PREZYGOTIC REPRODUCTIVE ISOLATION BETWEEN *MIMULUS GUTTATUS* AND *MIMULUS GLAUCESCENS*

A Thesis

Presented

to the Faculty of

California State University, Chico

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

in

Biological Sciences

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by

Jean-Phillippe W. Bergmann

Spring 2013

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ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to the following people, without whom this thesis never would have been possible.

-To the BCCER for your financial support, which greatly helped in acquiring greenhouse supplies and fuel for driving up and down Honey Run.

-To my advisor and friend Dr. Chris Ivey, for hours of engaging scientific discussion and entertaining all of my ideas. For molding this project into some semblance of sense, and for convincing me that *Mimulus* is indeed the best study system.

-To my colleague and friend Nicole Habecker and her husband Joe, for taking on the other half of this study, and for throwing the best potlucks ever.

-To Nate and Leah for allowing us to watch pollinators on the side of road in front of their house, while people driving by looked at us like we were crazy.

-To Carey Bruns, for assisting me in looking crazy on the side of the road, while waiting hours upon hours in the sun and rain for insects to visit us.

-To my wife Linda, family, and friends who incessantly asked me how my thesis was going, and faithfully put up with me along the way.

-And finally to my son Oscar, for always being the best distraction ever, and reminding me that there is more to life than just speciation.

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PREZYGOTIC REPRODUCTIVE ISOLATION BETWEEN

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Reproductive isolation is critical to speciation and therefore biodiversity. The immediate goal of such studies is to identify which barriers contribute most to overall reproductive isolation between species, in order to gain a broader understanding of the process of speciation. *Mimulus guttatus* and *M. glaucescens* have postzygotic barriers to hybridization, but their prezygotic barriers remain unstudied. I examined potential premating prezygotic barriers such as phenology, pollinator behavior and floral morphology, as well as possible post-pollination prezygotic barriers such as pollen adhesion, germination and pollen tube growth rates between species. We found no significant barriers to hybridization between species for any traits, although we did identify subtle differences in herkogamy and corolla tube width. We conclude that prezygotic barriers to reproduction between *M. guttatus* and *M. glaucescens* are weak, and contrast with evidence for postzygotic barriers such as reduced seed set and delayed flowering in hybrids. Additional research should focus on microhabitat preferences and a

population genetic analysis to determine whether introgression can occur between natural populations.

CHAPTER I

INTRODUCTION

The process of speciation is perhaps the most compelling aspect of the study of evolution. Natural selection is generally accepted as the mechanism of speciation, however the means by which new species are prevented from simply introgressing into generic homogeneity remains a compelling and exciting area of research. Species are broadly defined as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups" (Mayr 1963), which reduces to the singular question: what causes reproductive isolation and how is it maintained?

Barriers to hybridization can occur both prezygotically and postzygotically (Dobzhansky 1937), but any one barrier alone may not reduce gene flow sufficiently to maintain species boundaries. The total reduction in gene flow between two species results from an accumulation of barriers, where stronger barriers contribute more to the process of speciation than weak barriers. While multiple barriers normally contribute to overall isolation, prezygotic barriers can have a strong impact because they occur earlier in an organism's reproductive cycle (Ramsey 2003). For example, a study of *Mimulus cardinalis* and *M. lewisii* found that in regions where the species occurred sympatrically 97.6% of pollinator bouts were specific to one species or the other (Ramsey et al. 2003). In examples such as these, in which most of the isolation occurs before hybrid formation, the importance of barriers that could occur later in the reproductive cycle is greatly reduced. Therefore, postzygotic mechanisms can only influence reproductive isolation

when prezygotic barriers are incomplete. The circumstances by which these combinations of barriers arise are not fully understood, but examining how individual pre and postzygotic barriers combine to cause reproductive isolation between species allows us to determine which factors are most important in maintaining new species and bio-diversity.

Mimulus guttatus and *M. glaucescens* are closely related species (Beardsley 2004) occurring in Butte County, that have been found to exhibit postzygotic barriers to reproduction such as reduced seed set, inability to germinate F_1 seeds, and hybrid breakdown in experimental crosses (Vickery 1964). Not much is known, however, about the role of prezygotic barriers in maintaining their species identities. This study examines the contribution of several prezygotic reproductive barriers between two closely related yellow monkey-flowers, *Mimulus guttatus* and *M. glaucescens*, with the goal of revealing what factors maintain their discrete species identities.

CHAPTER II

LITERATURE REVIEW

The Role of Reproductive Isolation in Speciation

The importance of biodiversity to the stability and functioning of ecosystems is a subject of much concern in the world today (Purvis 2000). A necessary step in comprehending and preserving biodiversity is understanding speciation: the mechanism by which biodiversity arises. Darwin's *Origin of Species* (1859) sparked an interest in speciation which has been incredibly fruitful throughout the history of evolutionary study, spawning many great works such as Fischer's *The Genetical Theory of Natural Selection* (1930), Dobzhanky's *Genetics and the Origin of Species* (1937), and Mayr's *Systematics and the Origin of Species* (1942), which is perhaps the most widely used reference on what actually defines a species.

In his book, Mayr (1942) introduced the Biological Species Concept (BSC), and defined a species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups." Since Mayr's (1942) proposition of the BSC, there have been at least twenty-four recognized definitions of what constitutes a species (de Queiroz 2007). Regardless of the debate about how a species should be delimited, all viewpoints on the issue agree that speciation requires disruption of gene flow, or reproductive isolation.

Reproductive isolation is necessary for new species to evolve, but evolutionary biology has only begun to understand the mechanisms and interactions of how

reproductive barriers can combine to produce new distinct species (Dobzhansky 1937). The process of allopatric speciation – populations of a species becoming geographically isolated and subjected to differing selective pressures, supplemented by random mutations, and genetic drift – gives rise to genetic divergence and new species (Dobzhansky 1937, Mayr 1959). But what conditions are required to maintain these new species upon secondary contact? In short, genetic divergences accumulated within allopatry become barriers to introgression upon secondary contact, combining to reproductively isolate newly formed sister species. By studying barriers to reproduction among closely related species we can identify which traits are the most important in limiting gene flow during the early stages of speciation, and answer questions about how combinations of ecological and physiological barriers produce reproductively isolated species (Kay 2006).

Relative Contribution of Barriers

Individual barriers often convey only partial isolation between species. Barriers act in concert with one another, necessitating comprehensive studies that include as many barriers as possible. Ultimately the goal of any reproductive isolation study is to understand the relative contributions of each barrier to the overall isolation between species.

Barriers to reproduction disrupt gene flow sequentially during two organisms potential mating opportunities. Each barrier has an opportunity to decrease gene flow, with subsequent barriers acting upon the remaining gene flow left over from previous barriers. In this way, multiple barriers contribute to the total reproductive isolation

between species. These barriers can be divided into two categories, prezygotic and postzygotic, or those that occur before and after fertilization. Prezygotic barriers are considered to be more efficient contributors to reproductive isolation because they occur earlier in an organism's life history than postzygotic barriers, and are often quite strong individually, (Ramsey et al. 2003, Kay 2006). Presumably, the strongest barrier should be the most important in driving speciation.

Estimating Reproductive Isolation

Estimates of total reproductive isolation, and the relative contribution of combined barriers have historically followed methods set forth by Coyne and Orr (1989), which were later extended by Ramsey (2003). Total reproductive isolation (*T*) was given on a scale from 0 to 1, with 1 representing complete isolation, and 0 representing no reproductive isolation. These indices are of limited use because they do not account for disassociative mating. Furthermore the indices produced are non linear and cannot be easily compared among barriers to evaluate the magnitude of isolation (Sobel and Chen, unpublished). New formulae have recently been developed to address this issue, and now provide a linear way to examine total reproductive isolation and its relative contribution of its individual components.

Using the new formulae (Sobel and Chen unpublished) the amount of reproductive isolation (*RI*) conveyed by each barrier can be estimated as $RI = 1 - 2 * \left(\frac{H}{H + C} \right)$, where *H* is equal to the success of heterospecific matings, and *C* is equal to the success of conspecific matings. Values in this index range from -1 to 1,

where -1 indicates disassociative mating, 0 indicates random mating, and 1 indicates complete reproductive isolation. Additionally, these values are related in a linear fashion so that a barrier with a value of 1 is twice as strong as a barrier with a value of 0.5.

The actual contribution (*AC*) of each barrier can be calculated as $AC_n = RI_n\left(1 - \sum_{i=1}^{n-1} AC_i\right)$, where $AC_1=RI_1$. Total reproductive isolation (*T*) can be found as the sum of each barrier's *AC*, or $T = \sum_{i=1}^{m} AC_i$, and each barrier's relative contribution (*RC*) to overall reproductive isolation can be expressed as $RC_n = \frac{AC_n}{T}$

CHAPTER III

MATERIALS AND METHODS

Study System

The genus *Mimulus* is characterized by a wide range of morphological, physiological, and genetic variation (Vickery 1978). *Mimulus* was described by Grant (1924) as a group of 120 species in ten sections, the majority of which occur in North America. The Simiolus section of the genus is characterized by species with a high level of plasticity, and relatively low genetic distances (Beardsley et al. 2004). *Mimulus guttatus* is the most widespread, and likely the most diverse species in the section, ranging over most of the North American continent (Grant 1924). *Mimulus glaucescens*, a close relative, is rare in comparison and restricted to Northern California, primarily located in the Nevada, Butte, Tehama county (Vickery 1964). These species were selected because of two attributes: their close evolutionary relationship, and their proximity to each other in Northern California, both of which are features which make them ideal subjects to study reproductive isolation (Kay 2006).

Previous investigations of the reproductive compatibility between *M. guttatus* and *M. glaucescens* have suggested strong barriers to gene exchange between the species (Vickery 1964). These barriers were seen in the F_1 hybrid as reduced seed set, and in the F_2 as loss of vigor (measured as the length of time to plant flowering and plant height at flowering as compared to parental generation), and sterility. Based on these results,

combined with their morphological differences, the two have long been considered distinct species (Vickery 1964).

Phenological Isolation

To estimate the contribution of phenology to reproductive isolation, co-flowering field populations were located along Honey Run Road in Butte Creek canyon, Chico CA. The two species' close proximity in the canyon was ideal for studying their isolation while in near-sympatry, and also minimized environmental differences that could affect flowering phenology. The populations sampled were located within 400 meters of each other (Table 1). At the *M. guttatus* site, four 0.5m^2 quadrats were placed at non-random locations that had high plant densities, in order to ensure enough flowers to be representative of the entire population. The population of *M. glaucescens* was small enough that the entire population was included in data collection.

At the onset of flowering (April 20 2011), the number of flowering stems were counted between once and twice a week, until both populations had finished flowering and senesced (July 15 2011). Proportion of seasonal flowering was calculated as the number of flowering stems at a given date divided by the total number of senesced stems for the whole season. This provided a way to examine both the duration and dynamics of peak flowering – the date at which populations displayed the highest proportion of flowering over the entire season. Resulting proportion data for each species was compiled into a cumulative distribution and compared between populations using the Kolmogorov-Smirnov two-sample test with α=0.95.

Ecological Isolation due to Pollinator Behavior

To estimate reproductive isolation due to pollinator behavior, I observed floral visitor behavior in mixed-species arrays near natural populations of each species. Observations were made in the spring of 2010 in the Big Chico Creek Ecological Reserve (BCCER) near a population of *M. glaucescens*, and in 2011 in Butte Creek canyon near a population of *M. guttatus*. Arrays consisted of 36 plants (18 of each species) from natural populations of each species. The BCCER array consisted of local *M. glaucescens* plants arranged with *M. guttatus* plants collected from Coal Canyon Rd, whereas the Butte Creek canyon arrays were composed of *M. guttatus* plants from onsite, and *M. glaucescens* plants from populations along Honey Run Road and Centerville Road within 2 km from the study site (Table 1). Plants from these natural populations were transplanted into 10-centimeter square pots and placed into a 6 x 6 grid, in which alternating species were spaced at 0.5m in a checkerboard pattern. Corollas were removed from open flowers as needed so that each plant had only one open flower to avoid unequal floral display sizes. Thus, floral visitors should have had an equal chance of encountering flowers of either species. Arrays were placed approximately thirty meters away from natural populations of *M. guttatus* (2011) and *M. glaucescens* (2010), to ensure that naturally occurring pollinators would be nearby. Visitor taxon, number of visits, bout sequence (the sequence of plants visited by a single pollinator in a single visit), and duration of time spent at each flower was recorded.

Although specimens were collected and later identified, difficulty identifying visitors "on the wing" necessitated that visitors be placed into the following categories:

large bee fly, small bee fly, small fly, hover fly, honeybee, striped large bee, small black bee, big black bee, *Osmia sp*., paper wasp, small black beetle, and Elaterid beetle (more specific visitor identities can be found in Table 2). Visitation was observed and described in "visitation sequences": the sequence of flowers entered by a visitor during a single visit to the array. Observations were recorded in the following manner: When a visitor entered a corolla tube the visitor identity was recorded as well as flower species, and its duration inside the tube was timed with a stop-watch. If the pollinator moved to another flower this was recorded as a transition within the same visitation sequence and the time spent in the new flower was also recorded. When the pollinator left the array the visitation sequence was finished. Only visitation sequences containing transitions were used for comparison of visitor behavior, while visitation sequences containing only one flower were discarded. Each transition in a visitation sequence could then be classified as either conspecific or heterospecific. Data were analyzed using ANOVA including visitation sequence as a random effect.

Mechanical Isolation

If mechanical isolation contributed to species divergence, differences in morphology might be apparent. Morphological differences that could influence pollinator behavior or pollen placement were measured between *M. guttatus* and *M. glaucescens* flowers using plants grown from seed collected in eight populations in northern California (Table 1). These included corolla lower lobe width, corolla tube opening width, anther and pistil length, and herkogamy. Corolla width was measured as the widest point on the lower lobe of the corolla. The width of the corolla tube opening

was measured at the distal opening of the corolla tube, where the upper and lower lobes join. Anther length was measured from the bottom of the corolla tube to the tip of the longest anther, and herkogamy was measured as the distance from the tip of the longest anther to the widest point of the closed stigma lobes. Pistil length was then estimated as the sum of anther length and herkogamy. Traits were compared between species using *t*-tests.

Table 1. Sources of *Mimulus guttatus* and *M. glaucescens* seeds grown in the CSUC greenhouse for use in floral measurements and post-pollination prezygotic isolation experiments. The two Honey Run populations were also surveyed for phenological comparison.

Species	Site	Lat/Long	Elevation
M. guttatus	Cherry Hill Meadows	40°06'N, 121°29'W	1435 m
	Coal Canyon Road	39°36'N, 121°36'W	147 m
	Dry Creek	39°37'N, 121°37'W	79 m
	Honey Run	39°44'N, 121°40'W	165m
	Table Mountain	39°35'N, 121°32'W	362 m
M. glaucescens	Honey Run	39°44' N, 121°40'W	167 m
	Salmon Hole	39°46'N, 121°44'W	147 m
	Sandstone Glade	39°51'N, 121°42'W	250 m
	Simmon's Loop	39°49'N, 121°42'W	530 m

Post-Pollination Prezygotic Isolation

To investigate the contribution of barriers that may limit hybridization after pollination but before fertilization, pollen adhesion, pollen germination, and pollen tube growth rate were compared between conspecific and hybrid hand-pollinations. Each of these assays was conducted in a semi *in vitro* experiment. To be sure that results were not due to localized population traits, and were in fact general species traits, two pairs of populations of each species from different regions of Butte Creek canyon were used. The two pairs were separated by 3.2km, with populations within pairs roughly 400 meters

apart. This resulted in three cross categories: Within-population crosses (conspecific), between-population crosses (conspecific) and heterospecific (hybrid) crosses.

Each assay used plants grown from seeds collected in four populations growing in Butte Creek canyon except the pollen tube growth rate comparisons, which were supplemented with plants from the morphology component of this study. For each component listed below, 400 plants (100 from each of the four populations) were grown in the greenhouse at CSU Chico State, with supplemental light from 400W sodium vapor lamps (18 hour photoperiod). Plants were grown in two-inch pots filled with potting soil and placed in trays. Flowers to be used as pollen recipients were emasculated in bud by pulling the corolla and anthers off with forceps as soon as corollas were mature enough to remove. Recipients were pollinated by a single donor each, chosen at random from one of the four populations. Each assay was analyzed with ANOVA, and groups of crosses were tested for equal variance using Bartlett's test of homogeneity of variances.

Adhesion

Differences in pollen adhesion between species could contribute to reproductive isolation by reducing the success of pollination in mixed pollen loads. If so, conspecific pollen should be more adept at adhering to the stigma than heterospecific pollen. A technique modified from Zinkl (1999) was used to examine adhesion, in which pollen deposition was carefully quantified. Pollen was shaken from anthers onto a clean microscope slide, and, while observing under a dissection microscope, excess pollen was removed until approximately 100 grains remained. The number of pollen grains on the slide was recorded. The pistil of the pollen recipient was then removed from the flower and the stigma was used to sweep the pollen grains from the slide. Pollen grains

remaining on the slide were counted and subtracted from the original number of grains on the slide to accurately determine the number of pollen grains deposited.

Pollen grains were allowed to adhere for one minute before stigmas were placed in a microcentrifuge tube with 200 µl of 50µM KPO_4 with 1% Tween-20, vortexed for 5 seconds, and then centrifuged for one minute at 7200 rpm. Pistils were then fixed in a solution of 9:1 ethanol:glacial acetic acid. Stigmas were stained with basic fuchsin dye and viewed at 100x. Proportion of grains deposited that adhered was compared among cross categories using one-way ANOVA.

Germination

To examine pollen germination as a reproductive barrier, pollen germination percentages were obtained using a technique modified from Martin (1959) and Goodwillie (1997). Plants were grown consistent with the methods outlined above. Pollinations were performed by inserting clean micro-forceps roughly a millimeter into a mature anther, then placing the pollen coated forceps between lobes of recipient stigmas. In this manner, 100-500 pollen grains were transferred to the recipient stigma. Pollen was allowed to adhere and germinate for 6-12 hours, before pistils were harvested and fixed in a 3:1 solution of 95% ethanol and glacial acetic acid for 24 hours. Pistils were then rinsed with deionized water and stored in 70% ethanol until observation. After clearing in 70°- 80°C 1 M NaOH for 2 minutes, pistils were rinsed in deionized water and stained in decolorized 0.1% aniline blue in 0.033 M K_3PO_4 for 24 hours. Pistils were then mounted on slides with a drop of aniline blue, gently squashed with a coverslip, and viewed under epifluorescence at 100x with a compound microscope. Proportion of pollen grains germinated was compared among cross categories using ANOVA.

Pollen tube growth rate

Pollen tube growth rates were compared using plants grown as described above. To perform pollinations, a single anther from the assigned pollen donor was gently pressed into the stigma of recipient flowers, which had been emasculated in bud. After five minutes of germination, the styles were cut cleanly from pistils with a sharp razor with stigmas attached, and placed on 1% agar enriched with 0.3 M sucrose, 1.6 mM H_3BO_3, 1% Bacto Agar, 2 mM $CaCl_2$, and 1 mM $MgSO_4 \cdot 7H_2O$.

Style lengths were measured to the nearest 0.1mm using a dissection microscope fitted with a graduated ocular lens. Plates were then monitored, and the time until pollen tubes were observed emerging from the cut end of the style was recorded. Rate of pollen tube growth was then calculated as the time for pollen tubes to exit style divided by length of cut style. Rates were compared among categories of cross type using ANOVA.

CHAPTER IV

RESULTS

Phenological Isolation

Both species began flowering on April 20, 2011. *Mimulus glaucescens* flowered for 59 days, reached its peak (highest proportion of flowering stems over the entire season) of flowering on May 11, and all flowers had senesced by June 22. *M. guttatus* flowered for 80 days, reached its peak of flowering on June 15 and lasted until July 8. As a consequence, *M. glaucescens* flowering completely overlapped with *M. guttatus,* whereas *M. guttatus* had 21 days of isolated flowering at the end of its season (Fig. 1). Although the two species did not have identical flowering durations, the two flowering distributions did not significantly deviate from each other (Kolmogorov-Smirnov, $D^{+}=0.33$, $p>0.05$) suggesting that phenological differences do not contribute to reproductive isolation.

Ecological Isolation due to Pollinator Behavior

Over two seasons and 67 total hours of observation of experimental arrays, 533 individual flowers were visited in 312 recorded visitation sequences. Of those flowers visited, 261 (49%) were *M. guttatus* and 272 (51%) were *M. glaucescens*. The combined total visitation rate was 7.85 individual flower visits/hour, with *M. guttatus* flowers receiving 3.84 visits/hour and *M. glaucescens* flowers receiving 4.01 visits/hour (Table 2).

Visitors moved between plants on 223 occasions (Table 3). One-hundred one of those between plant movements (45.3%) were conspecific, and 122 (54.7%) were

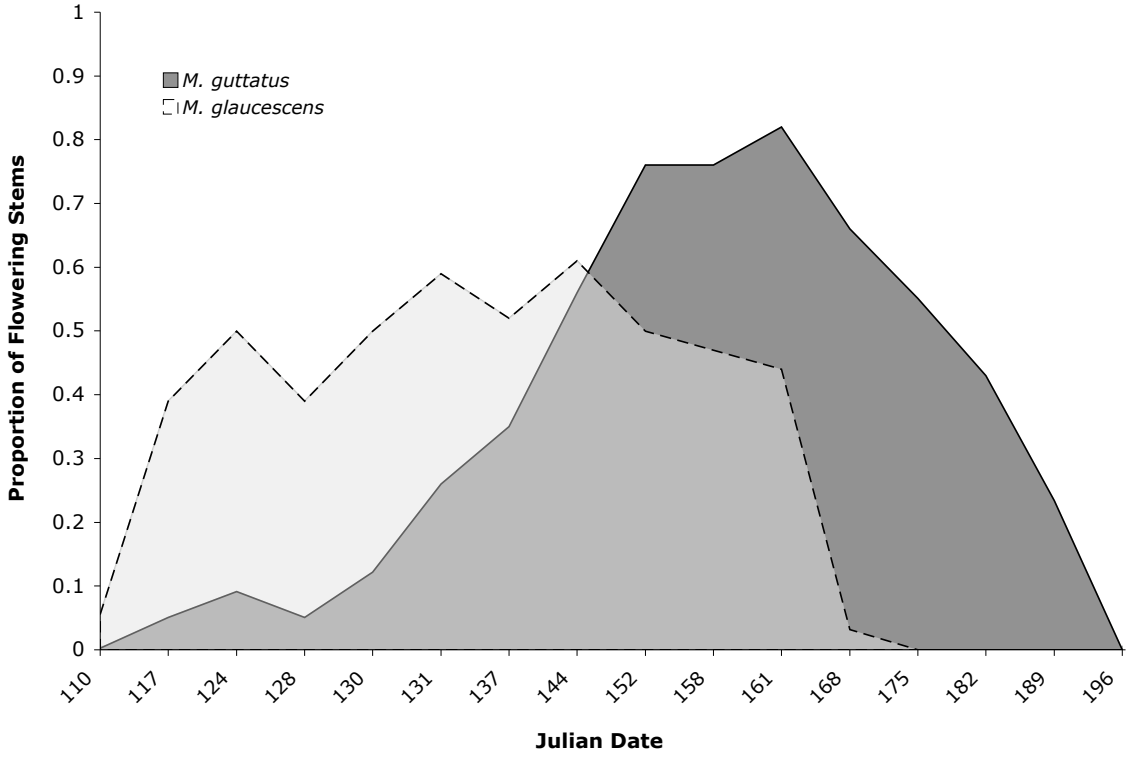

Figure 1. Proportion of seasonal total flowering stems in one population each of *Mimulus glaucescens* and *M. guttatus* in Butte Creek Canyon, Chico California from April 20 to July 8 2011. Proportion of flowering was calculated as number of flowering stems at each date divided by total senesced stems at the end of flowering.

heterospecific. Fifty-one conspecific visits occurred between *M. guttatus* and 50 between *M. glaucescens* individuals. Of the heterospecific visits, 60 were from *M. guttatus* to *M. glaucescens*, and 62 were from *M. glaucescens* to *M. guttatus*. Visitors moved from *M. glaucescens* to *M. guttatus* an average (SE) of 55.9% (4.8) of the time, and from *M. guttatus* to *M. glaucescens* and average (SE) of 54.0% (4.7) of the time, however the

difference was not statistically significant ($F_{1,1} = 0.08$, $P = 0.8$), therefore these values were not included in estimates of reproductive isolation.

Table 2. The number and average duration of *Mimulus guttatus* and *M. glaucescens* visits by insects in mixed arrays in BCCER and Butte Creek Canyon during 2010 and 2011. Visitation rate for each type of visitor calculated as number of visits divided by 67 total hours of observation. Average duration of visit (seconds) shown in parentheses.

	Number of flowers visited		Visitation rate (visits/hr)		
Visitor Identity	*M. guttatus*	*M. glaucescens*	*M. guttatus*	*M. glaucescens*	Total
Coleoptera					
Acmaeodera sp.	1 (120)	1 (8)	0.01	0.01	0.02
Anthaxia sp.	21 (223.8)	20 (268.5)	0.31	0.30	0.61
Cantharidae sp.	-	1 (95)	-	0.01	0.01
Elateridae	1 (50)	2 (255)	0.01	0.03	0.04
Gnathacmaeops pratensis	5 (49.6)	5 (53.2)	0.07	0.07	0.14
Diptera					
Black Fly	1 (2)	7 (86.4)	0.01	0.10	0.11
Bombylius major	4 (14.3)	1 (2)	0.06	0.01	0.07
House Fly	3 (8.7)	2 (342)	0.04	0.03	0.07
Small Fly	5 (25.2)	5 (51.8)	0.07	0.07	0.14
Syrphidae (Large)	27 (12.8)	26 (9.4)	0.40	0.39	0.79
Syrphidae (Small)	15 (34.3)	27 (14.5)	0.22	0.40	0.62
Hemiptera					
Cicadellidae	-	1 (20)	-	0.01	0.01
Small Hemipteran	-	2 (300)	-	0.03	0.03
Hymenoptera					
Apis mellifera	18 (41.6)	13 (10.1)	0.27	0.19	0.46
Bombus sp.	9 (5)	2 (5.5)	0.13	0.03	0.16
Lasioglossum sp.	109 (35.9)	124 (46.5)	1.63	1.85	3.48
Osmia sp.	39 (6.2)	29 (8.3)	0.58	0.43	1.01
Polistes sp.	1 (1)	3 (3.3)	0.01	0.04	0.05
Striped Large Bee	1 (10)	1 (2)	0.01	0.01	0.02
Striped Small Bee	1 (5)	-	0.01	-	0.01
Totals	261 (32.27)	272 (79.08)	3.84	4.01	7.85

Table 3. Summary of insect visitor movements between *Mimulus guttatus* and *M. glaucescens* flowers in mixed arrays. Visitors were recorded if they entered the corolla tube of a flower and visually identified. Observations took place at the BCCER and Butte Creek Canyon during 2010 and 2011. Species name in the heterospecific transition columns refer to the initial species visited during a transition event.

	Transition Category				
	Conspecific		Heterospecific		
Visitor ID	*M. guttatus*	*M. glaucescens*	*M. guttatus*	*M. glaucescens*	Total
Apidae	0	0	1	0	1
Apis mellifera	8	3	2	4	17
Bombus sp.	3	0	0	0	3
Bombylius major	1	0	1	0	2
Buprestidae	1	0	0	1	2
Diptera	0	1	0	0	1
Elateridae	0	1	0	0	1
Lasioglossum sp.	24	29	27	31	111
Osmia sp.	10	6	17	16	49
Polistes sp.	0	1	0	1	2
Syrphidae (Large)	3	2	6	6	17
Syrphidae (Small)	1	7	6	3	17
Totals	51	60	50	62	223

Mechanical Isolation

Corolla tube opening width of first node flowers was 5.5% greater in *M. guttatus* than *M. glaucescens* (Table 4). *Mimulus guttatus* had 13.6% greater anther-stigma separation than *M. glaucescens*. No other characters measured differed significantly between species.

Post-Pollination Prezygotic Isolation

No significant differences were found among all possible crosses – within population and out of population conspecific, and heterospecific (hybrid) – in any of the components of post-pollination/prezygotic stage of reproduction (Table 5). Therefore these components do not appear to contribute to overall reproductive isolation between

species. Bartlett's test results showed equal variances among all crosses in each assay (germination: $k^2 = 7.4183$, $df = 3$, $p > 0.05$, adhesion: $k^2 = 1.1016$, $df = 5$, $p > 0.05$, pollen tube growth rate: $k^2 = 4.1595$, $df = 3$, $p > 0.05$).

Table 4. Mean (SD) of floral traits of *M. guttatus* and *M. glaucescens* from multiple northern California populations (see Table 1) grown in a greenhouse. Traits in bold differ significantly between species at alpha = 0.05 in a two-tailed t-test.

	Species				
Character	*Mimulus glaucescens*	n	*Mimulus guttatus*	n	*t*
Corolla tube opening width	**12.32 (1.72)**	**59**	**13.02 (1.39)**	**44**	**2.22**
Corolla lower lobe width	28.86 (5.02)	59	28.57 (3.51)	42	-0.34
Anther length	22.52 (3.72)	46	21.04 (3.58)	28	1.69
Pistil length	26.37 (4.13)	46	25.32 (3.76)	28	-1.09
Herkogamy	**3.83 (1.21)**	**59**	**4.39 (0.84)**	**44**	**2.80**

Prezygotic Reproductive Isolation

None of the traits measured, with the possible exception of floral morphology, differed significantly between species or between hybrid vs. conspecific crosses. Therefore, based on the barriers measured, there appears to be no significant barrier to gene flow between species.

Table 5. Mean (SE) percent pollen adhesion, percent germination and pollen tube growth rate from hand-pollinations among *Mimulus glaucescens* and *M. guttatus* plants grown in the greenhouse. Also included are results from one-way ANOVA comparing means among types of crosses within each species.

Component	Species	F	P	Cross								
				Within-populations			Between-populations			Hybrid		
					n	Grains deposited		n	Grains deposited		n	Grains deposite
Pollen Adhesion (%)	*M. glaucescens*	$F_{5,88} = 1.66$	0.15	64.7 (7.82)	11	1,193	62.1 (9.60)	6	645	56.32 (7.86)	20	2,336
	M. guttatus			37.0 (7.76)	16	1,741	52.6 (7.83)	13	1,461	49.4 (4.95)	28	3,069
Pollen Germination (%)	*M. glaucescens*	$F_{5,111} = 0.85$	0.52	14.7 (1.93)	15	6,848	15.2 (2.53)	20	4,512	16.7 (1.83)	26	8,563
	M. guttatus			20.1 (2.53)	15	3,846	14.3 (1.73)	10	2,786	18.9 (2.46)	31	10,062
Pollen Tube Growth Rate (mm/min)	*M. glaucescens*	$F_{5,71} = 1.28$	0.28	0.019 (0.002)	7	--	0.019 (0.001)	11	--	0.020 (0.001)	17	--
	M. guttatus			0.024 (0.002)	9	--	0.022 (0.002)	12	--	0.021 (0.001)	21	--

CHAPTER V

DISCUSSION

Studies of reproductive isolation attempt to identify what traits reduce gene flow, and thereby mediate the process of speciation. Increasingly, the importance of measuring the combined effect of multiple barriers has been recognized, as usually a single barrier is not sufficient for complete isolation (Ramsey et al. 2003; Kay 2006). Even so, there is little general agreement about which traits are likely to evolve first, perhaps owing to the relative paucity of comprehensive studies (Widmer 2009; Sobel et al. 2010). In the genus *Mimulus,* a variety of prezygotic traits have been implicated in speciation (Schemske & Bradshaw 1999; Diaz and Macnair 1999; Martin and Willis 2007). Surprisingly, therefore, I find little evidence for prezygotic isolation between the taxa studied herein.

The mechanisms measured in this study were demonstrated to impose reproductive isolation in other species of *Mimulus*. Previous studies of other species found strong isolation resulting from ecogeographic differences, flowering asynchrony, pollinator fidelity, and pollen competition. However, in contrast to the taxa in my project, these earlier studies involved species that differed starkly in floral characteristics or mating system (Diaz and Macnair 1999; Martin and Willis 2007). My results suggest that these characteristics do not significantly differ between *M. guttatus* and *M. glaucescens*, thereby providing an opportunity to identify other mechanisms that maintain boundaries between closely related *Mimulus*.

Schemske and Bradshaw (1999) found that differences in floral morphology associated with pollinator preferences maintained isolation between *Mimulus cardinalis* and *M. lewisii*. I found no evidence for pollinator discrimination between *M. guttatus* and *M. glaucescens,* perhaps because of similarities in their floral characteristics. Close measurement of flowers revealed that corolla tubes were 5.5% wider and herkogamy 13.6% greater in *M. guttatus*. Although these differences were relatively modest, they may contribute to some reduction in gene flow. Contrasts in herkogamy have been found to reduce pollen movement between other species by preventing heterospecific pollen deposition or enforcing self-pollination with low herkogamy, in some cases prior to anthesis (Smith and Rausher 2007; Martin and Willis 2007). Similarly, differences in corolla tube width can limit visitation by certain pollinators (Castellanos et al. 2004). I was unable to estimate the extent to which differences in floral morphology limit gene flow between *M. guttatus* and *M. glaucescens*, so this may be a stage worthy of attention for future studies. Given the small scale of the difference involved (< 1 mm), and the apparent lack of species discrimination by pollinators, I predict any impact on reproductive isolation to be relatively small.

Differences in pollen-pistil compatibility or other aspects of post-pollination prezygotic performance can also limit gene flow. Diaz and Macnair (1999), for example, found that while there was no difference in pollen germination, differences in pollen tube growth rate provided a complete barrier to hybridization between *M. guttatus* and *M. nasutus* in which *M. guttatus* pollen had a competitive advantage in mixed pollen loads. This difference was attributed to the species' mating strategies. Fast pollen growth is often selected for in outcrossing species such as *M. guttatus*, where competition for

limited ovules can be high (Stephenson and Bertin 1983). Differences in pollen tube growth rate between species can also reflect differences in style length between species. Kay (2006), for example, found that pollen tubes of the shorter-styled *Costus scaber* failed to reach the ovaries of the long-styled *C. pulverulentus* in interspecific crosses. In contrast, pollen of *C. pulverulentus* has lower rates of adhesion and germination when used in interspecific crosses involving *C. scaber*. Thus, post-pollination prezygotic mechanisms can be asymmetrical, both in magnitude and in the traits involved (Kay 2006; Martin and Willis 2007; Yost and Kay 2009). *M. guttatus* and *M. glaucescens* have similar flowers which may explain why no reproductive isolation due to pollen-pistil interactions was observed.

In the absence of the barriers discussed above, it follows that if *M. guttatus* and *M. glaucescens* are distinct species, other barriers must be limiting gene flow. Habecker (2012) investigated postzygotic performance in hybrid seed set, seed germination, phenology, total flower production, biomass, ovule counts, and pollen viability. Significant reproductive isolation was found in F_1 seed set, and reproductive phenology of hybrids (Table 6), where within-population crosses of *Mimulus glaucescens* had 37.5 % higher F_1 seed set than hybrids, showing a reduction in female fitness of hybrids. In hybrids where *M. guttatus* was the maternal parent, flowering was delayed by 21.7% compared to intraspecific crosses, possibly indicative of reduced fitness in hybrids.

In addition Habecker (2012) used herbarium records and modeling and reported a significant amount of ecogeographic isolation between *M. guttatus* and *M. glaucescens* (Table 6). The study predicted that up to 45% of *M. glaucescens*' gene flow and 55% of *M. guttatus*' gene flow were restricted due to habitat differences between species. These

differences were attributed primarily to historical geologic conditions, and mirrored the Jepson ecogeographic regions of California (Hickman 1993).

Ecogeographic effects on reproductive isolation are well documented. One study found that elevational differences conferred 58.7% of the reproductive isolation between *Mimulus cardinalis* and *Mimulus lewisii* (Ramsey et al. 2003). Ecogeographic factors such as soil composition and moisture can also influence phenology, and have been reported to coincide with divergence in the onset and duration of flowering between species (Galloway 1995, Inouye 2003, Hall & Willis 2006, Kiang 1978, Peñuelas 2004). Species in areas of higher precipitation and soil moisture tend to lengthen their flowering duration, especially in Mediterranean climates (Peñuelas 2004) such as Butte Creek Canyon. Hall and Willis (2006) found that drier soils select for earlier flowering in *M. guttatus*, and that continually moist sites select for later flowering. They concluded that differences in flowering imposed by soil moisture were contributing to reproductive isolation between populations.

With these findings in mind, it is worth exploring the extent to which *M. guttatus* and *M. glaucescens* have adapted to differing environments. Some evidence of this comes from Banchero's (1987) observation that *M. glaucescens* tends to occur annually along canyon walls that dry sooner than the soils in which *M. guttatus* is found. He speculated that *M. glaucescens'* glaucous bracts are an adaptation to the drier more ephemeral habitats in which it occurs. While *M. guttatus* can also grow as a strict annual in drying soils, it is frequently found growing as a facultative perennial in soils that stay moist throughout the year, giving it a wider distribution than *M. glaucescens* (Banchero 1987, Vickery 1959, Lowry and Willis, 2010, Hickman 1993, Grant 1924).

If the assertions that *M. guttatus* and *M. glaucescens* tend to occur in habitats with different conditions are correct, it may be reasonable to suspect that greater reproductive isolation occurs in nature than was found in our study. A fine scale study of habitat including geologic and hydrologic characterization of population habitats would be helpful in resolving the extent to which habitat differences contribute to reproductive isolation in these species. Additionally, while this study did not find significant phenological differences between species, it is worth noting that only one population from each species was surveyed. Long-term phenological comparisons of multiple populations from a broader geographic area are needed to draw stronger conclusions about their reproductive isolation due to flowering asynchrony.

Another worthy scenario to consider is that *M. guttatus* and *M. glaucescens* may be introgressing more than was previously assumed. In laboratory crosses, hybridization success is high in maternal *M. guttatus* with no reduction in seed set, and a 37.5% reduction in seed set for *M. glaucescens* (Habecker 2012). However gene transfer has not been measured between natural populations located close to one another. Using molecular techniques such as allozyme and restriction fragment length polymorphism analysis, Nason et al. (1992) were able to distinguish first, second and later generation hybrids in natural populations of closely related species of oak (*Quercus kelloggii* and *Q. wislenzenii* var. *frutescens*), manzanita (*Arctostaphylos patula* and *A. viscida*) and iris (*Iris fulva* and *I. hexagona*), allowing for accurate estimates of introgression. A survey using these techniques could be used to determine the degree to which gene exchange is occurring between *M. guttatus* and *M. glaucescens*.

CHAPTER VI

CONCLUSIONS

Prezygotic barriers to reproduction are efficient in preventing hybridization because they limit opportunities for gene flow prior to the formation of zygotes. Although this study looked at several stages of prezygotic reproduction, none were found to impart a significant barrier to hybridization between *M. guttatus* and *M. glaucescens*. In contrast, a related study investigating postzygotic hybrid performance estimated between 62-68% reduction in gene flow from breakdown in hybrid fitness, and predicted a strong role of geography in prezygotically isolating *M. guttatus* and *M. glaucescens* (Habecker 2012). Further research regarding boundaries between these species should include a long-term phenology survey including more populations, a microhabitat comparison as well as a population genetics survey to estimate introgression.

Literature Cited

Beardsley, P. M. and S. E. Schoenig and J. B. Whittall, and R. G. Olmstead. 2004. Patterns of evolution in Western North American *Mimulus* (Phrymaceae). Am. J. Bot. 91:474-489.

Brown, W. L., and E. O. Wilson. 1956. Character displacement. Systematic Biology 5:49-64.

Callahan, J. T. 1984. Long-term ecological research. BioScience 34:363-367.

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

Coyne, J., and H.A. Orr. 1989. Patterns of speciation in *Drosophila*. Evolution 43:362-381.

Coyne, J., and H.A. Orr. 1997. "Patterns of speciation in *Drosophila*" revisited. Evolution 51:295-303.

Darwin, C.1859. The origin of species by means of natural selection. Reprinted by The Modern Library, Random House, New York.

de Queiroz, K. 2007. Species concepts and species delimitation. Syst. Biol. 56:879-886.

Dobzhansky, T. 1937. Genetics and the Origin of Species. Columbia Univ. Press, New York.

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

Galloway, L.F. 1995. Response to natural environmental heterogeneity: maternal effects and selection on life-history characters and plasticities in *Mimulus guttatus*. Evolution 49:1095-1107.

Gathmann, A., and T. Tscharntke. 2002. Foraging ranges of solitary bees. Journal of Animal Ecology 71:757-764.

Goodwillie, C. 1997. The genetic control of self-incompatibility in *Linanthus parviflorus* (Polemoniaceae). Heredity 79:424-432.

Grant, A.L. 1924. A monograph of the genus *Mimulus*. Annals of the Missouri Botanical Garden 11:99-388.

Guédot, C., J. Bosch, and W.P. Kemp. 2009. Relationship between body size and homing ability in the genus *Osmia* (Hymenoptera; Megachilidae). Ecological Entomology 34:158–161.

Grossenbacher, D.L., and J.B. Whittall. 2011. Increased floral divergence in sympatric monkeyflowers. Evolution 65:2712–2718

Habecker, N. 2012. Ecogeographical and intrinsic postzygotic isolation between Mimulus Glaucescens and Mimulus Guttatus. (Master's Thesis) Retrieved from CSU, Chico Research Station.

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

Heslop-Harrison, Y., and K.R. Shivanna. 1977. The receptive surface of the angiosperm stigma. Annals of Botany 41:1233-1258.

Heslop-Harrison, Y. 1981. Stigma characteristics and angiosperm taxonomy. Nordic Journal of Botany 1:401-420.

Heslop-Harrison, Y. 2000. Control gates and micro-ecology: the pollen-stigma interaction in perspective. Annals of Botany 85:5-13.

Hickman, J. C (ed.) 1993. The Jepson manual: higher plants of California. University of California Press, Berkeley.

Husband, B.C., and H.A. Sabara. 2003. Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (Onagraceae). New Phytol. 161:703-713.

Inouye, D.W., F. Saavedra, and W. Lee-Yang. 2003. Environmental influences on the phenology and abundance of flowering by *Androsace septentrionalis* (Primulaceae). Am. J. Bot. 90:905-910.

Johnson, S. D., and K. E. Steiner. 2000. Generalization versus specialization in plant pollination systems. TREE 15:140-143.

Kay, K. M. 2006. Reproductive isolation between two closely related hummingbird-pollinated neotropical gingers. Evolution. 60:538-552.

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

Kraus, F.B., S. Wolf, and R.F.A. Moritz. 2009. Male flight distance and population substructure in the bumblebee *Bombus terrestris*. Journal of Animal Ecology 78:247–252.

Levin, D. 1985. Reproductive character displacement in *Phlox*. Evolution 39:1275-1281.

Lowry, D. B., R. C. Rockwood, and J. H. Willis. 2008a. Ecological reproductive isolation of coast and inland races of *Mimulus guttatus*. Evolution 69: 2196 – 2214.

Lowry, D. B., J. L. Modliszewski, K. M. Wright, C. A. Wu, and J. H. Willis. 2008b. The strength and genetic basis of reproductive isolating barriers in flowering plants. Phil. Trans. R. Soc. B. 363: 3009 – 3021.

Lowry, D. B., and J. H. Willis. 2010. A widespread chromosomal inversion polymorphism contributes to a major life-history transition, local adaptation, and reproductive isolation. PLoS Biol. 8:1-14.

Martin, F. M. 1959. Staining and observing pollen tubes in the style by means of fluorescence. Stain Technol. 34:436-437.

Mayr, E. 1942. Systematics and the origin of species. Columbia Univ. Press, New York.

Mayr, E. 1959. Isolation as an evolutionary factor. Proc. Am. Philos. Soc. 103:221-230.

Nason, J.D., N.C. Ellstrand, and M.L. Arnold. 1992. Patterns of hybridization and introgression in populations of oaks, manzanitas, and irises. American Journal of Botany 79:101-111.

Peñuelas, J., I. Filella, X. Zhang, L. Llorens, R. Ogaya, F. Lloret, P. Comas, M. Estiarte, and J. Terradas. 2004. Complex spatiotemporal phenological shifts as a response to rainfall changes. New Phytologist 161:837-846.

Plitmann, U., and D. A. Levin. 1983. Pollen-pistil relationships in the Polemoniaceae. Evolution 37:957-967.

Purvis, A., and A. Hector. 2000. Getting the measure of biodiversity. Nature 405:212-219.

Ramsey, J., H. D. Bradshaw, and D. W. Schemske. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). Evolution 57:1520-1534.

Ritland, C., and K. Ritland. 1989. Variation of sex allocation among eight taxa of the Mimulus guttatus species complex (Scrophulariaceae). Am. J. Bot. 76:1731–1739.

Schemsky, D.W. 1999. Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). Proc. of the Nat. Acad. Sci. 96:11910-11915.

Smith, R. A., M. D. Rausher. 2007. Close clustering of anthers and stigma in *Ipomoea hederacea* enhances prezygotic isolation from *Ipomoea purpurea*. New Phytologist 173:642-647

Smith, R. A., M. D. Rausher. 2008. Experimental evidence that selection favors character displacement in the ivyleaf morning glory. The American Naturalist 171:1-9.

Vickery, R.K., 1959. Barriers to gene exchange within *Mimulus guttatus* (Scrophulariaceae). Evolution 13:300-310.

Vickery, R.K., 1964. Barriers to gene exchange between members of the *Mimulus guttatus* complex (Scrophulariaceae). Evolution 18:52-69.

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

Zinkl, G. M., B.I. Zwiebel, D.G. Grier, and D. Preuss. 1999. Pollen-stigma adhesion in *Arabidopsis*: a species-specific interaction mediated by lipophilic molecules in the pollen exine. Development 126:5431-5440.