</i> | | ‡ | | | ‡ | | | | ‡ | | ‡ | | | | | | | | | | ‡ | ‡ | ‡ |
| Gentianaceae | <i>Centaurium tricanthum</i> | ‡ | ‡ | ‡ | ‡ | ‡ | | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | | ‡ | ‡ | ‡ | ‡ |
| Ranunculaceae | <i>Delphinium uliginosum</i> | ‡ | ‡ | | | ‡ | | | | ‡ | | | | | | | ‡ | ‡ | ‡ | ‡ | ‡ | | | |
| Onagraceae | <i>Epilobium ciliatum</i> | ‡ | ‡ | | | ‡ | | | | | | | | | | | ‡ | | | | | | | |
| Campanulaceae | <i>Heterocodon rariflorum</i> | ‡ | ‡ | | ‡ | ‡ | | | | | | | | | | | ‡ | | | | | | | |
| Asteraceae | <i>Lessingia micradenia</i> | ‡ | ‡ | ‡ | | ‡ | | | ‡ | ‡ | | ‡ | | ‡ | | ‡ | ‡ | | | | | | | |
| Fabaceae | <i>Lotus micranthus</i> | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | | | ‡ | ‡ | ‡ | | ‡ | | ‡ | ‡ | ‡ | | ‡ | ‡ | | ‡ | |
| Phrymaceae | <i>Mimulus guttatus</i> | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ |
| Phrymaceae | <i>Mimulus laynaea</i> | ‡ | ‡ | | | ‡ | | | | | | | | | | | ‡ | | | | | | | |
| Phrymaceae | <i>Mimulus nudatus</i> | ‡ | ‡ | ‡ | ‡ | ‡ | | ‡ | | | | | | | | | ‡ | ‡ | ‡ | | | ‡ | | ‡ |
| Boraginaceae | <i>Plagiobothrys stipitatus</i> | ‡ | ‡ | ‡ | ‡ | ‡ | | ‡ | | | | | | | | | ‡ | | | | | | ‡ | |
| Ranunculaceae | <i>Ranunculus californicus</i> | ‡ | | | | ‡ | | | | | | | | | | | | | | | | | | |
| Lamiaceae | <i>Scutellaria siphocampyloides</i> | | ‡ | | | ‡ | | | | ‡ | | ‡ | | | | | ‡ | ‡ | | | | | | ‡ |
| Asteraceae | <i>Senecio clevelandii</i> | ‡ | | | | ‡ | | | | | | | | | | | | | ‡ | | | | | |
| Lamiaceae | <i>Stachys albens</i> | | ‡ | ‡ | ‡ | ‡ | | | | ‡ | | | | | | | | ‡ | | | | | ‡ | |
| Fabaceae | <i>Trifolium obtusiflorum</i> | ‡ | ‡ | | | ‡ | ‡ | | | ‡ | | ‡ | | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | | | |
| Liliaceae | <i>Triteleia peduncularis</i> | ‡ | ‡ | ‡ | ‡ | ‡ | | | | ‡ | | ‡ | | | ‡ | | ‡ | ‡ | ‡ | ‡ | ‡ | | ‡ | ‡ |
| Liliaceae | <i>Zigadenus venenosus</i> | ‡ | ‡ | ‡ | ‡ | ‡ | | ‡ | | ‡ | ‡ | ‡ | | | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ | ‡ |

BIBLIOGRAPHY

- Abelson, R. P. and D. A. Prentice. 1997. Contrast tests of interaction hypothesis. *Psychological Methods* 2:315.
- Aguilar, R., L. Ashworth, L. Galetto, and M. A. Aizen. 2006. Plant reproductive susceptibility to habitat fragmentation: review and synthesis through a meta-analysis. *Ecology Letters* 9:968-980.
- Aizen, M. and D. Vázquez. 2006. Flowering phenologies of hummingbird plants from the temperate forest of southern South America: is there evidence of competitive displacement? *Ecography* 29:357-366.
- Aizen, M. A. and L. D. Harder. 2007. Expanding the limits of the pollen-limitation concept: effects of pollen quantity and quality. *Ecology* 88:271-281.
- Aizen, M. A., K. B. Searcy, and D. L. Mulcahy. 1990. Among-and within-flower comparisons of pollen tube growth following self-and cross-pollinations in *Dianthus chinensis* (Caryophyllaceae). *American Journal of Botany* 77:671-676.
- Aizen, M. A. and D. P. Vázquez. 2006. Flowering phenologies of hummingbird plants from the temperate forest of southern South America: is there evidence of competitive displacement? *Ecography* 29:357-366.
- Albrecht, M., B. Schmid, Y. Hautier, and C. B. Müller. 2012. Diverse pollinator communities enhance plant reproductive success. *Proceedings of the Royal Society B: Biological Sciences* 279:4845-4852.
- Alonso, C., C. M. Herrera, and T. L. Ashman. 2012. A piece of the puzzle: a method for comparing pollination quality and quantity across multiple species and reproductive events. *New Phytologist* 193:532-542.
- Alonso, C., C. M. Navarro, G. Arceo-Gómez, G. Meindl, V. Parra-Tabla and T.-L. Ashman. 2013. Among species differences in pollen quality and quantity limitation: implications for endemics in biodiverse hotspots. *Ann. Bot.* 112:1461-1469.
- Alonso, C., J. C. Vamosi, T. M. Knight, J. A. Steets, and T. L. Ashman. 2010. Is reproduction of endemic plant species particularly pollen limited in biodiversity hotspots? *Oikos* 119:1192-1200.

- Anderson, S. H., D. Kelly, J. J. Ladley, S. Molloy, and J. Terry. 2011. Cascading effects of bird functional extinction reduce pollination and plant density. *Science* 331:1068-1071.
- Arathi, H. and J. Kelly. 2004. Corolla morphology facilitates both autogamy and bumblebee pollination in *Mimulus guttatus*. *International Journal of Plant Sciences* 165:1039-1045.
- Arathi, H., A. Rasch, C. Cox, and J. K. Kelly. 2002. Autogamy and floral longevity in *Mimulus guttatus*. *International Journal of Plant Sciences* 163:567-573.
- Arceo-Gómez, G. and T. L. Ashman. 2011. Heterospecific pollen deposition: does diversity alter the consequences? *New Phytologist* 192:738-746.
- Arceo-Gómez, G. and T.-L. Ashman. 2014. Co-flowering community context influences female fitness and alters the adaptive value of flower longevity in *Mimulus guttatus*. *The American Naturalist* 183:E50-E63.
- Armbruster, W. S., L. Antonsen, and C. Pélabon. 2005. Phenotypic selection on *Dalechampia blossoms*: honest signaling affects pollination success. *Ecology* 86:3323-3333.
- Ashman, T. I. A. L. and D. Schoen. 1997. The cost of floral longevity in *Clarkia tembloriensis*: an experimental investigation. *Evolutionary Ecology* 11:289-300.
- Ashman, T. L. and D. J. Schoen. 1994. How long should flowers live? *Nature* 371:788-790.
- Ashman, T.-L. and G. Arceo-Gómez. 2013. Toward a predictive understanding of the fitness costs of heterospecific pollen receipt and its importance in co-flowering communities. *American Journal of Botany* 100:1061-1070.
- Ashman, T.-L., T. M. Knight, J. A. Steets, P. Amarasekare, M. Burd, D. R. Campbell, M. R. Dudash, M. O. Johnston, S. J. Mazer, and R. J. Mitchell. 2004. Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. *Ecology* 85:2408-2421.
- Baker, A. M., S. C. Barrett, and J. D. Thompson. 2000. Variation of pollen limitation in the early flowering Mediterranean geophyte *Narcissus assoanus* (Amaryllidaceae). *Oecologia* 124:529-535.
- Bartomeus, I., J. Bosch, and M. Vilà. 2008. High invasive pollen transfer, yet low deposition on native stigmas in a *Carpobrotus*-invaded community. *Annals of Botany* 102:417.
- Bascompte, J. and P. Jordano. 2007. Plant-animal mutualistic networks: the architecture of biodiversity. *Annual Review of Ecology, Evolution and Systematics* 38:567-593.
- Bascompte, J., P. Jordano, C. Melián, and J. Olesen. 2003. The nested assembly of plant-animal mutualistic networks. *Proceedings of the National Academy of Sciences of the United States of America* 100:9383-9387.

- Bell, J., J. Karron, and R. Mitchell. 2005. Interspecific competition for pollination lowers seed production and outcrossing in *Mimulus ringens*. *Ecology* 86:762-771.
- Bowman, R. N. 1987. Cryptic self-incompatibility and the breeding system of *Clarkia unguiculata* (Onagraceae). *American Journal of Botany* 74:471-476.
- Bradshaw, H., K. G. Otto, B. E. Frewen, J. K. McKay, and D. W. Schemske. 1998. Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). *Genetics* 149:367-382.
- Brown, B. and R. Mitchell. 2001. Competition for pollination: effects of pollen of an invasive plant on seed set of a native congener. *Oecologia* 129:43-49.
- Brown, B. J., R. J. Mitchell, and S. A. Graham. 2002. Competition for pollination between an invasive species (purple loosestrife) and a native congener. *Ecology* 83:2328-2336.
- Burd, M. 1994. Bateman's principle and plant reproduction: the role of pollen limitation in fruit and seed set. *The Botanical Review* 60:83-139.
- Busch, J. W. 2005. The evolution of self-compatibility in geographically peripheral populations of *Leavenworthia alabamica* (Brassicaceae). *American Journal of Botany* 92:1503-1512.
- Campbell, D. and A. Motten. 1985. The mechanism of competition for pollination between two forest herbs. *Ecology* 66:554-563.
- Carr, D. E. and M. R. Dudash. 1997. The effects of five generations of enforced selfing on potential male and female function in *Mimulus guttatus*. *Evolution* 51:1797-1807.
- Caruso, C. M. 2000. Competition for pollination influences selection on floral traits of *Ipomopsis aggregata*. *Evolution* 54:1546-1557.
- Caruso, C. M. and M. Alfaro. 2000. Interspecific pollen transfer as a mechanism of competition: effect of *Castilleja linariaefolia* pollen on seed set of *Ipomopsis aggregata*. *Botany* 78:600-606.
- Caruso, C. M., S. B. Peterson, and C. E. Ridley. 2003. Natural selection on floral traits of *Lobelia* (Lobeliaceae): spatial and temporal variation. *American Journal of Botany* 90:1333-1340.
- Conner, J. K., R. Davis, and S. Rush. 1995. The effect of wild radish floral morphology on pollination efficiency by four taxa of pollinators. *Oecologia* 104:234-245.
- Conner, J. K. and S. Rush. 1996. Effects of flower size and number on pollinator visitation to wild radish, *Raphanus raphanistrum*. *Oecologia* 105:509-516.

- Conner, J. K., S. Rush, and P. Jennetten. 1996. Measurements of natural selection on floral traits in wild radish (*Raphanus raphanistrum*). I. Selection through lifetime female fitness. *Evolution* 50:1127-1136.
- Cruzan, M. B. and S. C. Barrett. 1993. Contribution of cryptic incompatibility to the mating system of *Eichhornia paniculata* (Pontederiaceae). *Evolution* 47:925-934.
- Dafni, A. 1992. *Pollination ecology: a practical approach*. Oxford University Press.
- Davila, Y. C., E. Elle, J. C. Vamosi, L. Hermanutz, J. T. Kerr, C. J. Lortie, A. R. Westwood, T. S. Woodcock, and A. C. Worley. 2012. Ecosystem services of pollinator diversity: a review of the relationship with pollen limitation of plant reproduction. *Botany* 90:535-543.
- Davis, H. G., C. M. Taylor, J. G. Lambrinos, and D. R. Strong. 2004. Pollen limitation causes an allee effect in a wind-pollinated invasive grass (*Spartina alterniflora*). *Proceedings of the National Academy of Sciences of the United States of America* 101:13804-13807.
- Demchik, S. M. and T. A. Day. 1996. Effect of Enhanced UV-B radiation of pollen quantity, quality, and seed yield in *Brassica rapa* (Brassicaceae). *American Journal of Botany* 83:573-579.
- Dole, J. 1990. Role of corolla abscission in delayed self-pollination of *Mimulus guttatus* (Scrophulariaceae). *American Journal of Botany* 77:1505-1507.
- Dudash, M. R. and D. E. Carr. 1998. Genetics underlying inbreeding depression in *Mimulus* with contrasting mating systems. *Nature* 393:682-684.
- 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.
- Ehrlén, J. and O. Eriksson. 1995. Pollen limitation and population growth in a herbaceous perennial legume. *Ecology* 76:652-656.
- Elle, E. and R. Carney. 2003. Reproductive assurance varies with flower size in *Collinsia parviflora* (Scrophulariaceae). *American Journal of Botany* 90:888-896.
- Ellstrand, N. C., A. M. Torres, and D. A. Levin. 1978. Density and the rate of apparent outcrossing in *Helianthus annuus* (Asteraceae). *Systematic Botany* 3:403-407.
- Etcheverry, A., J. Protomastro, and C. Westerkamp. 2003. Delayed autonomous self-pollination in the colonizer *Crotalaria micans* (Fabaceae: Papilionoideae): structural and functional aspects. *Plant Systematics and Evolution* 239:15-28.
- Fang, Q. and S.-Q. Huang. 2013. A directed network analysis of heterospecific pollen transfer in a biodiverse community. *Ecology* 94:1176-1185.

- Feinsinger, P., W. Busby, and H. Tiebout. 1988. Effects of indiscriminate foraging by tropical hummingbirds on pollination and plant reproductive success: experiments with two tropical treelets (Rubiaceae). *Oecologia* 76:471-474.
- Feinsinger, P., K. Murray, S. Kinsman, and W. Busby. 1986. Floral neighborhood and pollination success in four hummingbird-pollinated cloud forest plant species. *Ecology* 67:449-464.
- Feinsinger, P. and H. Tiebout III. 1991. Competition among plants sharing hummingbird pollinators: laboratory experiments on a mechanism. *Ecology* 72:1946-1952.
- Feldman, T., W. F Morris, and W. G Wilson. 2004. When can two plant species facilitate each other's pollination? *Oikos* 105:197-207.
- Fenster, C. B. and K. Ritland. 1994. Evidence for natural selection on mating system in *Mimulus* (Scrophulariaceae). *International Journal of Plant Sciences* 155:588-596.
- Fetscher, A. E. and J. R. Kohn. 1999. Stigma behavior in *Mimulus aurantiacus* (Scrophulariaceae). *American Journal of Botany* 86:1130-1135.
- Fisher, R. F. 1980. Allelopathy: A potential cause of regeneration failure. *Journal of Forestry* 78:346-350.
- 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.
- Fishman, L. and R. Wyatt. 1999. Pollinator-mediated competition, reproductive character displacement, and the evolution of selfing in *Arenaria uniflora* (Caryophyllaceae). *Evolution* 53:1723-1733.
- Flanagan, R. J., R. J. Mitchell, and J. D. Karron. 2011. Effects of multiple competitors for pollination on bumblebee foraging patterns and *Mimulus ringens* reproductive success. *Oikos* 120:200-207.
- Flanagan, R. J., R. J. Mitchell, D. Knutowski, and J. D. Karron. 2009. Interspecific pollinator movements reduce pollen deposition and seed production in *Mimulus ringens* (Phrymaceae). *American Journal of Botany* 96:809-815.
- Fleming, T. H. and J. N. Holland. 1998. The evolution of obligate pollination mutualisms: senita cactus and senita moth. *Oecologia* 114:368-375.
- Freestone, A. and B. Inouye. 2006. Dispersal limitation and environmental heterogeneity shape scale-dependent diversity patterns in plant communities. *Ecology* 87:2425-2432.
- Freestone, A. L. and S. Harrison. 2006. Regional enrichment of local assemblages is robust to variation in local productivity, abiotic gradients, and heterogeneity. *Ecology Letters* 9:95-102.

- Galen, C. and T. Gregory. 1989. Interspecific pollen transfer as a mechanism of competition: consequences of foreign pollen contamination for seed set in the alpine wildflower, *Polemonium viscosum*. *Oecologia* 81:120-123.
- Galen, C. and M. L. Stanton. 1989. Bumble bee pollination and floral morphology: factors influencing pollen dispersal in the alpine sky pilot, *Polemonium viscosum* (Polemoniaceae). *American Journal of Botany* 76:419-426.
- Galen, C. and M. L. Stanton. 2003. Sunny-side up: flower heliotropism as a source of parental environmental effects on pollen quality and performance in the snow buttercup, *Ranunculus adoneus* (Ranunculaceae). *American Journal of Botany* 90:724-729.
- García-Camacho, R. and Ø. Totland. 2009. Pollen limitation in the alpine: a meta-analysis. *Arctic, Antarctic, and Alpine Research* 41:103-111.
- Gardner, M. and M. Macnair. 2000. Factors affecting the co-existence of the serpentine endemic *Mimulus nudatus* Curran and its presumed progenitor, *Mimulus guttatus* Fischer ex DC. *Biological Journal of the Linnean Society* 69:443-459.
- Gay, G., C. Kerhoas, and C. Dumas. 1987. Quality of a stress-sensitive *Cucurbita pepo* L. pollen. *Planta* 171:82-87.
- Ghazoul, J. 2006. Floral diversity and the facilitation of pollination. *Journal of ecology* 94:295-304.
- Gilman, R. T., N. S. Fabina, K. C. Abbott, and N. E. Rafferty. 2012. Evolution of plant–pollinator mutualisms in response to climate change. *Evolutionary Applications* 5:2-16.
- Gomez, J. M., M. Abdelaziz, J. Lorite, A. Jesús Muñoz-Pajares, and F. Perfectti. 2010. Changes in pollinator fauna cause spatial variation in pollen limitation. *Journal of ecology* 98:1243-1252.
- Goodwillie, C., S. Kalisz, 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.
- Goulson, D. 1999. Foraging strategies of insects for gathering nectar and pollen, and implications for plant ecology and evolution. *Perspectives in Plant Ecology, Evolution and Systematics* 2:185-209.
- Grossenbacher, D. L. and J. B. Whittall. 2011. Increased floral divergence in sympatric monkeyflowers. *Evolution* 65:2712-2718.
- Haig, D. and M. Westoby. 1988. On limits to seed production. *The American Naturalist* 131:757-759.

- Hamrick, J. and M. Godt. 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 351:1291-1298.
- Harder, L. D. and M. A. Aizen. 2010. Floral adaptation and diversification under pollen limitation. *Philosophical Transactions of the Royal Society B: Biological Sciences* 365:529-543.
- Harrison, S., J. Maron, and G. Huxel. 2000. Regional turnover and fluctuation in populations of five plants confined to serpentine seeps. *Conservation Biology* 14:769-779.
- Hastie, T. and R. Tibshirani. 1990. *Generalized additive models*. Chapman and Hall/CRC.
- Hegland, S. J. and L. Boeke. 2006. Relationships between the density and diversity of floral resources and flower visitor activity in a temperate grassland community. *Ecological Entomology* 31:532-538.
- Hegland, S. J., A. Nielsen, A. Lázaro, A. L. Bjerknes, and Ø. Totland. 2009. How does climate warming affect plant-pollinator interactions? *Ecology Letters* 12:184-195.
- Hereford, J. 2009. A quantitative survey of local adaptation and fitness trade-offs. *The American Naturalist* 173:579-588.
- Herrera, C. M. 1988. Variation in mutualisms: the spatiotemporal mosaic of a pollinator assemblage. *Biological Journal of the Linnean Society* 35:95-125.
- Herrera, C. M. 1987. Components of pollinator quality: comparative analysis of a diverse insect assemblage. *Oikos* 50:79-90.
- Holtsford, T. P. and N. C. Ellstrand. 1992. Genetic and environmental variation in floral traits affecting outcrossing rate in *Clarkia tembloriensis* (Onagraceae). *Evolution* 46:216-225.
- Hughes, A. R., B. D. Inouye, M. T. Johnson, N. Underwood, and M. Vellend. 2008. Ecological consequences of genetic diversity. *Ecology Letters* 11:609-623.
- Jakobsson, A., B. Padrón, and A. Traveset. 2008. Pollen transfer from invasive *Carpobrotus* spp. to natives—A study of pollinator behaviour and reproduction success. *Biological Conservation* 141:136-145.
- Johnson, M. T. J. and J. R. Stinchcombe. 2007. An emerging synthesis between community ecology and evolutionary biology. *Trends in Ecology and Evolution* 22:250-257.
- Johnston, M. O. 1991. Natural selection on floral traits in two species of *Lobelia* with different pollinators. *Evolution* 45:1468-1479.

- 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.
- Kalisz, S., D. Vogler, B. Fails, M. Finer, E. Shepard, T. Herman, and R. Gonzales. 1999. The mechanism of delayed selfing in *Collinsia verna* (Scrophulariaceae). *American Journal of Botany* 86:1239-1247.
- Kearns, C. A. and D. W. Inouye. 1993. *Techniques for pollination biology*. University of Texas Press.
- Kelly, J. and H. Arathi. 2003. Inbreeding and the genetic variance in floral traits of *Mimulus guttatus*. *Heredity* 90:77-83.
- Knight, T. M. 2003. Floral density, pollen limitation, and reproductive success in *Trillium grandiflorum*. *Oecologia* 137:557-563.
- Knight, T. M., J. A. Steets, J. C. Vamosi, S. J. Mazer, M. Burd, D. R. Campbell, M. R. Dudash, M. O. Johnston, R. J. Mitchell, and T.-L. Ashman. 2005. Pollen limitation of plant reproduction: pattern and process. *Annual Review of Ecology, Evolution, and Systematics* 36:467-497.
- Kohn, J. and N. Waser. 1985. The effect of *Delphinium nelsonii* pollen on seed set in *Ipomopsis aggregata*, a competitor for hummingbird pollination. *American Journal of Botany* 72:1144-1148.
- Kruszewski, L. J. and L. F. Galloway. 2006. Explaining outcrossing rate in *Campanulastrum americanum* (Campanulaceae): Geitonogamy and cryptic self-incompatibility. *International Journal of Plant Sciences* 167:455-461.
- Kunin, W. E. 1997. Population size and density effects in pollination: pollinator foraging and plant reproductive success in experimental arrays of *Brassica kaber*. *Journal of ecology* 85:225-234.
- Kwak, M. M. and O. Jennersten. 1991. Bumblebee visitation and seedset in *Melampyrum pratense* and *Viscaria vulgaris*: heterospecific pollen and pollen limitation. *Oecologia* 86:99-104.
- Larson, B. M. and S. C. Barret. 2000. A comparative analysis of pollen limitation in flowering plants. *Biological Journal of the Linnean Society* 69:503-520.
- Larson, D., R. Royer, and M. Royer. 2006. Insect visitation and pollen deposition in an invaded prairie plant community. *Biological Conservation* 130:148-159.
- Lázaro, A., R. Lundgren, and Ø. Totland. 2009. Co flowering neighbors influence the diversity and identity of pollinator groups visiting plant species. *Oikos* 118:691-702.

- Ledesma, N. and N. Sugiyama. 2005. Pollen quality and performance in strawberry plants exposed to high-temperature stress. *Journal of the American Society for Horticultural Science* 130:341-347.
- Leonard, A. S., A. Dornhaus, 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.
- Littell, R. C., W. W. Stroup, and R. J. Freund. 2002. SAS for linear models. SAS Publishing.
- Lloyd, D. G. and D. J. Schoen. 1992. Self- and cross-fertilization in plants. I. Functional dimensions. *International Journal of Plant Sciences* 153:358-369.
- Lopezaraiza-Mikel, M., R. Hayes, M. Whalley, and J. Memmott. 2007. The impact of an alien plant on a native plant-pollinator network: an experimental approach. *Ecology Letters* 10:539-550.
- Lu, Y. 2000. Effects of density on mixed mating systems and reproduction in natural populations of *Impatiens capensis*. *International Journal of Plant Sciences* 161:671-681.
- Marshall, D. L., J. J. Avritt, S. Maliakal-Witt, J. S. Medeiros, and M. G. M. Shaner. 2010. The impact of plant and flower age on mating patterns. *Annals of Botany* 105:7-22.
- Martin, N. 2004. Flower size preferences of the honeybee (*Apis mellifera*) foraging on *Mimulus guttatus* (Scrophulariaceae). *Evolutionary Ecology Research* 6:777-782.
- McLernon, S. M., S. D. Murphy, and L. W. Aarssen. 1996. Heterospecific pollen transfer between sympatric species in a midsuccessional old-field community. *American Journal of Botany* 83:1168-1174.
- Memmott, J., P. G. Craze, N. M. Waser, and M. V. Price. 2007. Global warming and the disruption of plant-pollinator interactions. *Ecology Letters* 10:710-717.
- Mitchell, R. J., R. J. Flanagan, B. J. Brown, N. M. Waser, and J. D. Karron. 2009. New frontiers in competition for pollination. *Annals of Botany* 103:1403-1413.
- Moeller, D. A. 2004. Facilitative interactions among plants via shared pollinators. *Ecology* 85:3289-3301.
- Montgomery, B. R. and B. J. Rathcke. 2012. Effects of floral restrictiveness and stigma size on heterospecific pollen receipt in a prairie community. *Oecologia* 168:449-458.
- Moragues, E. and A. Traveset. 2005. Effect of *Carpobrotus* spp. on the pollination success of native plant species of the Balearic Islands. *Biological Conservation* 122:611-619.
- Morales, C. L. and A. Traveset. 2008. Interspecific pollen transfer: magnitude, prevalence and consequences for plant fitness. *Critical Reviews in Plant Sciences* 27:221-238.

- Morgan, M. T. and W. G. Wilson. 2005. Self-fertilization and the escape from pollen limitation in variable pollination environments. *Evolution* 59:1143-1148.
- Mosquin, T. 1971. Competition for pollinators as a stimulus for the evolution of flowering time. *Oikos* 22:398-402.
- Motten, A. F. and J. L. Stone. 2000. Heritability of stigma position and the effect of stigma-anther separation on outcrossing in a predominantly self-fertilizing weed, *Datura stramonium* (Solanaceae). *American Journal of Botany* 87:339-347.
- Muchhala, N., Z. Brown, W. S. Armbruster, and M. D. Potts. 2010. Competition drives specialization in pollination systems through costs to male fitness. *The American Naturalist* 176:732-743.
- Murcia, C. and P. Feinsinger. 1996. Interspecific pollen loss by hummingbirds visiting flower mixtures: effects of floral architecture. *Ecology* 77:550-560.
- Murphy, S. D. 2000. Field testing for pollen allelopathy: a review. *Journal of chemical ecology* 26:2155-2172.
- Murphy, S. D and L. Aarssen. 1995. In vitro allelopathic effects of pollen from three *Hieracium* species (Asteraceae) and pollen transfer to sympatric Fabaceae. *American Journal of Botany* 82:37-45.
- Murphy, S. D. and L. W. Aarssen. 1995. Reduced seed set in *Elytrigia repens* caused by allelopathic pollen from *Phleum pratense*. *Canadian Journal of Botany* 73:1417-1422.
- Nattero, J., A. Cocucci, and R. Medel. 2010. Pollinator-mediated selection in a specialized pollination system: matches and mismatches across populations. *Journal of Evolutionary Biology* 23:1957-1968.
- Neiland, M. and C. Wilcock. 1999. The presence of heterospecific pollen on stigmas of nectariferous and nectarless orchids and its consequences for their reproductive success. *Protoplasma* 208:65-75.
- 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.
- Olesen, J. M. and P. Jordano. 2002. Geographic patterns in plant-pollinator mutualistic networks. *Ecology* 83:2416-2424.
- Petanidou, T., A. S. Kallimanis, J. Tzanopoulos, S. P. Sgardelis, and J. D. Pantis. 2008. Long-term observation of a pollination network: fluctuation in species and interactions, relative invariance of network structure and implications for estimates of specialization. *Ecology Letters* 11:564-575.

- Porcher, E. and R. Lande. 2005. The evolution of self-fertilization and inbreeding depression under pollen discounting and pollen limitation. *Journal of Evolutionary Biology* 18:497-508.
- Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, and W. E. Kunin. 2010. Global pollinator declines: trends, impacts and drivers. *Trends in Ecology and Evolution* 25:345-353.
- Potts, S. G., B. Vulliamy, A. Dafni, G. Ne'eman, and P. Willmer. 2003. Linking bees and flowers: how do floral communities structure pollinator communities? *Ecology* 84:2628-2642.
- Price, M. V., N. M. Waser, R. E. Irwin, D. R. Campbell, and A. K. Brody. 2005. Temporal and spatial variation in pollination of a montane herb: a seven-year study. *Ecology* 86:2106-2116.
- Primack, R. B. 1985. Longevity of individual flowers. *Annual Review of Ecology and Systematics* 16:15-37.
- Pyke, G. H. 1978. Optimal foraging: movement patterns of bumblebees between inflorescences. *Theoretical Population Biology* 13:72-98.
- Qu, R., X. Li, Y. Luo, M. Dong, H. Xu, X. Chen, and A. Dafni. 2007. Wind-dragged corolla enhances self-pollination: a new mechanism of delayed self-pollination. *Annals of Botany* 100:1155-1164.
- Ramsey, M. and G. Vaughton. 2000. Pollen quality limits seed set in *Burchardia umbellata* (Colchicaceae). *American Journal of Botany* 87:845-852.
- Robertson, A., C. Mountjoy, B. Faulkner, M. Roberts, and M. Macnair. 1999. Bumble bee selection of *Mimulus guttatus* flowers: the effects of pollen quality and reward depletion. *Ecology* 80:2594-2606.
- Rosenthal, R. and R. L. Rosnow. 1985. Contrast analysis: focused comparison in the analysis of variance. Cambridge University Press.
- Ryan, S. E. and L. S. Porth. 2007. A tutorial on the piecewise regression approach applied to bedload transport data. US Department of Agriculture, Forest Service, Rocky Mountain Research Station Fort Collins, CO.
- Sandring, S. and J. Agren. 2009. Pollinator-mediated selection on floral display and flowering time in the perennial herb *Arabidopsis lyrata*. *Evolution* 63:1292-1300.
- Sargent, R. D. and D. D. Ackerly. 2008. Plant-pollinator interactions and the assembly of plant communities. *Trends in Ecology and Evolution* 23:123-130.

- Sargent, R. D., S. W. Kembel, N. C. Emery, E. J. Forrestel, and D. D. Ackerly. 2011. Effect of local community phylogenetic structure on pollen limitation in an obligately insect-pollinated plant. *American Journal of Botany* 98:283-289.
- Sargent, R. D. and S. P. Otto. 2006. The role of local species abundance in the evolution of pollinator attraction in flowering plants. *The American Naturalist* 167:67-80.
- SAS Institute. 2010. SAS/IML software. Version 9.2. SAS Institute, Cary, North Carolina, USA.
- Schemske, D. W. and C. C. Horvitz. 1984. Variation among floral visitors in pollination ability: a precondition for mutualism specialization. *Science* 225:519-521.
- Schuett, E. M. and J. C. Vamosi. 2010. Phylogenetic community context influences pollen delivery to *Allium cernuum*. *Evolutionary Biology* 37:19-28.
- Sletvold, N., J. M. Grindeland, and J. Ågren. 2010. Pollinator-mediated selection on floral display, spur length and flowering phenology in the deceptive orchid *Dactylorhiza lapponica*. *New Phytologist* 188:385-392.
- Smith, R. A. and M. D. Rausher. 2008. Experimental evidence that selection favors character displacement in the ivyleaf morning glory. *The American Naturalist* 171:1-9.
- Snow, A. and T. Spira. 1991. Differential pollen-tube growth rates and nonrandom fertilization in *Hibiscus moscheutos* (Malvaceae). *American Journal of Botany* 78:1419-1426.
- Stinchcombe J. R., C. Weinig, M. Ungerer, K. M. Olsen, C. Mays, S. S. Halldorsdottir, M. D. Purugganan and J. Schmitt. 2004. A latitudinal cline in flowering time in *Arabidopsis thaliana* modulated by the flowering time gene FRIGIDA. *Proceedings of the National Academy of Sciences* 13:4712-4717.
- Strauss, S. Y., J. A. Lau, and S. P. Carroll. 2006. Evolutionary responses of natives to introduced species: what do introductions tell us about natural communities? *Ecology Letters* 9:357-374.
- Strauss, S. Y. and P. Murch. 2004. Towards an understanding of the mechanisms of tolerance: compensating for herbivore damage by enhancing a mutualism. *Ecological Entomology* 29:234-239.
- Sukhada, D. and Jayachandra. 1980. Pollen allelopathy: a new phenomenon. *New Phytologist* 84:739-746.
- Sun, S.-G., Y.-H. Guo, R. Gituru, and S.-Q. Huang. 2005. Corolla wilting facilitates delayed autonomous self-pollination in *Pedicularis dunniana* (Orobanchaceae). *Plant Systematics and Evolution* 251:229-237.
- Thomann, M., E. Imbert, C. Devaux, and P.-O. Cheptou. 2013. Flowering plants under global pollinator decline. *Trends in Plant Science* 18:353-359.

- Thompson, J. D. 2001. How do visitation patterns vary among pollinators in relation to floral display and floral design in a generalist pollination system? *Oecologia* 126:386-394.
- Thomson, J., B. Andrews, and R. Plowright. 1981. The effect of a foreign pollen on ovule development in *Diervilla lonicera* (Caprifoliaceae). *New Phytologist* 90:777-783.
- Tjoelker, M., J. Craine, D. Wedin, P. Reich, and D. Tilman. 2005. Linking leaf and root trait syndromes among 39 grassland and savannah species. *New Phytologist* 167:493-508.
- Toms, J. D. and M. L. Lesperance. 2003. Piecewise regression: a tool for identifying ecological thresholds. *Ecology* 84:2034-2041.
- Totland, Ø. 2001. Environment-dependent pollen limitation and selection on floral traits in an alpine species. *Ecology* 82:2233-2244.
- Totland, Ø., H. L. Andersen, T. Bjelland, V. Dahl, W. Eide, S. Houge, T. R. Pedersen, and E. U. Vie. 1998. Variation in pollen limitation among plants and phenotypic selection on floral traits in an early-spring flowering herb. *Oikos* 82:491-501.
- Vamosi, J. C., T. M. Knight, J. A. Steets, S. J. Mazer, M. Burd, and T.-L. Ashman. 2006. Pollination decays in biodiversity hotspots. *Proceedings of the National Academy of Sciences of the United States of America* 103:956-961.
- Vamosi, J. C., J. A. Steets, and T.-L. Ashman. 2013. Drivers of pollen limitation: macroecological interactions between breeding system, rarity, and diversity. *Plant Ecology & Diversity* 6:1-10.
- Vanhoeacker, D., J. Ågren, and J. Ehrlén. 2006. Spatio-temporal variation in pollen limitation and reproductive success of two scape morphs in *Primula farinosa*. *New Phytologist* 169:615-621.
- Vellend, M. 2006. The consequences of genetic diversity in competitive communities. *Ecology* 87:304-311.
- Vellend, M. and M. A. Geber. 2005. Connections between species diversity and genetic diversity. *Ecology Letters* 8:767-781.
- Vickery Jr, R. 1978. Case studies in the evolution of species complexes in *Mimulus*. *Evolutionary biology* 11:405-507.
- Vogler, D. W. and S. Kalisz. 2001. Sex among the flowers: the distribution of plant mating systems. *Evolution* 55:202-204.
- Waser, N. L. Chittka, M. Price, N. Williams, and J. Ollerton. 1996. Generalization in pollination systems, and why it matters. *Ecology* 77:1043-1060.

- Waser, N. L. and M. Fugate. 1986. Pollen precedence and stigma closure: a mechanism of competition for pollination between *Delphinium nelsonii* and *Ipomopsis aggregata*. *Oecologia* 70:573-577.
- Waser, N. M. 1978. Competition for hummingbird pollination and sequential flowering in two Colorado wildflowers. *Ecology* 59:934-944.
- Watkins, L. and D. A. Levin. 1990. Outcrossing rates as related to plant density in *Phlox drummondii*. *Heredity* 65:81-89.
- Weber, A. and A. Kolb. 2012. Local plant density, pollination and trait–fitness relationships in a perennial herb. *Plant Biology* 15:335-343.
- Weller, S. and R. Ornduff. 1989. Incompatibility in *Amsinckia grandiflora* (Boraginaceae): distribution of callose plugs and pollen tubes following inter-and intramorph crosses. *American Journal of Botany* 76:277-282.
- Weller, S. G. and R. Ornduff. 1977. Cryptic self-incompatibility in *Amsinckia grandiflora*. *Evolution* 31:47-51.
- Wesselingh, R. A. 2007. Pollen limitation meets resource allocation: towards a comprehensive methodology. *New Phytologist* 174:26-37.
- Wiebes, J. 1979. Co-evolution of figs and their insect pollinators. *Annual Review of Ecology and Systematics* 10:1-12.
- Wilcock, C. and R. Neiland. 2002. Pollination failure in plants: why it happens and when it matters. *Trends in Plant Science* 7:270-277.
- Willis, J. H. 1993. Effects of different levels of inbreeding on fitness components in *Mimulus guttatus*. *Evolution* 47:864-876.
- Wolf, A., P. Brodmann, and S. Harrison. 1999. Distribution of the rare serpentine sunflower, *Helianthus exilis* (Asteraceae): the roles of habitat availability, dispersal limitation and species interactions. *Oikos* 84:69-76.
- Zhu, H. and A. U. Mallik. 1994. Interactions between kalmia and black spruce: isolation and identification of allelopathic compounds. *Journal of Chemical Ecology* 20:407-421.
- Zimmerman, M. 1981. Optimal foraging, plant density and the marginal value theorem. *Oecologia* 49:148-153.