WHAT CONSTRAINS DIRECTIONAL SELECTION

ON COMPLEX TRAITS IN THE WILD?

BY

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I am indebted to many of my fellow students for enriching my five-years of graduate school, intellectually and socially. I thank the members of the Ecology and Evolutionary Biology Genetics group (professors, post-docs, and students) for the helpful criticisms on talks, papers, and grant applications.

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I dedicate this work to my father (Francisco Mojica), mother (Emelita Mojica) and sister (Julie Ann Mojica). Thank you for your infinite love, support, and encouragement. I would also

**ABSTRACT**

The fact that abundant genetic variation persists within populations despite strong directional selection on complex traits is one of the unresolved conundrums in evolutionary biology. In this dissertation, I employed a multi-faceted approach combining classical and modern genomic methods with field studies to identify the factors that may reduce total selection on a complex trait. I investigated the causes and consequences of phenotypic and genotypic variation in flower size using the wild flower *Mimulus guttatus* (yellow monkeyflower) as the model system. Flower size in *Mimulus guttatus* exhibits abundant genetic variation amidst strong directional selection in the wild. To understand directional selection, we must consider the "invisible fraction" (the proportion of individuals that die before expressing the trait), which is typically unmeasured in correlative studies. Chapter 1 demonstrates that viability selection prior to trait expression can change the direction and magnitude of selection. In Chapter 2, I identified fitness trade-offs (viability and fecundity) and varying selection at the spatial and temporal scale as factors that can reduce the effect of directional selection in the wild. Using NILs (Nearly Isogenic Lines), I demonstrate that alleles increasing flower size also increase fecundity but they reduce survivorship. Furthermore, I also detected fluctuating selection by year and on a spatial scale of meters. Finally, correlated selection on corolla width may limit the evolutionary response to directional selection for flower size. I demonstrated in Chapter 3 using a multi-year phenotypic manipulation experiment that corolla width is under indirect selection due to its genetic correlation with other traits that influence fitness in the field (e.g. rate of development, reproductive capacity, vegetative size).

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**INTRODUCTION**

Natural selection drives phenotypic diversification. Because of its fundamental role in evolutionary change, documenting the strength and direction natural of selection in wild populations has been a major goal for evolutionary biologists. The development of statistical methods by (Lande, 1979, Lande & Arnold, 1983) to estimate natural selection on quantitative traits paved the way for extensive field studies. The scientific literature now documents natural selection on behavioral, morphological, physiological, and life-history traits from many taxa.

The synthesis of the studies on natural selection reveals that directional selection, where one end of the phenotypic range is favored relative to the other, is common (Endler, 1986, Kingsolver et al., 2001). The prevalence and power of directional selection to drive rapid microevolutionary change (Grant & Grant, 1989, Reznick & Ghalambor, 2001) implies that phenotypic traits under strong consistent and continuous directional selection should exhibit low genetic variation. However, traits under directional selection remain genetically variable. What constrains directional selection?

The *Mimulus guttatus* species complex offers a great system to address this question. *Mimulus guttatus* (Phrymaceae) is a self-compatible, monoecious plant that can outcross and self-fertilize. It possesses the qualities of a true genetic model system, but is also amenable to field research. These characteristics include diverse native variation in morphology (e.g., corolla size), short generation time (2-3 months), small size (approximately 5 x 13 cm at adult stage), and reproductive flexibility (can be easily outcrossed and selfed).

My dissertation employs complementary approaches to investigate how natural selection acts on the quantitative trait of flower size (corolla width), operationally defined as the span of lower corolla lip. There is immense variation in flower size within and between populations and

species of the *M. guttatus* complex. *Mimulus nasutus* is a highly selfing species which has an average flower size of 3 mm. In contrast, the perennial sub-species of *M. guttatus*, which is predominantly outcrossing, has an average flower size of 45 mm. Within the Browder Ridge population of *Mimulus guttatus*, a single contiguous population in central Oregon, flower size varies from 10 mm to 50 mm (Julius Mojica, personal observation). Hall and Willis (2006) reported that flower size differs between two *Mimulus guttatus* population located on the Oregon's western Cascades (mean = 12 mm) and Oregon's Pacific coast (mean = 29 mm). The genetic dissection of flower size difference has been investigated via QTL mapping at different taxonomic levels: between species (Fishman et al., 2002), between geographic populations of the same species (Hall et al., 2006), and most recently within local populations (Lee, 2010).

In different ways, the three chapters of my thesis address the factors limiting strong directional selection on flower size of *Mimulus guttatus*. Chapter 1 and the resulting article (Mojica and Kelly, 2010) investigates the importance of determining the nature invisible fraction (proportion of individuals that die before expressing the trait) in predicting evolutionary change based on estimates of selection from the wild. The majority of estimates for natural selection come from correlative studies. Generally, investigators identify individuals within a field population, measure traits, and then monitor these individuals to determine their reproductive success. Natural selection is inferred from the association of trait values (e.g. flower size) with reproductive success (e.g. seed set).

The correlative approach is straightforward and broadly applicable, but the resulting selection estimates are prone to several well-known biases (Mitchell-Olds & Shaw, 1987, Rausher, 1992, Wade & Kalisz, 1990, Willis, 1996). Direct interpretation of correlative estimates requires that included individuals are a random sample of the population. To be

included in the calculation of a selection differential or gradient, an individual must express the trait and then yield an estimate for fitness. Individuals that die before expressing the trait, e.g. before flowering, will be excluded. If the likelihood of flowering is correlated with the 'latent' value for the trait—formally the genotypic value for the trait (Falconer & Mackay, 1996)—then a component of selection is missed. Grafen (1988) referred to this population of individuals that die before expressing the trait of interest as the "invisible fraction." This fraction may be a causal factor that can limit the evolutionary response to directional selection.

Chapter 2 documents the role of two factors (fitness trade-offs and alternating selection) in limiting the effect directional selection, and hence maintaining additive genetic variation, for flower size. Flower size is a genuine quantitative trait within the Iron Mountain population of *M. guttatus* (IM is located in Oregon, USA). The variation in flower size is caused by environmental effects combined with many QTLs (Lee, 2010). This trait is under strong natural selection at IM (Willis, 1996, Mojica & Kelly, 2010), which is expected to deplete genetic variation. Despite this, abundant additive genetic variation persists and the quantity and nature of this variation cannot be explained by mutation-selection balance (Kelly, 2003, Kelly & Willis, 2001). Balancing selection emerges as a viable alternative explanation, but what specific form or forms of balancing selection are important? I implement a transplant experiment of NILs (Nearly Isogenic Lines) that contain flower size QTLs from an intra-population mapping experiment (Lee, 2010) to determine the form(s) of balancing selection on this trait.

Chapter 3 utilizes phenotypic manipulation to determine if flower size is a direct target of selection. Phenotypic and genetic correlations between traits may cause correlated selection—a factor that may limit directional selection. Trait-fitness associations in the wild can result from direct selection on a trait or due to indirect selection (correlated selection). The distinction

between direct and indirect target of selection is necessary because the response to the rate of

selection (i.e. change in mean phenotype) is dependent on the relative direction between indirect

and direct selection.

Chapter 3 describes phenotypic manipulation experiments that determine whether corolla

width (a measure of flower size) is under direct selection. I manipulate the trait by trimming

tissue from the corolla. I hypothesize that corolla width is under direct selection and therefore

reducing corolla (through trimming of the lower corolla lip) will both reduce the mechanical

function (as pollen trap for indirect pollination/reproductive assurance) and aesthetic function

(for pollinator attraction). Trimming of the corolla directly isolates the effect of corolla

morphology by eliminating associations with other traits (like plant size, rate of development,

etc).

**Chapter 1**

**Viability selection prior to trait expression is an**

**essential component of natural selection**


Measuring natural selection in wild populations is a major endeavor for evolutionary biologists. Since the publications of Lande (1979) and Lande and Arnold (1983), estimates of natural selection on behavioral, morphological, physiological, and life-history traits have accumulated from many taxa (Endler, 1986, Kingsolver et al., 2001). This abundance of data permits testing of broad taxonomic hypotheses. For example, strong positive correlations between body size and fecundity have been documented in many species (Kingsolver et al., 2001). Interpreted literally, these estimates suggest a mechanism for Cope's rule, the tendency for lineages to evolve larger body size over time (Cope, 1896, Bonner, 1988).

In plants, many studies have considered selection on flower size. The survey of Kingsolver and colleagues (2001) includes nine estimates of selection, seven of which indicate significant positive directional selection on flower size. Moreover, the Kingsolver et al. (2001) review includes only a fraction of the abundant literature suggesting that natural selection favors larger flowers (Medel et al., 2003, Wright & Stanton, 2007, Armbruster et al., 2005, Sandring & Agren, 2009). Despite apparently overwhelming directional selection for larger flowers, populations retain high genetic variation in flower size. Artificial selection can greatly increase mean flower size (Worley & Barrett, 2000, Lendvai & Levin, 2003, Delph et al., 2004, Lehtila & Brann, 2007, Kelly, 2008), indicating that natural populations are not at maximum values for this trait.

The majority of estimates for natural selection come from correlative studies. Generally, investigators identify individuals within a field population, measure traits, and then monitor these individuals to determine their reproductive success. Natural selection is inferred from the association of trait values (e.g. flower size) with reproductive success (e.g. seed set). The overall selection on a quantitative trait is most naturally estimated as $s$, the selection differential (Falconer & Mackay, 1996), which equals the covariance of trait values with relative fitness (typically positive for flower size). S includes both direct selection on the measured trait, as well as indirect selection on correlated traits. Many studies report selection gradients, which describe the "direct selection" on a character. Selection gradients are the partial regression coefficients obtained when a fitness component is regressed on multiple traits simultaneously (Lande & Arnold, 1983). Like selection differentials, flower size gradients are usually positive. However, there is greater variability in estimates for these quantities. Selection gradients on floral size measurements depend strongly on the identity of other plant traits that are included in the multiple regression (see DISCUSSION).

While the correlative approach is straightforward and broadly applicable, selection estimates are prone to several well-known biases (Mitchell-Olds & Shaw, 1987, Rausher, 1992, Wade & Kalisz, 1990, Willis, 1996). Direct interpretation of correlative estimates requires that included individuals are a random sample of the population. To be included in the calculation of a selection differential or gradient, an individual must express the trait and then yield an estimate for fitness. Individuals that die before expressing the trait, e.g. before flowering, will be excluded. If the likelihood of flowering is correlated with the 'latent' value for the trait—formally the genotypic value for the trait (Falconer and Mackay 1996)—then a component of

selection is missed.  Grafen (1988) referred to this population of individuals that die before expressing the trait of interest as the "invisible fraction."

The difficulty of detecting viability selection prior to trait expression is relevant to any correlative selection analysis of adult traits.  However, the quantitative importance of this effect is not widely appreciated. Bennington (1995) showed that selection on plant height of jewelweed was significantly altered when the invisible fraction is taken into account. Sinervo and McAdam (2008) assessed the survival of side-blotched lizards before sexual maturity and revealed non-random mortality prior to clutch-size expression. Hadfield (2008) has recently considered a variety of invisible fraction scenarios and questions whether any non-manipulative method can accurately estimate selection when individuals die before trait expression.

Here, I describe a genotypic manipulation experiment demonstrating that viability selection on the invisible fraction can be strong enough to completely reverse the overall direction of selection.  The focus of this work is *Mimulus guttatus* (yellow monkeyflower) and research on this species is a substantial component of the flower size / natural selection literature. At least six previous studies report estimates from a multitude of populations and years of study (Willis, 1996, Hall & Willis, 2006, Fenster & Ritland, 1994a, van Kleunen & Ritland, 2004, Fishman & Willis, 2008, Murren et al., 2009).  In all cases, the overall selection on floral size traits appears to be significantly, and often strongly, positive.

I employ a field transplant experiment of small, medium, and large flowered genotypes of *M. guttatus* to estimate both viability and fecundity selection on flower size. This approach allows us to "visualize" the invisible fraction through prior knowledge about the phenotypes of the transplanted individuals. As expected from previous studies, I find that fecundity is positively related to flower size among adult plants.  However, the genotypic transplant allows us to

estimate the relative viability of flower size genotypes prior to expressing the trait in the field.  I find that viability is negatively related to the genotypic value for flower size.  When survivorship and female reproductive success are both taken into account, natural selection generally favors smaller flowers.

**Methods**

*Study system*

The yellow monkeyflower, *Mimulus guttatus* (Phrymaceae), is a self-compatible, mostly outcrossing, monoecious plant that inhabits a wide range of habitats from alpine (annual) to coastal (perennial). The study was carried out from 2007 to 2009 within a single annual alpine population at the Browder Ridge trailhead (N 43 degrees 57.594, W 124 degrees 07.847; Oregon, USA). At this site, *Mimulus* is annual or winter annual.  Seeds germinate either in the autumn (and persist as seedlings under the snow) or during the spring immediately following snowmelt in late May or early June. They develop rapidly and the peak of flowering is typically from mid-to-late June.  These predominantly bumblebee pollinated plants set seed by mid-July in most years (personal observation).

*Genotypic field transplant experiment*

I transplanted seedlings from three distinct genotypic groups into the field site.  Each group was a random selection of plants from one of three divergent populations produced via nine generations of artificial selection on corolla width, a measure of flower size (Kelly, 2008). Low, Control, and High populations were initiated from a common source population derived from a single natural population located at Iron Mountain in central Oregon, U.S.A. (Willis,

1996). Importantly, the selection experiment was conducted using very large population sizes: 200 adult plants were selected in each population to constitute the next generation. As a consequence, the High, Low, and Control plants are actually genotypic classes. Each class retains high internal genetic variation (see Table 3 of Kelly 2008). The difference between High, Low, and Control plants in mean flower size is due to the cumulative effects of many Quantitative Trait Loci (QTLs). Lee (2009) mapped flower size QTL on all 14 *M. guttatus* chromosomes (most chromosomes have multiple QTLs) using parental plants from these same Low and High populations.

I pooled seed from approximately 70 distinct families within each population. Seedlings were germinated in separate flats in the University of Oregon greenhouse during May in three successive years (2007, 2008 and 2009). I transplanted 14-day-old seedlings ($n_{2007}$= 675, $n_{2008}$= 750, $n_{2009}$= 450) into five five-meter transects at the Browder Ridge site in the first week of June (coincident with the seedling stage of the native *Mimulus*). Browder Ridge is close to the Iron Mountain site (source population) and experiences approximately the same climatic environment. Any transplants that died within a week of transplant were replaced to reduce the effect of transplant shock on survivorship data. I monitored transplants subsequently and recorded the day when the first flower opened (flowering time) and measured corolla width in mm using a steel ruler. At the end of the growing season (mid-July), I collected and counted all the seeds produced by each flowering plant.

*Estimation of phenotypic selection on flower size*

I used corolla width as the measure of flower size. After square-root transformation, this variable is normally distributed within genotypic classes and has been used in previous studies

(Kelly 2008 and references therein). For all plants that flowered, I estimated the effects of genotype, year, and their interaction on corolla width using 2-way ANOVA (normal residuals). Generalized linear models were used to assess year and genotype effects on survivorship and seed counts. For survivorship, the response variable is dichotomous (0/1) and I use the standard logit link function. Seed number of surviving plants (fecundity) was highly right-skewed and I employed an overdispersed Poisson model with the Log link function. I used the same statistical model for total female fitness, which is the seed production of all transplants (including zero values for plants that died before flowering). Finally, I also used the Poisson model for the regression of fecundity onto observed flower sizes with year included as a categorical predictor. The estimates from this model yield a selection gradient for corolla width. These analyses were conducted using JMP v8 (SAS corporation).

## Results

*Phenotypic variation among flower size genotypes*

The significant differences in flower size among Low, Control, and High genotypes that had been previously documented in the greenhouse (see Table 2 of Kelly 2008) were reiterated in the field ($F_{2,580} = 157.3$, $p < 0.001$). Across years, the estimated mean corolla width was 2.96 among Lows (Standard Deviation = 0.34, n = 406), 3.55 among Controls (SD = 0.40, n = 153), and 4.30 among Highs (SD = 0.51, n=30). The phenotypic distribution of Control plants is representative of the ancestral Iron Mountain population and the native plants at Browder Ridge. Relative to the Control distribution, the mean floral size of High plants was about 1.9 SD greater than the Control mean, while the mean floral size of the Lows was about 1.5 SD less (Fig. 1.1A).

11
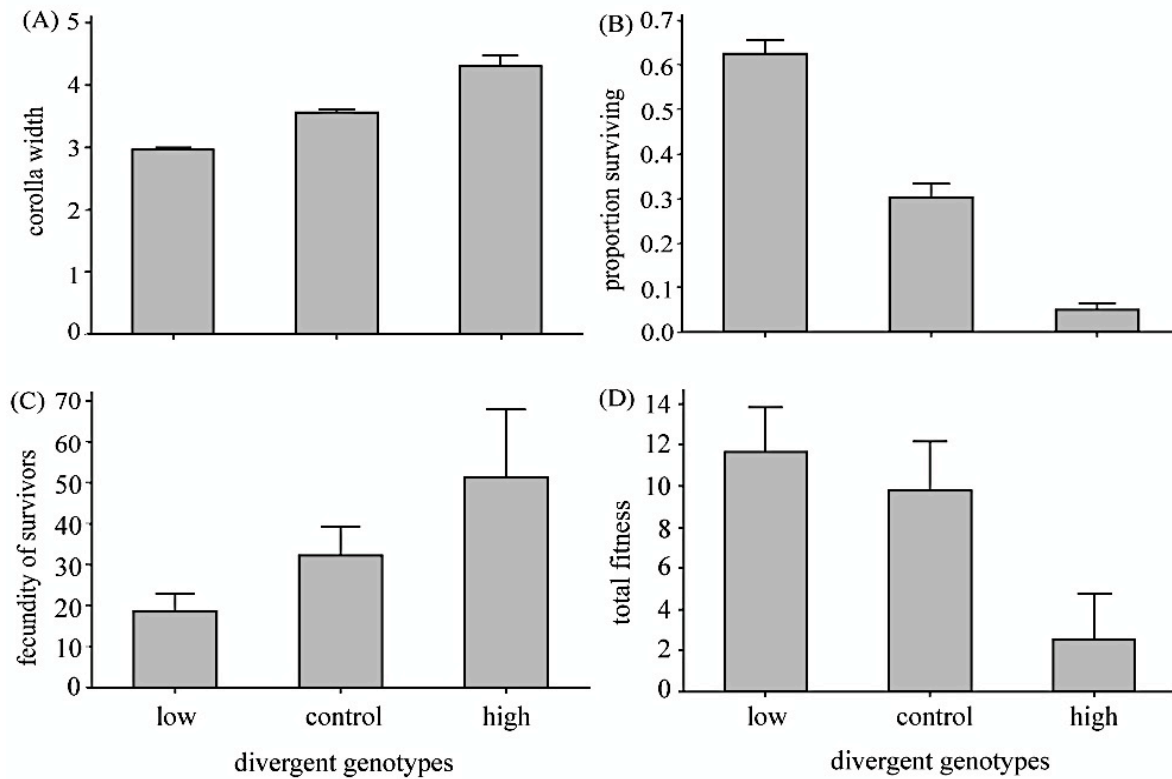


Figure 1.1. Phenotypic description of selection lines. Means are reported for (A) corolla width, (B) survivorship to flowering, (C) fecundity of survivors, and (D) total fitness for each *Mimulus guttatus* genotype, pooled across years. Corolla width (panel A) is square-root transformed and total fitness (panel D) is the average of absolute seed set of all transplants of a genotype. Error bars are 95% confidence intervals of each mean.

*Viability and fecundity selection on flower size*

Death due to transplant shock was low across three years and unrelated to genotype. After transplant establishment, mortality was uniformly low for all genotypes until the final 'dry down' in each field season. All plants eventually desiccated as snowmelt diminished, although the timing of this final drought differed among years (July 20-30 in 2007, August 5-15 in 2008, and July 25-August 4 in 2009). However, the number of plants that matured fast enough to flower and set seed varied greatly with genotype and year. Across years, survivorship to flowering of Low genotypes was 12 fold higher than that of High genotypes (Fig. 1.1B). Control genotypes consistently showed intermediate survivorship to flowering. Likelihood ratio tests confirm the significant viability effects of Genotype ($X^2$ = 486.6, d.f. = 2, p < 0.001), of Year ($X^2$ = 68.0, d.f. = 2, p < 0.001), and of Genotype-by-Year interaction ($X^2$ = 29.6, d.f. = 4, p < 0.001). Despite the interaction, the rank order of genotypes was consistent across years (Low survivorship > Control > High).

Among plants surviving to flower, fecundity was positively related to flower size genotype (Fig. 1.1C; $X^2$ = 8.25, d.f. = 2, p = 0.016). There was also a large effect of year ($X^2$ = 59.0, d.f. = 2, p < 0.001). The Genotype-by-Year interaction was non-significant and dropped from the model. The combined effects of viability and fecundity were evaluated by considering total seed production per transplant (Table 1.1). There were significant effects of genotype ($X^2$ = 39.3, d.f. = 2, p < 0.001) and year ($X^2$ = 112.7, d.f. = 2, p < 0.001) on this measure of total female fitness. The interaction was non-significant and was excluded the model, but the rank order of estimated genotype means for total fitness was not completely consistent across years. The High genotype was always lowest, but Control had the highest mean in 2009 (Table 1.1).

Figure 1.2 illustrates the relationship between observed flower sizes and fecundity. The Poisson regression model estimates expected seed set as $C*Exp(0.93\ z)$, where z is the corolla width (square root transformed) and C is a constant dependent on year (the positive relationship is highly significant: $X^2 = 54.0$, d.f. = 1, p < 0.001). The univariate selection gradient, i.e. the linear regression of relative fitness onto trait value, is 0.93. Gradients can be standardized in a variety of ways (Hereford et al., 2004) given the trait mean ($\bar{z} = 3.18$) and standard deviation ($\sigma_z = 0.217$ after factoring out differences among years). The variance standardized gradient is $(0.217)(0.93) = 0.20$. The mean standardized gradient is $(3.18)(0.93) = 2.96$. These values are comparable to gradient estimates from other plants and animals for size-related traits (see figures 1-2 of Kingsolver and Pfennig 2004; figure 3 of Hereford et al 2004).
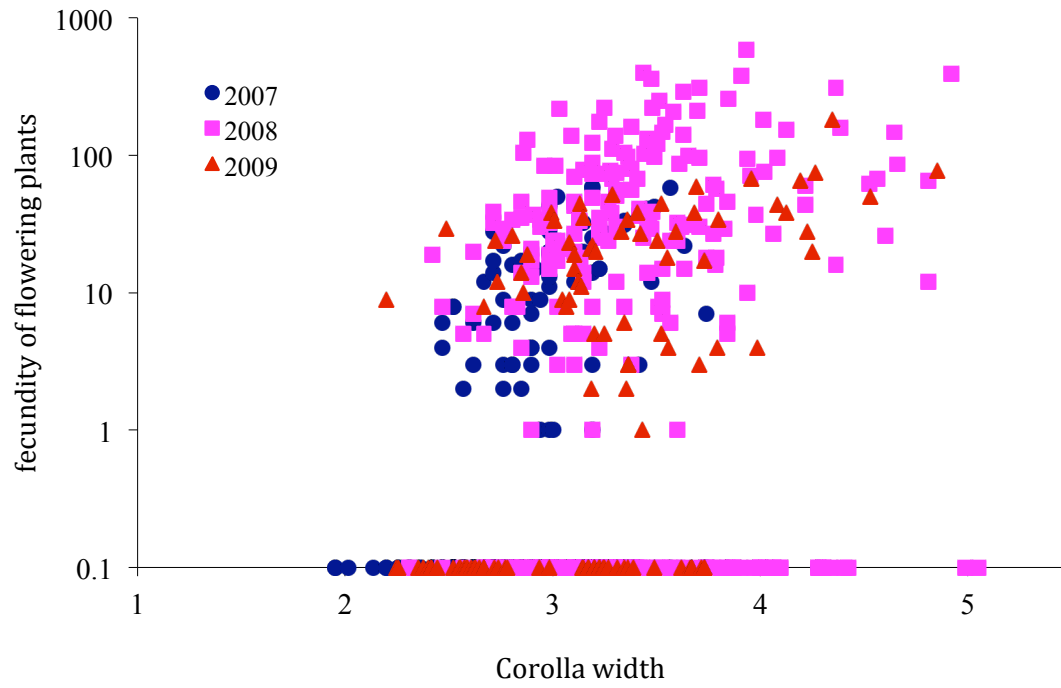
Figure 1.2. The linear regression of survivor fecundity onto corolla width of *Mimulus guttatus* is depicted. Corolla width is square-root transformed.

Table 1.1. The average seed set of each *Mimulus guttatus* genotype in each year of study is given (n is the sample size, sem is the standard error of the mean and sd is the standard deviation).

| genotype | n | mean | sem | sd |
|---|---|---|---|---|
| year 2007 | | | | |
| low | 259 | 3.66 | 0.65 | 10.46 |
| control | 159 | 1.08 | 0.49 | 6.15 |
| high | 257 | 0.01 | 0.01 | 0.12 |
| | | | | |
| year 2008 | | | | |
| low | 269 | 23.70 | 3.33 | 54.62 |
| control | 257 | 17.57 | 3.68 | 59.02 |
| high | 224 | 6.25 | 2.50 | 37.36 |
| | | | | |
| year 2009 | | | | |
| low | 150 | 3.82 | 0.81 | 9.91 |
| control | 150 | 5.61 | 1.57 | 19.22 |
| high | 150 | 1.27 | 0.74 | 9.02 |

**Discussion**

*Seeing the invisible fraction*

Figures 1.1-1.2 demonstrate the potentially dominant effects of selection on the invisible fraction, the collection of individuals that die prior to trait expression (Grafen, 1988). Viability and fecundity selection on flower size were conflicting in each of the three years of the study, but only the latter process would be evident in a correlative study of natural selection. I observed a strong positive relationship between fecundity and flower size (Fig. 1.2), a result consistent both with the extensive field data from this species and from flowering plants generally (see INTRODUCTION). However, here I was also able to measure pre-flowering selection by using genotypic groups known *a priori* to differ in flower size. The mortality data (Figure 1.1B) identifies strong viability selection prior to trait expression. The invisible fraction is composed disproportionately of large flowered genotypes that did not mature fast enough to reach flowering. As a consequence, the overall direction of selection was usually for smaller flowers (Table 1). Given that the intrinsic features of the invisible fraction are usually unknown, the assumption that natural selection habitually favors larger flowers should be viewed with caution.

In this experiment, the differential mortality of flower size genotypes was determined by differences in development rate. Across genotypes, mortality was minimal until the terminal drought period in each year. During this drought interval, all plants died. Survivorship to flowering was highest for Low genotypes because they mature faster than Control genotypes. Control genotypes reach flowering faster than High genotypes. Across years, mean days to first flower was 45.1 for Lows (SD = 17.0, n = 424), 55.9 for Controls (SD = 15.3, n = 170), and 68.6 for Highs (SD = 16.1, n = 31). Flower size exhibits a positive genetic correlation with time to flower mainly because plants that delay flowering have greater vegetative biomass when they do

flower (see Figure 6 of Kelly 2008). Larger flowers have greater reproductive capacity, producing more pollen and more ovules (Kelly, 2008) and may also have greater pollinator recruitment (Sandring & Agren, 2009, Martin, 2004). However, the delay in flowering can be costly in alpine field sites such as Browder Ridge where the availability of water diminishes over the season and drought is the primary cause of mortality.

The fact that flower size is a direct function of plant size in annual monkeyflowers suggests a broader relevance of these results. Strong positive correlations between body size and fecundity are frequently observed in both plants and animals (Kingsolver and Pfennig, 2004), but there are several reasons why the overall direction of selection may favor smaller or intermediate body sizes. Most relevant to the present study is the increased mortality of juveniles due to longer development. In animals, longer development means elongated exposure to predation, parasitism or starvation before reproductive maturity (Blanckenhorn 2000). Also, mate attraction traits such vocal call features are often correlated with adult size and such traits routinely exhibit strong positive correlations with mating success (Burrowes, 2000, Grether, 1996, Hews, 1990, Preziosi & Fairbairn, 1996). However, if attraction traits are genetically correlated with development time, then viability selection on the invisible fraction might provide a brake on sexual selection. At the very least, a failure to account for pre-adult viability can lead to a misleading characterization of natural selection.

*Genotypic manipulation as a tool for measuring natural selection*

My experimental design is a variant of the genotypic transplant method (Rausher, 1992; Willis, 1996). Measuring selection on individuals of known genotype, or of known ancestry within a breeding design, was suggested to address the problem of environmentally induced

covariances between traits and fitness measures (Stinchcombe et al., 2002, Rausher & Simms, 1989). Bias is reduced if trait breeding values are used to predict fitness instead of phenotypic values.  Breeding value estimates can be obtained by averaging individuals from a genotype or family.  Given that this sample must be random, the breeding value regression is also subject the problem of viability selection prior to trait measurement, although averaging likely reduces the magnitude of this difficulty (see Hadfield 2008 for a detailed consideration).  In this study, each of my 'genotypes' was a genetically diverse collection of plants classified *a priori* for flower size.  Flower size means observed in the field study followed my prior expectation (Low < Control < High).

This experiment does not identify causal relationships between particular traits and fitness because my genotypes differ in multiple features simultaneously, e.g. overall plant size, flower size, and development time (see Table 2 of Kelly 2008).  In principle, putative causal relationships can be distinguished statistically.  Selection gradients estimate the 'direct' effect of the trait on fitness while statistically controlling for other measured characters (Lande and Arnold 1983).  Fenster and Ritland (1994) measured overall plant size in a study of selection on floral traits of *M. guttatus*.  In two populations (Guenoc and Hough Springs), these authors obtained positive selection differentials on corolla width and corolla length, indicating a positive overall association of flower size with fecundity.  However, when overall plant size was included with other traits in a multiple regression predicting seed set, the selection gradients on the flower size traits became significantly negative.  It is possible that the data of Figure 1.2 might produce a negative selection gradient on corolla width if plant size or ovule number were included as additional predictors in the regression.

From a genetic perspective, the observed negative correlation between female reproductive success and viability likely represents an example of antagonistic pleiotropy. While genetic correlations can also result from linkage disequilibria (Falconer and Mckay, 1996), several features of this system favor pleiotropy. Most notably, there is a clear developmental connection between flower size and development rate. Antagonistic pleiotropy has been invoked as an explanation for the evolution of late-life fecundity (Rauser et al., 2006), age and size of sexual maturation (Basolo, 2008), senescence (Curtsinger et al., 1994, Williams & Day, 2003), and the maintenance of genetic variation (Charlesworth & Hughes, 2000). Given that flower size exhibits abundant genetic variation within the Iron Mountain population and that this variation cannot be explained by mutation-selection balance (Kelly, 2003, Kelly & Willis, 2001) , antagonistic pleiotropy emerges as a potentially important mechanism. Beyond *Mimulus*, this study contributes to the very limited field data on antagonistic pleiotropy.

Blackenhorn (2000) motivated his review of natural selection on body size with the question "What keeps organisms small?" This study provokes the opposite question: What keeps flowers large? Across years, the large flower genotypes consistently had the lowest overall fitness. The Low genotypes had the highest fitness average in two years, but the Controls were actually highest in one year. Environmental fluctuations may determine the evolutionary balance between viability and fecundity selection (Childs et al., 2004). Also, I admit that this study did not include one critical component of fitness, outcross siring success. Few studies have considered this variable, but the available data suggest that differential siring success may generate selection on flower size (van Kleunen & Burczyk, 2008, Bell, 1985, Stanton et al., 1986). Also, because *M. guttatus* is a self-compatible hermaphrodite, female fecundity may include both outcrossed and self-fertilized seed. If flower size influences the partitioning of

reproductive effort between outcrossing and selfing, this may be another avenue of natural selection on this trait.  Experiments are ongoing to determine the effect of flower size on outcross siring success and selfing rate.

**Chapter 2**

**Evidence for a combination of selective mechanisms maintaining quantitative genetic variation in natural populations**

Recent surveys of genomic variation in humans have identified molecular signatures of natural selection (Tishkoff et al., 2007, Sabeti et al., 2007). Counter to initial expectations, hard sweeps wherein a novel mutation is immediately favored and spreads to fixation, e.g. (Coop et al., 2009, Pritchard et al., 2010, Simonson et al., 2010), seem to be relatively infrequent. In contrast, polygenic adaptation (subtle changes in allele frequencies at many loci) may be quite common (Pritchard & Di Rienzo, 2010, Hancock et al., 2010). The infrequency of hard sweeps has prompted renewed focus on adaptation via selection on standing variation. Unfortunately, the signature of this kind of selection can be very subtle at the gene sequence level (Kelly, 2006, Pennings & Hermisson, 2006, Teshima et al., 2006) and direct experimental approaches are required. There has been extensive experimental work demonstrating the effect of gene sequence variants on complex traits of fruitflies (Dworkin et al., 2005) and yeast (Ehrenreich et al., 2010). In these systems however, we cannot measure the effects of genetic variants on survival and reproduction in the wild.

A small but growing collection of field experiments have directly demonstrated natural selection at the scale of QTLs, e.g. (Bradshaw & Schemske, 2003, Verhoeven et al., 2004, Lexer et al., 2003, Weinig et al., 2003, Li et al., 2003, Korves et al., 2007). The evolutionary interpretation of results from these studies is contingent on the source of genetic materials and on the field site (or sites) in which genotypes are evaluated. Typically, the genetic materials are recombinants between divergent populations or species, as in *Mimulus* (Bradshaw & Schemske,

2003, Hall & Willis, 2006); *Helianthus* (Lexer et al., 2003); *Avena* (Gardner & Latta, 2006),

*Hordeum* (Verhoeven et al., 2004), and *Boechera* (Anderson et al., 2011). The genetic

differences segregating in these experiments are the products of past adaptation. As a result, data

from these experiments thus speak to the genetic basis of divergence. Questions about the

maintenance of variation are most directly addressed by considering intra-population variation.

It is thus notable that field selection studies of intra-population variation have focused mainly on

Mendelian traits (Dobzhansky & Levene, 1948, Ford, 1971, Subramaniam & Rausher, 2000,

Greaves et al., 1977).

Flower size is a genuine quantitative trait within the Iron Mountain population of *M.*

*guttatus* (IM is located in Oregon, USA). Variation is caused by environmental effects combined

with many QTLs (Lee, 2010). This trait is under strong natural selection at IM (Willis, 1996,

Mojica & Kelly, 2010), which is expected to deplete genetic variation. Despite this, abundant

additive genetic variation persists and the quantity and nature of this variation cannot be

explained by mutation-selection balance (Kelly, 2003, Kelly & Willis, 2001). Balancing

selection emerges as a viable alternative explanation, but what specific form or forms of

balancing selection are important? Simple overdominance (heterozygote superiority across

environments) is the standard example, but other models are probably more important for

quantitative traits like flower size. These include spatial and temporal variation in selection

(fitness differs in time and space), genotype by environment interaction (trait expression is

dependent on the environment), antagonistic pleiotropy (negative genetic correlation of viability

and fecundity), and frequency dependent selection (fitness is a function of genotype frequencies).

QTL-level estimates for fitness effects may be necessary to determine if and how natural

selection maintains genetic variation. Quantitative genetic methods, which are based on the

aggregate effect of QTLs, effectively identify balancing selection mechanisms only if selection is consistent across the QTLs affecting a trait. For example, consistent antagonistic pleiotropy predicts a negative genetic correlation between fitness components (Radwan, 2008). Previous field studies of the IM population of *M. guttatus* identify such a correlation (Mojica & Kelly, 2010). Fecundity is positively related to flower size because larger flowers produce more ovules and pollen and might be more attractive to pollinators. Survival to flowering is much lower in large flowered genotypes than in small flowered genotypes because they fail to flower before the onset of summer drought typical of the Mediterranean climate of western North America. Unfortunately, it is difficult to know if this viability/fecundity trade-off is critical to maintaining variation in flower size because the flower size classes used in this study differed due to combined effects of many QTLs. Theoretical studies indicate that locus-specific details (e.g. dominance relationships among alleles) are critical to whether antagonistic pleiotropy maintains polymorphism and it is possible that many of the loci contributing to flower size variation do not exhibit a viability/fecundity trade-off. We need to determine if this tradeoff exists at individual QTL.

We measured survivorship and fecundity effects at the QTL level by transplanting and monitoring four nearly isogenic genotypes in the field. These genotypes were created by introgressing alleles that increase flower size at two unlinked QTLs into the uniform genetic background of a single inbred line (IM62) by repeated backcrossing. The IM62 allele is labeled L (low) while the alternative is H (high) at each locus. This yielded four genotypes: HH, LL, HL, and LH, where the first letter denotes allele at the QTL on chromosome 1 and the second letter is allele at the QTL on chromosome 8. In order to avoid the effects of inbreeding depression in our field experiments, we produced hybrid isogenic genotypes by crossing the four

IM62 genotypes to a different inbred line from the same population (IM767). Experimental genotypes were transplanted as seedlings into the Oregon field site in three successive years (2008-2010); HH and LL in each year, HL in two seasons (2009-2010) and LH only in 2010. Soil water availability varies between the two locations within the site. The "Wet" location receives snowmelt later into the summer than the "Dry" location.

## Methods

### *Mapped QTL*

Nearly isogenic lines (NILs) containing QTL 1 and QTL 8 were bred from an $F_1$ individual derived from a cross between a large flowered and a small flowered parent. The parents were sampled from an artificial selection experiment which was founded by genotypes sampled from Iron Mountain (Kelly, 2008). The F1 individual was selfed to create an $F_2$ mapping population as well as backcrossed (BC) for four generations to IM62, the genome sequenced *Mimulus guttatus* line. We identified potential QTLs in the $F_2$ mapping population, and subsequently identified a line carrying both QTLs 1 and 8 in the fourth generation NIL population. This individual was selfed and we confirmed effects on flower size at these loci in the IM62 background by measuring flower size in the $BC_4F_2$ progeny. We identified flanking markers for both QTLs through progeny testing of recombinant sub-NILs generated in the $BC_4F_3$ through $BC_4F_5$ generations. The NILs placed in the field are descendants of a fixed homozygous double QTL line from the $BC_4F_2$ generation propagated for seed. All field genotypes were confirmed with flanking markers.

Alternative alleles are $H_1$ and $L_1$ for the QTL on linkage group 1 and $H_8$ and $L_8$ for the linkage group 8 QTL (the H allele increases flower size relative to the IM62 allele). In order to

avoid the effects of inbreeding depression in our field experiments, we produced the transplanted nearly-isogenic genotypes by crossing four IM62 genotypes ($H_1H_8/H_1H_8$, $L_1L_8/L_1L_8$, $H_1L_8/H_1L_8$, and $L_1H_8/L_1H_8$) to a different inbred line from the same population (IM767). We designate the IM767 alleles $X_1$ and $X_8$, respectively. The X allele at each QTL may be functionally equivalent to one of the alternatives (L or H) but not to both, and it may differ from both. In the main paper, the four genotypes $H_1H_8/X_1X_8$, $L_1L_8/X_1X_8$, $H_1L_8/X_1X_8$, and $L_1H_8/X_1X_8$, are denoted HH, LL, HL, and LH, respectively.

*Field Transplant*

Seeds were sown in 53 cm x 27 cm x 5.7 cm seeding flats two weeks before the transplant. Seedlings were transplanted ($n_{2008}= 400$, $n_{2009}= 900$, $n_{2010}= 1600$) into five-meter transects at the Browder Ridge site (Oregon, USA; 44º220 7.319400 N, 122º20 57.8400 W) in June of each year. This site is within 10 km of the IM population and is similar in habitat. Transplants were done at Browder Ridge to avoid contamination of the source IM population. At both sites, *M. guttatus* is annual and seeds germinate either in the autumn (and persist as seedlings under the snow) or during the spring immediately following snowmelt in late May or early June. They develop rapidly and the peak of flowering is typically from mid- to late June. Plants set seed and die by mid-July in most years due to low soil moisture caused by summer drought.

In each year, transects were established in two spatially distinct locations. The Wet and Dry locations are approximately 10 m apart; and a low density of native M. guttatus spans these sub-populations. Pollinating insects traverse the entire population regularly. In each year, transplants were monitored daily. We recorded the day when the first flower opened (flowering time) and measured corolla width for each flower produced. At the end of the growing season (mid-July), we collected and counted seed from all transplants that survived to flower.

*Statistical analysis*

Corolla width was square root transformed for analysis. We used the standard general linear model (normal residuals) for corolla width. Generalized linear models were applied to other dependent variables: survival was treated as a binary (logit link function) while seed counts (both fecundity of survivors and total fitness) were treated with the overdispersed Poisson model (log link function). Hypothesis tests were conducted using likelihood ratios ($x^2$) for generalized linear model tests calculated with JMP v8 ©.

## Results

Given that the genotypes assayed as well as environmental conditions differed among years, we first compared genotypes within years (Fig 2.1). In 2008, HH exhibited significantly lower survivorship ($X^2_{[1]} = 15.4$, p<0.0001) than LL. Among survivors, HH had larger flowers ($F_{1,51} = 20.87$, p<0.0001), higher days to flower ($F_{1,55} = 5.48$, p = 0.02), and higher fecundity ($X^2_{[1]} = 758$, p<0.0001). Total fitness (seed set of all transplants including zeros for those that died prior to flowering) was slightly but significantly higher for HH than LL ($X^2_{[1]} = 36.1$, p<0.0001). In 2009, survivorship differed significantly among genotypes ($X^2_{[2]} = 48.6$, p<0.0001) with HL lowest and LL highest. Differences in observed flower sizes were marginally non-significant owing to generally low survival. Fecundity of survivors did differ significantly ($X^2_{[2]} = 152$, p<0.0001) due mainly to low seed set of HH genotypes. Total fitness for LL was much greater than for the HL and HH in 2009 ($X^2_{[2]} = 2176$, p<0.0001).

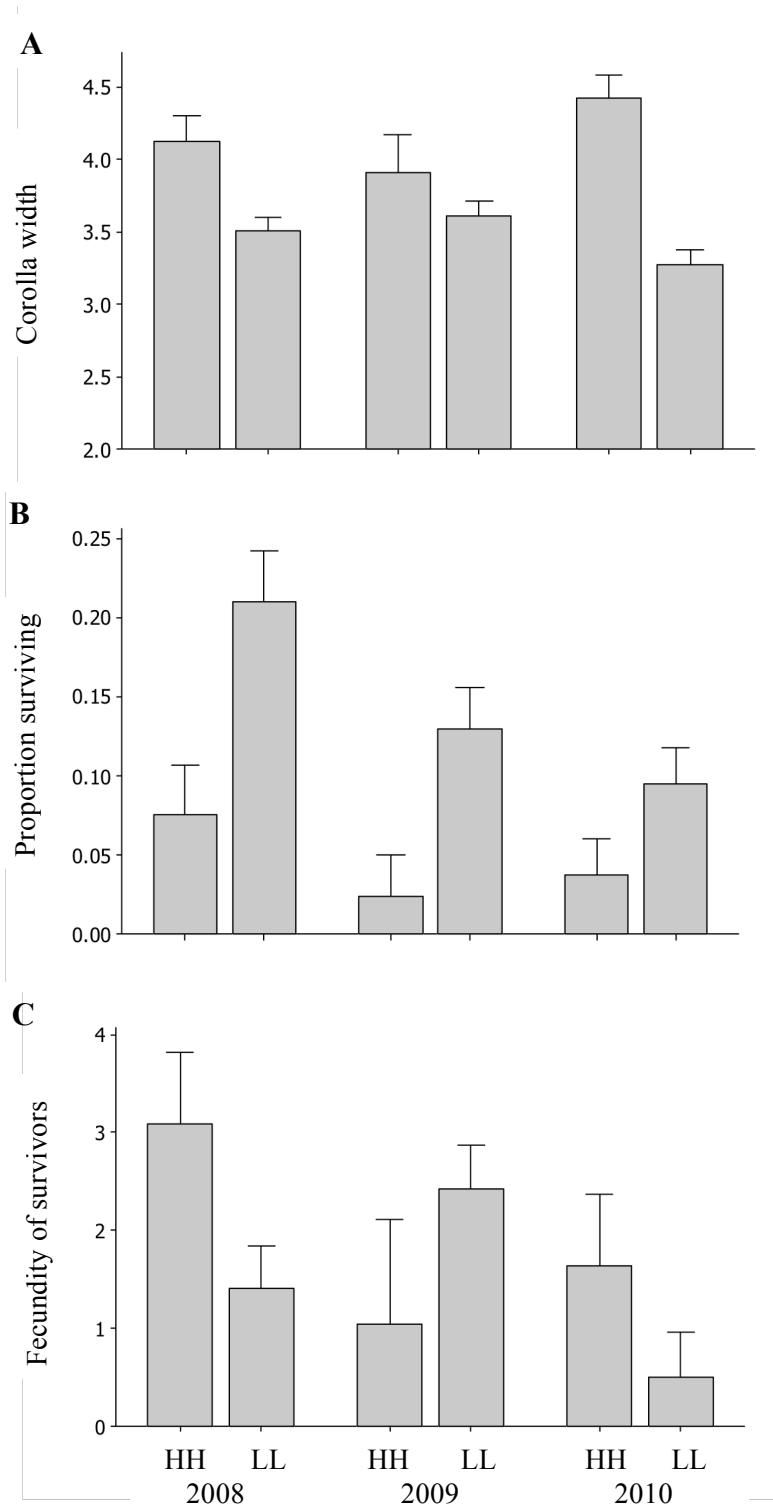Figure 2.1. Means are reported for (A) corolla width, (B) proportion surviving, and (C) fecundity of survivors for each *Mimulus guttatus* genotype (HH and LL), in each year of transplant. (A) Corolla width is square root transformed ($\sqrt{mm}$). (B) Proportion surviving is the number of individuals that survived to flower. (C) Fecundity of survivors is ln (seed set +1). Error bars are 95 % confidence intervals of each mean.

In 2010, survival differed among genotypes ($X^2_{[3]} = 29.6$, p<0.0001) – HL was significantly higher than LL, while LH was significantly lower than LL. Among survivors, flower size differed in a predictable way given genotype (Figure 2.2; $F_{3,85} = 39.37$, p<0.0001). Fecundity of survivors also differed strikingly ($X^2_{[3]} = 117$, p<0.0001) with LH > (HL, HH) > LL. For total fitness, $X^2_{[3]} = 204$ (p<0.0001) with HL > (LL, HH) > LH. Because all four genotypes were assayed in 2010, we can treat each locus as a factor and test for an interaction (epistasis). For survival, the high allele at QTL8 had a significant and negative direct effect ($X^2_{[1]} = 26.1$, p<0.0001), but the interaction of high alleles was significantly positive ($X^2_{[1]} = 4.37$, p=0.04). In other words, survivorship of HH individuals was higher than predicted given the direct (single locus) effects of each H allele. For fecundity of survivors, the direct effects of H alleles at each locus were significantly positive (QTL1: $X^2_{[1]} = 61.6$, p<0.0001; QTL8: $X^2_{[1]} = 34.0$, p<0.0001) but the interaction was significantly negative ($X^2_{[1]} = 32.7$, p<0.0001). For total fitness (Table 2.1), the direct effect of the H allele was significantly positive at QTL1 ($X^2_{[1]} = 65.0$, p<0.0001) and significantly negative at QTL8 ($X^2_{[1]} = 138.7$, p<0.0001). The interaction was marginally non-significant ($X^2_{[1]} = 3.05$, p=0.08).
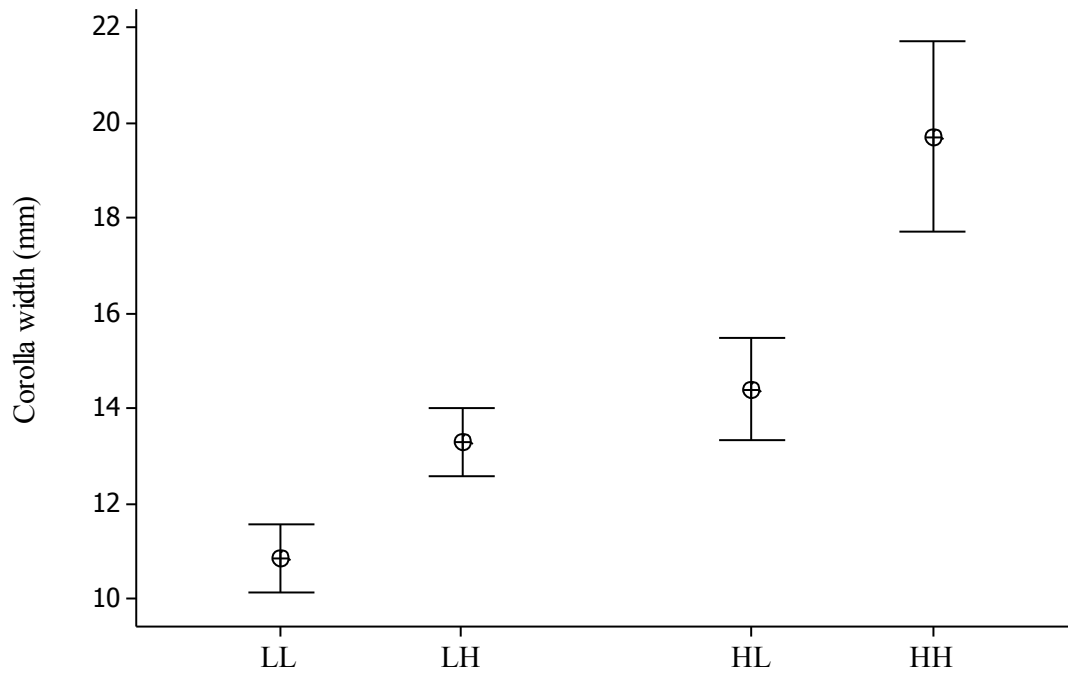
Figure 2.2. The mean and 95% confidence interval for flower size of each genotype in 2010.

Table 2.1. Temporal and spatial variation in total fitness among nearly isogenic genotypes. Total fitness is the average of absolute seed set of all transplants of a genotype. SEM is the standard error of the mean, SD is the standard deviation, and N is the sample size.

| Year | Location | Genotype | Mean | SD | N |
|---|---|---|---|---|---|
| 2008 | Wet | HH | 0.29 | 2.41 | 120 |
| | | HL | -- | -- | -- |
| | | LH | -- | -- | -- |
| | | LL | 1.93 | 10.29 | 120 |
| | Dry | HH | 12.45 | 41.22 | 80 |
| | | HL | -- | -- | -- |
| | | LH | -- | -- | -- |
| | | LL | 6.80 | 28.25 | 80 |
| 2009 | Wet | HH | 0.50 | 4.33 | 150 |
| | | HL | 0.46 | 5.63 | 150 |
| | | LH | -- | -- | -- |
| | | LL | 8.13 | 26.10 | 150 |
| | Dry | HH | 0.00 | 0.00 | 150 |
| | | HL | 0.00 | 0.00 | 150 |
| | | LH | -- | -- | -- |
| | | LL | 1.00 | 11.61 | 150 |
| 2010 | Wet | HH | 0.83 | 6.50 | 240 |
| | | HL | 1.82 | 13.20 | 240 |
| | | LH | 0.50 | 4.81 | 240 |
| | | LL | 0.98 | 10.54 | 240 |
| | Dry | HH | 0.19 | 1.68 | 160 |
| | | HL | 0.02 | 0.24 | 160 |
| | | LH | 0.00 | 0.00 | 160 |
| | | LL | 0.34 | 4.27 | 160 |

**Discussion**

*Antagonistic pleiotropy*

There was a clear general tendency for H alleles to reduce survival to flowering, but to increase fecundity of survivors. The LL vs HH contrast was estimated in each year. High alleles in combination reduced survivorship to flowering by as much as seven fold ($X^2 = 44.6$, df= 1, p <0.0001; Fig. 1b). Fecundity selection (seed set of survivors) was in the opposite direction of viability selection in two of three years. Among individuals that survived to flower, the average seed set was higher for HH than LL across years (Fig. 1c; $X^2 =11.9$, df =1, p <0.0006).

The antagonistic pleiotropy is a direct consequence of differences among genotypes in the timing of the transition from vegetative growth to flowering (Fig. 2.3). Days to flower is significantly different between HH and LL ($F_{1,147} = 10.34$, p <0.01) but this understates the magnitude of the effect. Most plants with delayed transition to flowering die of desiccation before they flower. They never express the trait of flower size in the field. When H genotypes do survive to flowering, they are typically larger plants with greater vegetative mass and larger flowers. Increased fecundity is likely due to higher ovule and pollen numbers per flower, perhaps combined with increased pollinator attraction.

The pattern of selection at the two QTLs is remarkably consistent with our previous measurements of survival and reproduction on small, medium, and large flowered classes of plants that differed at many loci. By and large, plants with high alleles at each flower size QTL were more fecund but less viable compared to those with low alleles. The trade-off of fitness components (Fig. 2.1) is a necessary but not a sufficient condition for
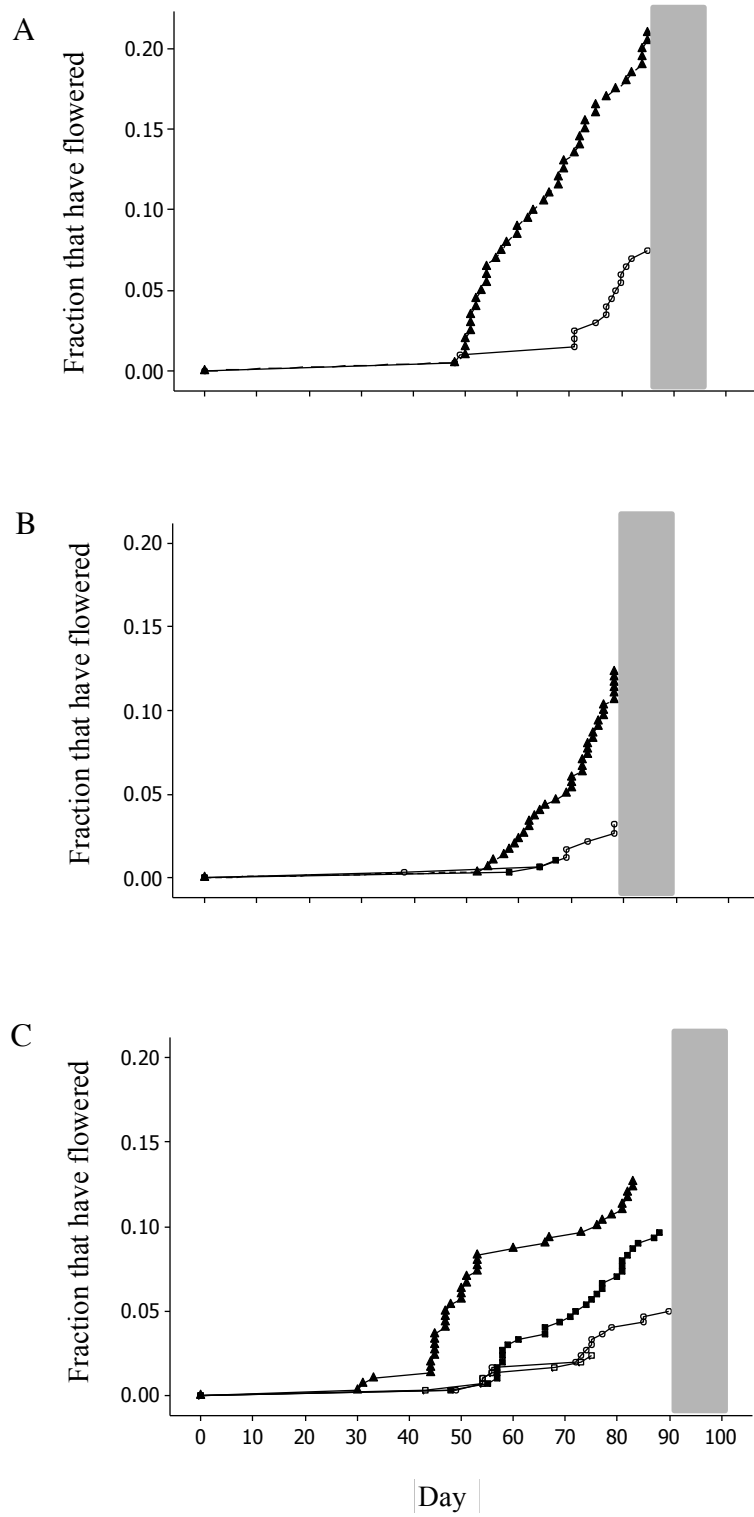
A

B

C

Day

Figure 2.3. Cumulative proportion of flowered HH (open circle), LL (shaded triangle), HL (shaded square), and LH (open square) *Mimulus guttatus* genotypes per day across three years: (A) 2008; (B) 2009; and (C) 2010. Grey bar indicates interval of final drought phase in each year.

antagonistic pleiotropy to maintain genetic variation.  Particular dominance relationships between alleles are necessary for a stable polymorphism in simple models (Radwan, 2008).  We cannot evaluate these relationships with the present data, because our design includes only two alternative genotypes per QTL.  However, theory predicts that polymorphism is more likely if per locus viability and fecundity effects are large, as they are in this study.

*Spatial and Temporal variation in selection*

Temporal fluctuations in the strength and direction of selection are evident from the year-to-year analysis reported above.  Table 2.1 reports total fitness estimates per genotype by location and year.  For the LL/HH contrast, we can simultaneously test for effects of genotype, location, and year, and their interactions (Appendix I).  All factors and their interactions are significant for survivorship (Table S1 of Appendix), but LL was consistently superior to HH (Fig. 2.1; Table 2.1).  Fecundity selection was heterogeneous with highly significant genotype x year and genotype x location interactions (Table S2 of Appendix). Finally, there were highly significant effects of genotype ($X^2$ =354, df =1, p <0.0001), year ($X^2$ =690, df =2, p <0.0001), and location ($X^2$ =18.4, df =1, p <0.0001) on total fitness. Interactions among these factors were also highly significant (Appendix I).

The significant interactions involving location and year included reversals.  Comparing LL to HH, the direction of fecundity selection reversed in 2009 relative to 2008 and 2010 (Fig. 2.1; interaction $X^2$ =425, df =2, p <0.0001). The overall direction of selection on the chromosome 1 QTL apparently reversed between 2009 and 2010 with L favored in 2009 and H in 2010 (Table 2.1).  Total fitness reversed between areas in 2008 with HH better in the dry

location and LL better in the wet location.  Fecundity selection was also spatially variable with H alleles usually more favorable in the Wet location.

*Maintenance of genetic variation*

This study provides direct evidence for necessary features of at least three distinct balancing selection mechanisms: antagonistic pleiotropy, spatial variation in fitness, and temporal variation in fitness.  The necessary conditions for 'protected polymorphism,' wherein selection acts to prevent either allele at a locus from going extinct, with antagonistic pleiotropy or temporal fluctuations are thought to be quite stringent (Radwan, 2008, Hedrick, 1999).  However, if both are operating combined with mutation and gene flow, variation is likely to persist at elevated levels regardless of genetic details specific to particular loci.  The conditions for polymorphism with spatially variation selection are less stringent, and with limited gene flow between patches, it is a powerful mechanism to maintain variation.  Local adaptation, the most compelling signature of polymorphism by spatial selection, has been demonstrated at the scale of meters in plant populations (Schmitt & Gamble, 1990).

The paucity of data comparable to that presented here likely reflects an enduring skepticism about successfully measuring natural selection on intra-population QTLs.  By definition, such loci contribute in an incremental way to variation and the concern is that fitness differences between alternative genotypes are simply be too small to be detected.  A surprising and important result from this experiment is that phenotypic and fitness differences between QTL genotypes that were first identified in the greenhouse are actually magnified in the field.  Differences in developmental timing that are fairly subtle under benevolent, nutrient rich conditions (Scoville et al., 2011, Lee, 2010) become more pronounced under relatively harsh,

nutrient poor field conditions.  This is an encouraging outcome for future QTL-focused field studies.  It is more broadly relevant because a mechanistic understanding of the forces that maintain variation is essential to understanding diversification of populations, species, and higher taxa.

**Chapter 3**

**Using phenotypic manipulation to test for direct selection on corolla width in *Mimulus guttatus* (yellow monkeyflower)**


Trait-fitness associations in the wild can result from direct selection on a trait or due to indirect selection (correlated selection). The distinction between direct and indirect target of selection is necessary because the response to the rate of selection (i.e. change in mean phenotype) is dependent on the relative direction between indirect and direct selection. For example, the change in mean flower size may be retarded if indirect selection (larger flowers are less fit to escape drought because of slow rate of development) opposes direct selection (larger flowers are more fit due to higher rate of pollinator visitation).

The phenotypic selection analysis (Lande, 1979) and the methods developed thereafter (Lande & Arnold, 1983, Arnold & Wade, 1984) are used to estimate direct and indirect target of selection in the wild (Kingsolver et al., 2001, Endler, 1986). While these methods are straightforward and widely applicable, selection estimates from these correlative approaches are prone to several well-known biases (Mitchell-Olds & Shaw, 1987, Wade & Kalisz, 1990, Rausher, 1992). For example because of environmental covariance, trait-fitness correlation will not always imply that the variance in fitness is caused by the "trait" variance. As an alternative to the correlative approach, phenotypic manipulation allows both detection of natural selection and provides causal links between the trait of interest (eg. flower size) and fitness in the wild.

Flower size is one of the most phenotypically variable traits among angiosperms. In *Mimulus guttatus,* this complex trait is under strong natural selection in the wild (Willis, 1996, Hall & Willis, 2006, Fenster & Ritland, 1994a, van Kleunen & Ritland, 2004, Fishman & Willis,

2008, Murren et al., 2009).    The phenotypic variation in flower size is a result of the action of multiple genes with rare partially dominant alleles (Lee, 2010). Flower size QTLs (Quantitative Trait Loci) are highly pleiotropic, affecting vegetative mass, water use efficiency, rate of development and intrinsic reproductive capacity (Kelly, 2008). Estimates of selection in the wild suggests that this trait is under strong selection (Fishman & Willis, 2008, Willis, 1996, Fenster & Ritland, 1994a, Murren et al., 2009, Kingsolver et al., 2001). However, the tremendous flower size variation in the field does not corroborate the predicted phenotypic variation based on the strength of directional selection estimated in the wild. This limit to directional selection may be a result of correlated selection (Kingsolver & Diamond, 2011) on flower size with other traits that influence fitness in the field.

My objective is to determine if corolla width (a measure of flower size) is under direct selection by performing phenotypic manipulation through trimming. This method directly isolates the effect of corolla morphology by eliminating associations with other traits (like plant size, rate of development, etc.). Phenotypic manipulation has been used to assess constraints on adaptation (Rose & Lauder, 1996) or to study multivariate selection on trait associations (Campbell, 2009) For example, floral morphology manipulation studies have documented significant reduction in fitness in manipulated flowers (Nilsson, 1988, Andersson, 1994, Herrera, 2001, Maad, 2000, Castellanos et al., 2004, Boberg & Agren, 2009) suggesting the functional significance of corolla morphology. However, very few studies evaluated such manipulations in the wild (Boberg & Agren, 2009; Herrera, 2001)

Here I provide the result of a multi-year phenotypic manipulation of flower size in the field. I trim the lower corolla lip without causing a wound response. I hypothesize that corolla width is under direct selection and therefore reducing corolla (through trimming of the lower

corolla lip) will both the mechanical function (as pollen trap for indirect pollination/reproductive assurance) and aesthetic function (for pollinator attraction). Using the native alpine *Mimulus guttatus*, I show that trimming of the entire lower corolla lip consistently reduces seed set and outcrossing rate. Partial trimming (removal of the lateral lobe but not the landing platform for the bees) affects the aesthetic but not the mechanical function. This data suggests that corolla width is under indirect selection due to its tight correlation with other characters (eg. biomass, pollen and ovule number, or rate of development)

**Methods**

*Study system and manipulations*

The common yellow monkeyflower (*Mimulus guttatus*) is a self-compatible hermaphrodite that produces zygomorphic flower with distinct upper lip and a lower lip with two lateral lobes (Figure 3.1). A typical annual alpine *Mimulus guttatus* produces a single flower and highly reduced leaves. The annual growing season usually ranges from mid-May to mid-July (seedling to seed). I identified a total of 1012 *Mimulus guttatus* at flower bud stage at the Browder Ridge site in Oregon, USA ($N_{2007}$ = 398, $N_{2008}$ = 344, $N_{2009}$ = 270). My previous experiments (Mojica & Kelly, 2010) and other experiments in *Mimulus* (Fishman & Willis, 2008, Fenster & Ritland, 1994b) and other species (Caruso, 2004, Fenster & Ritland, 1994b, Lehtila & Brann, 2007, Thompson et al., 2002, Meagher et al., 2005) measured the widest width across the two lateral lobes of the lower lip of the flower as a measure of flower size. I directly manipulate flower size by trimming this part of the flower. At the time when the flower opens, I randomly assigned each plant as either trimmed (lower corolla lip removed) or control (untrimmed corolla). In field season 2008 and 2009, I added the partially trimmed (lateral lip removed). This category reduces flower size but retains the landing platform for the bees.
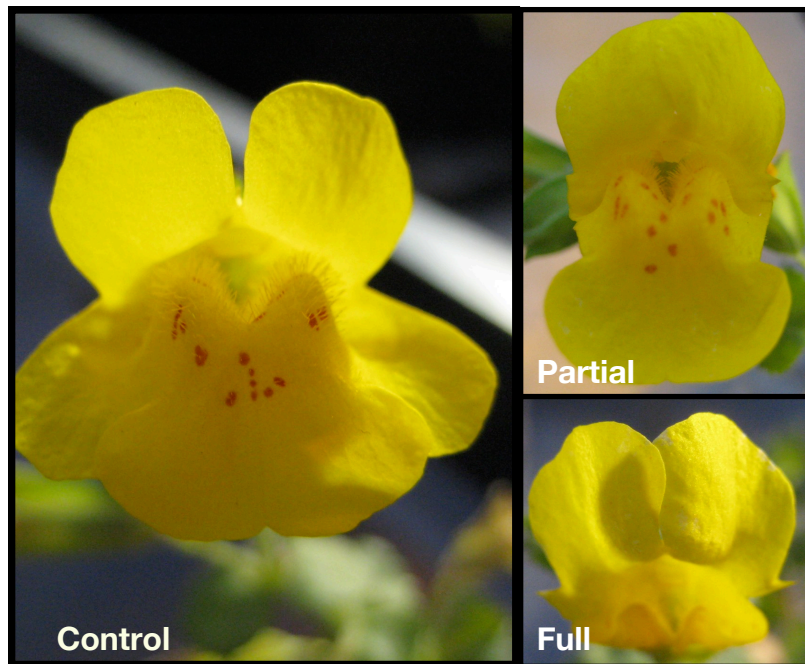
Figure 3.1. Phenotypic manipulation of *Mimulus guttatus* corolla.

I performed trimming and assigning of control native *Mimulus* plants from early-June to early July in each year of the study. I monitored the individuals and allowed natural pollination by bumblebees. I collected and counted the total seed set as a measure of female reproductive success.

*Procedural control*

In order to rule out the physiological effect of corolla trimming on seed set, I performed saturated hand pollination on separate group of native flowers in the field ($N_{2007}$ = 107, $N_{2008}$ = 153, $N_{2009}$ = 145) after which I randomly assigned each flower to a trimming treatment (partial or full) or control. I used surgical scissors to cut the entire lower corolla lip (fully trimmed) or to cut the lateral lobes (partially trimmed). I collected and counted all the seeds from each flower.

*Estimation of mating system parameters*

To estimate the rate of outcrossing of flowers, a random sample of 4-8 progeny per family were grown on 5x5 cm pots (one progeny per pot). I collected apical leaves and buds from each progeny for CTAB DNA extraction followed by PCR amplification for genotyping (see Marriage, 2009 for complete protocols). I genotyped each progeny and the maternal parent using five highly polymorphic microsatellite markers (AAT372, AAT 230, AAT300, AAT211, and AAT312). I used the MLTR program (parameters: multilocus outcrossing rate estimate based on 5 markers; standard errors of outcrossing rate is based upon 100 bootstraps with family resampling) to determine outcrossing rate per trimming category (Ritland, 2002).

*Statistical analyses*

I evaluated the effects of varying corolla morphology on reproductive failure, seed set, and outcrossing rate. I used $X^2$ goodness of fit to test the null hypothesis that the rate of reproductive failure is independent of trimming type. After log transformation, seed set is normally distributed within trimming groups for both hand pollinated and naturally pollinated flowers. I used the ANOVAs with log transformed seed set as the response variable and trimming type as the independent variable to determine the effect of the phenotypic manipulation per year. The difference in outcrossing rate was determined using MLTR's outcrossing rate comparison option. These analyses for both reproductive failure and seed set were done in Minitab® v.15.

**Results**

I found significant difference on fitness among naturally pollinated flowers in each year of the study (Figure 3.2B; 2007: $F_{1,396} = 14.01$, $p < 0.001$; 2008: $F_{2,116} = 4.38$, $p < 0.02$; 2009: $F_{2,226} = 17.38$, $p < 0.001$). In 2007, naturally pollinated fully trimmed flowers produced an average of 12.59 seeds (s.e. =1.68), which is significantly lower than control with 21.35 seeds (s.e. = 1.95). I observed the same trend in 2008 and 2009 between the seed set of fully trimmed flowers (mean$_{2008}$ = 4.73, s.e. = 1.16; mean$_{2009}$ = 28.71, s.e. = 3.5) vs. control flowers (mean$_{2008}$ =11.35, s.e. = 1.76; mean$_{2009}$ = 64.25, s.e. = 5.52). I did not find any significant difference on the average seed set between partially trimmed and control in 2008 and 2009 (mean$_{2008}$ = 11.87, s.e. = 2.27; mean$_{2009}$ = 52.25, s.e. = 4.58). The rate of reproductive failure (zero seed set) among naturally pollinated plants was highest in 2008 (65%) and lowest in 2009 (15%)
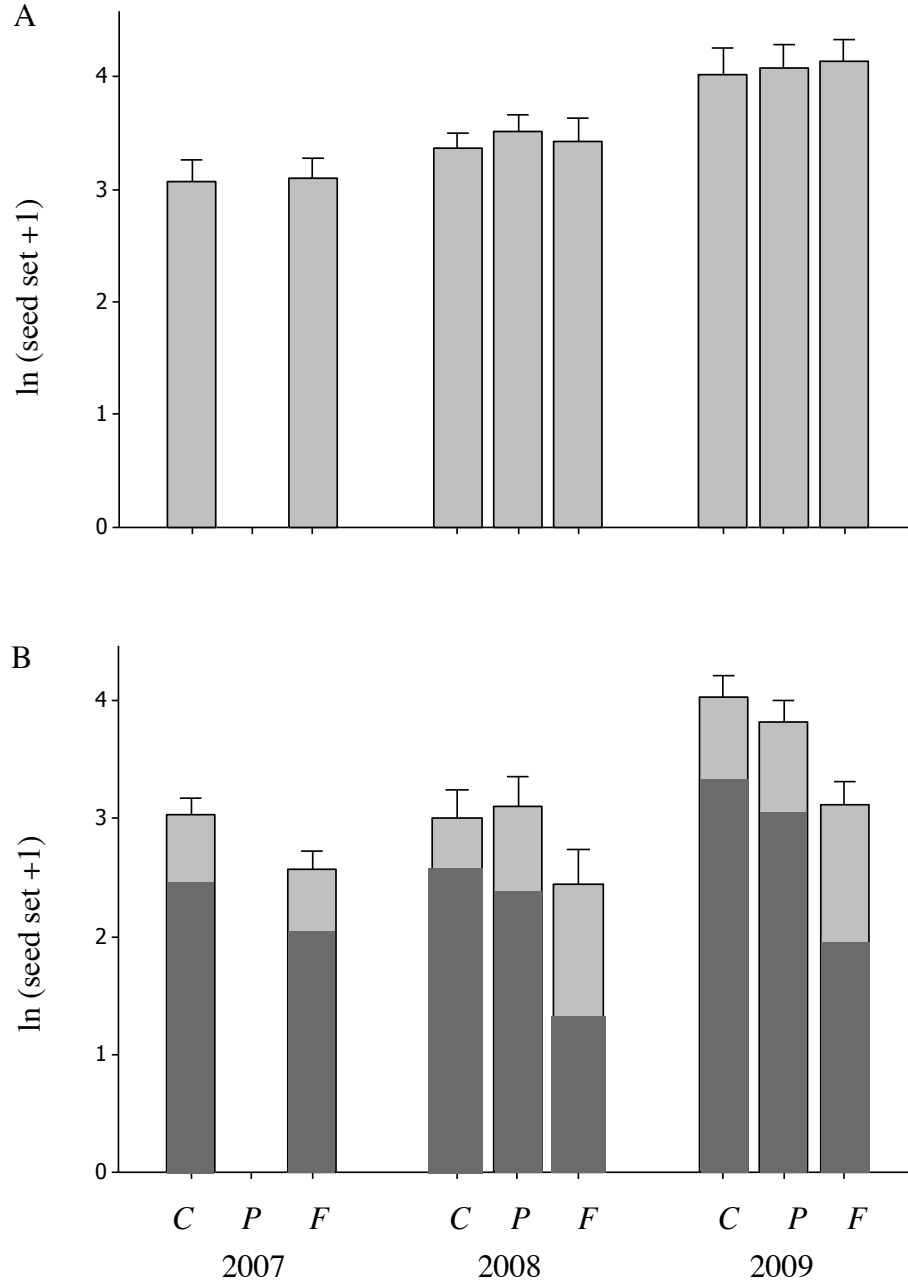
Figure 3.2. Total seed set between (A) hand pollinated and (B) naturally pollinated flowers across years. The trimming categories are: control *(C)*, partially trimmed *(P)*, and fully trimmed *(F)*. Dark gray bars in panel B indicate proportion of outcrossed seed set. Error bars are 95% confidence interval of the mean.

Failure to set seed was unrelated to treatment category except for year 2007 (37%) where full trimming had more zero seed set than expected under the null hypothesis (2007: $X^2 = 4.19$, df = 1, $p < 0.05$; 2008: $X^2 = 3.64$, df = 2, $p > 0.16$; 2009: $X^2 = 2.72$, df = 2, $p > 0.25$)

Progeny genotyping showed that full trimming significantly reduces the multilocus outcrossing rate $(t)$ relative to the control in most years except in 2007 ($t$ difference = 0.038, s.e. = 0.092). In 2008, full trimming of the lower corolla lip ($t = 0.568$, s.e. = 0.167) reduces outcrossing rate relative to the control ($t = 0.901$, s.e. = 0.026) by as much as 33%. I found a 15% reduction of outcrossed seed set in 2009 on fully trimmed flowers ($t = 0.755$, s.e. = 0.060) vs. control ($t = 0.907$, s.e. = 0.021). Partially trimmed flowers produced significantly higher outcrossing rate than fully trimmed flowers ($t_{2008} = 0.909$, s.e. = 0.059; $t_{2009} = 0.914$, s.e. = 0.033) but not vs. control (Figure 3.2A).

The procedural control data suggests that trimming of the corolla does not influence the intrinsic reproductive capacity of the flowers to produce seeds (Figure 3.2A). On average, seed set of each flower that received saturated hand pollination does not significantly differ among experimental categories in each year of the study despite large sample sizes (2007: $F_{1,105} = 0.13$, $p > 0.72$ ; 2008: $F_{2,150} = 1.88$, $p > 0.16$; 2009: $F_{2,131} = 0.48$, $p > 0.62$). Death of hand pollinated plants before fruit maturity was low across years and unrelated to the treatment category (2007: $X^2 = 1.48$, df = 1, $p > 0.48$; 2008: $X^2 = 3.16$, df = 2, $p > 0.20$; 2009: $X^2 = 0.18$, df = 2, $p > 0.48$).

**Discussion**

The corolla serves two distinct functions—mechanical, as a landing platform for pollinators and aesthetic, as a pollinator attractant. Corolla morphology plays a critical role in the pollination biology of *Mimulus guttatus*. The phenotypic manipulation in the field showed that relative to the untrimmed plants, full trimming of the lower corolla lip reduces fitness by as much as two fold and outcrossing rate by as much as 33%. However, partial trimming does not influence the number of seeds produced compared to control flowers suggesting the important role of the landing platform for attraction and effective pollination. Finally, I found no significant difference in seed set among the hand pollinated trimming categories in the field. This suggests that my phenotypic manipulation does not produce a "wound" response and any difference in seed set is a result of differential pollination success.

Sober (1984) used the analogy of a multilayered-sieve to explain the nature of simultaneous selection on multiple traits. He described a transparent barrel that contains several layers of sieve that *selects for* a different size of ball (each size is associated with a specific color). He argued that the size and color of the balls are analogous to correlated characters under selection. Ball size is under direct selection (*selection for* size) and color is an indirect target of selection (*selection of* color). Often in nature, however, I do not know what the "sieve" is selecting for.

Arathi and Kelly (2004) performed a series of experiments with the full trim and control categories in a greenhouse setting. They allowed *Mimulus* to be accessed by bumblebees or maintained in a pollinator free environment. Their experiments demonstrated that corolla morphology plays a critical mechanical role in both outcrossing and self-fertilization. In the absence of pollinators, the lower portion of the corolla facilitated autogamy by retaining pollen

released from the anthers. When pollinators were present, the corolla facilitated outcrossing prior to, during and after insect visitation. Correlational studies were also instrumental in proving the importance of corolla morphology in the reproductive success of plants. Willis (1996) and van Kleunen and Ritland (2004) showed that flower size is positively correlated with seed set. However, the presence of environmental covariance between traits and fitness measures can also bias field estimates for selection gradients. This bias can be eliminated by direct manipulation of the trait (eg. corolla trimming).

My data suggests that corolla width (a measure of flower size) is an indirect target of selection. In *Mimulus*, the *selection of* (Sober, 1984) corolla width is potentially due to its tight correlation to the rate of development. Field studies (Mojica & Kelly, 2010) and greenhouse studies (Kelly, 2008) on artificially selected population based on flower size showed that bigger flowers takes longer days to flower. The rate of development is a critical determinant of fitness especially in alpine environment where there is a limited time to grow and reproduce. The apparent selection of corolla width that is commonly detected in the field can be also due to the tight positive association of flower size to reproductive capacity (pollen and ovule number).

I did not find any evidence of pollen limitation after comparing seed set between hand-pollinated and naturally pollinated flowers in each year. Pollen limitation is common in many plant taxa (Ashman et al., 2004, Knight et al., 2005) and in *Mimulus guttatus* (Fishman and Willis 2008). The absence of pollen limitation can be a result of significant delayed selfing (Morgan & Wilson, 2005, Dole, 1992), which has been shown in this species (Arathi and Kelly 2004). Significant increase in selfing rate has been observed in just a few generations without pollinators in experimental populations of *Mimulus* (Bodbyl-Roels & Kelly, in press). Delayed

selfing is an important mechanism of reproductive assurance in self-compatible mixed-mating species when there is fluctuation pollinator availability (Lloyd, 1979, Kalisz et al., 2004).

The fitness difference that I detected between fully trimmed and control flowers is based only on seed set. When a selection gradient type study uses plant size, fruit number, or seed set the measure of plant fitness is restricted to female function. When the male and female fitness components are under different selection (Hodgins & Barrett, 2008) female reproductive success is not correlated with male reproductive success. Determining paternity of progenies from the field can be difficult because of the number of potential sires. An array experiment where the number of potential sires (assigned with a specific genotype) is restricted allows direct measurement of male fitness through outcross siring success.

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**APPENDIX I**

*Summary of analysis contrasting HH and LL genotype across years*

Survivorship (0/1) was treated as a binary response variable in a generalized linear model fit (logit link function) with genotype, location, and year as factors. The hypothesis test summary:

|  | df | $X^2$ | p |
|---|---|---|---|
| Genotype | 1 | 44.6 | 0.0000 |
| Location | 1 | 3.2 | 0.0749 |
| YEAR | 2 | 11.0 | 0.0040 |
| Genotype*Location | 1 | 5.1 | 0.0243 |
| Genotype*YEAR | 2 | 3.0 | 0.2227 |
| Location*YEAR | 2 | 60.8 | 0.0000 |

Fecundity of flowering plants was treated as an overdispersed Poisson response variable in a generalized linear model fit (log link function) with genotype, location, and year as factors. The hypothesis test summary:

|  | df | $X^2$ | p |
|---|---|---|---|
| Genotype | 1 | 11.9 | 0.0006 |
| Location | 1 | 27.8 | 0.0000 |
| YEAR | 2 | 218.1 | 0.0000 |
| Genotype*Location | 1 | 13.3 | 0.0003 |
| Genotype*YEAR | 2 | 425.4 | 0.0000 |
| Location*YEAR | 2 | 168.2 | 0.0000 |

Total fitness was treated as an overdispersed Poisson response variable in a generalized linear model fit (log link function) with genotype, location, and year as factors. The hypothesis test summary:

|  | df | $X^2$ | p |
|---|---|---|---|
| Genotype | 1 | 353.8 | 0.0000 |
| Location | 1 | 18.4 | 0.0000 |
| YEAR | 2 | 689.8 | 0.0000 |
| Genotype*Location | 1 | 128.2 | 0.0000 |
| Genotype*YEAR | 2 | 494.2 | 0.0000 |
| Location*YEAR | 2 | 1929.6 | 0.0000 |