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The Adaptive Nature of Stasis for Petal Number: Can Pollinator-Mediated Stabilizing Selection Explain Five-petaled Flowers?

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James Gilbert Mickley, PhD
University of Connecticut, 2017

Angiosperms display considerable petal number diversity. Yet, much of the massive eudicot clade, Pentapetalae, is fixed for five-petaled flowers, with widespread stasis within and among species. This stasis has been presumed adaptive and maintained by natural selection, namely by pollinator-mediated stabilizing selection or some other unknown mechanism. Little is known about selection and genetics of floral part number and the mechanisms underlying petal number stasis are unexplained in most plant groups. Despite broad patterns of stasis, some species maintain low levels of within-individual variation. To the extent that this variation is genetic, it can be used to test various mechanisms of stabilizing selection that might promote stasis on broader scales. By first testing for a genetic basis to variation in petal number, and then testing for pollinator-mediated stabilizing selection on this variation, as well as other mechanisms for canalization such as pleiotropy-mediated stabilizing selection, the sufficiency of adaptive stabilizing selection as a driver of petal number stasis can be evaluated.

Here, I conduct a selection experiment and show that natural within-individual variation for petal number has a genetic basis. Presence of this heritable variation provides leverage on which stabilizing selection can act, enforcing canalization and preventing this variation from increasing. Correlated responses in other floral parts to selection for increased, but not decreased, petal number show that pleiotropic constraints due to interactions with other floral part numbers cannot completely explain petal number stasis. Comparing levels of petal number

variation in natural populations between autogamous and outcrossing congeners show no relaxation of pollinator-mediated selection under autogamy, suggesting that pollinators are indiscriminate. Further evidence from a pollinator visitation experiment shows that pollinators exhibit no preference for five-petaled flowers, failing to explain fixation and stasis for five petals.

My work demonstrates that while variation in petal number is under genetic control, mechanisms controlling stasis remain elusive. A focus on pollinator preference as the main driver of stasis may be misguided, and I suggest that environmental factors, developmental constraints, pleiotropy, and historical contingency may be more important in explaining the adaptive nature of petal number.

The Adaptive Nature of Stasis for Petal Number: Can Pollinator-Mediated Stabilizing Selection

Explain Five-petaled Flowers?

James Gilbert Mickley

B.A., Kalamazoo College, **2008**

M.A., Stony Brook University, **2010**

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at the

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APPROVAL PAGE

Doctor of Philosophy Dissertation

The Adaptive Nature of Stasis for Petal Number: Can Pollinator-Mediated Stabilizing Selection
Explain Five-petaled Flowers?

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CHAPTER 1:

Heritability of Variation in Petal Number and Correlated Selection in *Phlox drummondii*

ABSTRACT

Though angiosperms vary in the number of all four floral organs, stasis is widespread, particularly for petal number in the large group Pentapetalae. This stasis has been considered adaptive, maintained by stabilizing selection. For selection to play a major role in maintaining stasis for petal number, genetic variation must be present. While one explanation for adaptive petal number stasis is pollinator-mediated stabilizing selection, other mechanisms generating stabilizing selection are possible. For example, floral traits are highly integrated, and pleiotropic effects among traits, such as the number of other floral organs might also maintain stasis. Some clades within Pentapetalae, particularly the Polemoniaceae, are known to have low levels of natural within-individual variation for petal number. To test whether this natural variation was due to genetic factors, a selection experiment was conducted using *Phlox drummondii* (Polemoniaceae), selecting for a higher proportion of four-petaled, six-petaled, and five-petaled flowers. Correlations between petal number and the other three floral organs were measured, as well as their correlated responses to selection on petal number. All four floral organ numbers were correlated, though carpel number was relatively decoupled from the number of the other three organs. Six-petaled flowers were more common than four-petaled flowers, both before and after selection. The heritability and response to selection for a higher proportion of six-petaled flowers was strong and triggered correlated responses in stamen and sepal number, implying shared genetic control. However, the response to selection for a higher proportion of four-petaled flowers was weaker, with no correlated responses. There was no response to selection for a higher proportion of five-petaled flowers. Data suggest that the gain of a petal may be under

different and stronger genetic control than the loss of a petal. These results show that there is a genetic basis to standing within-individual variation for changes in petal number, and that it is not merely developmental noise. That this heritable variation is only present at low levels in natural populations implies stabilizing selection is active. Pleiotropic consequences to increases in petal number could limit changes in petal number in one direction, but they may not fully explain stasis. The adaptive basis of petal number stasis remains unknown, though pollinator-mediated and tradeoff-mediated stabilizing selection may provide possible alternatives.

INTRODUCTION

Many components of plants are highly variable; however, flowers tend to be relatively invariant within a species. Yet, at higher taxonomic levels, there is tremendous diversity among species. This combination of diversity at higher taxonomic levels and invariance at lower levels has led floral traits to be used to delineate species, genera, and families (Grant 1949; Cronquist 1981). The numbers of sepals, petals, stamens or carpels within a flower or *merosity* (Ronse De Craene & Smets 1994) are particularly useful traits for characterizing higher taxa of plants such as families and genera because they are among the most consistent of all angiosperm characters (Cronquist 1981; Vlot et al., 1992; Givnish 2002; Herrera 2009), even spurring the use of floral formulae or floral diagrams as descriptors of families (Eichler 1875; Prenner et al., 2010; Ronse De Craene 2010). Despite the usefulness of merosity, the evolution, genetics, development, and ecology of floral organ number have been understudied compared with other areas of floral trait biology, such as symmetry (Giurfa et al., 1999; Endress 1999), organ identity (Coen & Meyerowitz 1991; Ó'Maoiléidigh et al., 2013; Soltis & Soltis 2013), and color (Epling & Dobzhansky 1942; Hopkins & Rausher 2012; Wessinger & Rausher 2012). Although generally consistent within clades, natural variation or *inconstancy* of floral organ number occurs to

varying extents in many species, both within species and individuals (Roy 1963; Huether 1969; Ellstrand 1983; Vlot et al., 1992; Doust 2001; Ronse de Craene 2016; Monniaux et al., 2015).

Research on merosity has focused on the corolla—not surprising given the corolla’s role in attracting pollinators. Though petal number was labile in early angiosperms, both petal number and its inconstancy decreased as angiosperms diverged (Stebbins 1950; Stebbins 1974; Ronse De Craene & Smets 1994; Soltis et al., 2003). Multiple clades evolved an invariant five petals or *pentamery*, most notably the aptly named Pentapetalae (Cantino et al., 2007; Wanntorp et al., 2007; Soltis et al., 2003; Ronse de Craene 2016). Pentapetalae comprises some 175 plant families and a vast portion of angiosperm diversity. Although some lineages within Pentapetalae (e.g. Brassicaceae) are fixed for other petal numbers (reviewed in Ronse de Craene 2016), most lineages are predominantly five-petaled. Pentamery has also evolved independently in a number of other clades, such as the Ranunculaceae and Sabiaceae; there are even a few examples in the monocots (Ronse de Craene 2016). Multiple convergences on corolla pentamery and its relative stasis raise questions about its evolutionary significance.

Stasis for corolla pentamery (or constancy in general) has been presumed to be adaptive (Stebbins 1974; Endress 2001a) for a variety of reasons: 1) pollinator preference for pentamery (Leppik 1953; Stebbins 1974; Zhao & Huang 2013; Monniaux et al., 2015) or against inconstancy (Herrera 2009), leading to stabilizing selection; 2) a necessary precondition for predictable development of more complex flowers (Endress 2001b; Ronse De Craene 2010); 3) pleiotropic effects from selection on other floral organ numbers due to phenotypic integration in the floral module (Byerley 2006; Herrera 2009); and 4) non-pollinator-mediated selection (Strauss & Whittall 2007) directly on petal number (e.g., petals have other functions, such as opening the flower bud, Monniaux et al., 2015). Removal of all petals on a flower lowers

visitation and seed set (Johnson et al., 1995; Kudoh & Whigham 1998). Therefore, I propose an additional reason that as petal number increases, floral water loss (Lambrecht 2013) or other costs might also increase and trade off with the ability of the flower to attract pollinators. These hypotheses are largely not mutually exclusive, and with few exceptions (Huether 1968; Byerley 2006; Zhao & Huang 2013), have not been well-tested.

Despite a focus on petal number, other floral organs vary in merosity too (Ronse de Craene 2016), and often this variation in merosity is correlated among floral whorls (Nobs 1963; Ellstrand 1983; Ronse de Craene 2016), though carpels tend to be slightly decoupled, supposedly because they are the most space-constrained organ when developing from a floral meristem (Ronse de Craene 2016), and because their number can have direct effects on fecundity (Byerley 2006). Floral traits are thought to be highly integrated and pleiotropic effects are common (Diggle 2014; Smith 2016). For example, organ identity is controlled by shared pathways in the ABCDE model (Coen & Meyerowitz 1991; Soltis & Soltis 2013). In the same manner that organ identity is determined, the genetic machinery responsible for generating a given petal number may be shared among other organs or even vegetative traits, and selection on one floral organ might act indirectly on others through pleiotropy.

Little is known about the genetics of floral organ number (reviewed in Ronse de Craene 2016), particularly to what extent pleiotropy governs merosity relationships. Like many other plant features, much of our understanding of genes with effects on merosity comes from *Arabidopsis thaliana*, a four-petaled species. These genes can be separated into several classes: 1) those that have general effects on meristem or floral meristem size (e.g., CLAVATA, WIGGUM; Running et al., 1998; Suzaki 2004; Ronse de Craene 2016), 2) those that increase the number of whorls (e.g., ULTRAPETALA; Ronse de Craene 2016), and 3) those that do not

affect meristem size or whorls (e.g., PERIANTHIA; Running & Meyerowitz 1996). Duplication of whorls has obvious implications for merosity and may be one avenue for large increases in petal number; however, it is of limited usefulness in explaining the stasis of pentamery (Ronse de Craene 2016). In contrast, changing floral meristem size affects the amount of space available for organ initiation, in turn changing the mechanical forces within the flower that govern the number of organs (Ronse de Craene 2016). These meristem size changes might be expected to affect multiple whorls within the flower, and indeed this is what happens in mutants (Crone & Lord 1993; Running et al., 1998), setting up mechanisms for correlated shifts in floral organ number. Some CLAVATA mutants also seem to act via heterochrony by increasing the time between developmental stages (Crone & Lord 1993). The third class of genes (PERIANTHIA) simply cause a reversion back to an ancestral pentamerous state in *A. thaliana* (Ronse de Craene 2016) when disabled. Some work has been done to elicit the genetic architecture of petal number or the numbers of other organs (Vlot & Bachmann 1991; Vlot et al., 1992; Monniaux et al., 2015; Pieper et al., 2015); however, it has not resulted in a set of candidate genes and has only pointed towards petal number being a polygenic trait. Given the tetramerous state of *A. thaliana*, it is difficult to apply this limited knowledge of the genetic basis of merosity to the broader question of pentamery and stasis.

Despite stasis for petal number in Pentapetalae, variation in petal number within individuals has been shown to be heritable in several studies (Huether 1968; Monniaux et al., 2015; Pieper et al., 2015), suggesting the potential for selection to change merosity (Herrera 2009). Though artificial selection on the number of a single floral organ has been attempted (Huether 1968; Vlot et al., 1992; Monniaux et al., 2015; Pieper et al., 2015; Byerley 2006), the correlated effects of this selection on other floral organs are unclear. If the number of organs per

whorl are integrated and under common genetic control, selection on the merosity of one organ may also change the others. These coordinated changes might be especially relevant to fitness given that changing stamen or carpel number can have consequences for the amount of pollen or ovules produced (Byerley 2006).

Here, I test for the necessary preconditions for selection (by pollinators or any other factors) to act on merosity, by selecting for a higher within-individual proportion of four-, five- and six-petaled flowers in the normally five-petaled *Phlox drummondii* (Polemoniaceae). This species has variation in the numbers of all floral organs (Lehmann 1987; Byerley 2006). I ask the following questions: 1) Can the proportion of abnormal four- and six-petaled flowers be increased via selection, indicating a heritable genetic basis and standing genetic variation available to selection? 2) Can the proportion of five-petaled flowers be increased via selection, indicating genetic variation for increased constancy and improved developmental buffering? 3) Does selection targeting changes in petal number result in correlated changes in the numbers of other floral organs, allowing for changes in merosity to be driven by floral organ integration? 4) Are there tradeoffs in floral size or the number of flowers produced by a plant associated with shifts in merosity that might indicate a developmental mechanism leading to pleiotropy? Given the scarcity of information on the genetics of floral organ number, answering these questions dispels the possibility of within-individual variation in petal number being simply developmental noise. This all provides a starting point towards addressing some of the hypotheses on stasis in petal number, particularly those dealing with the potential for selection on petal number and pleiotropic effects between floral organs or other floral traits.

METHODS

Study System

Phlox drummondii Hook. is an herbaceous annual within the Polemoniaceae native to central and southern Texas, growing primarily in roadsides and meadows (Grant 1959; Erbe & Turner 1962; Turner 1998). Seeds germinate in the fall or early spring, and flowering occurs between March and May. Flowers are pollinated by a variety of lepidopterans, primarily *Hyles lineata*, *Battus philenor*, (Grant & Grant 1965) and skippers (family Hesperidae) (Hopkins & Rausher 2014), though at least some genotypes are pseudo-self-fertile (selfing only after failure to outcross: Levin 1996). The species has been studied extensively, and its breeding and mating system (Levin 1975; Levin 1978; Levin 1993; Bixby & Levin 1996; Levin 1996; Ruane 2008), phenotypic plasticity (Schlichting & Levin 1984; Schlichting 1989b), pollination (Grant & Grant 1965; Majetic et al., 2014), floral biology (Levin 1969; Levin & Brack 1995; Kelly & Levin 2000; Lendvai & Levin 2003; Hopkins & Rausher 2012), and merosity (Lehmann 1987; Byerley 2006) have been well-characterized, providing a strong base for further research using this species. The species is amenable to greenhouse growing conditions with a generation time of 3–4 months.

Normally, like most species in the Polemoniaceae, *P. drummondii* has flowers with five sepals, five petals, five stamens, and a tricarpellate gynoecium with three corresponding stigma lobes (Grant 1959; Lehmann 1987; Byerley 2006). Petals are fused into a tube and stamens are partially attached to the corolla tube, though both of these fusions are postgenital (occurring after primordia initiation). Like other members of the Polemoniaceae (Huether 1968; Huether 1969; Ellstrand 1983; Ellstrand et al., 1984; Ellstrand & Mitchell 1988), *P. drummondii* has natural variation in numbers of all floral organs (Ellstrand 1983; Lehmann 1987; Byerley 2006). Most

variation in number consists of either the gain or loss of a single organ within a whorl, though more extreme variants occasionally occur.

Design of Selection Experiment

Wild-collected *P. drummondii* seeds were purchased in 2010 from Native American Seed (Junction, TX; Byerley 2006). Seeds were started in November 2010 in 60 four-inch pots containing a peat and perlite growing mix (Fafard #2, Sunagro Horticulture, Agawam, MA). Each pot received six seeds; 84% germinated within 1–2 weeks. Seedlings were randomly thinned to one per pot after their first true leaves emerged.

Plants began to flower approximately 75 days after sowing. When most plants were flowering, all flowers were removed from plants, dissected, and the number of sepals, petals, stamens and carpels counted. Subsequent flowers were removed for ease of counting and to prolong flower production. Flower removal and the counting of floral organs was repeated approximately twice a week for seven weeks and then plants were allowed to self-pollinate and seeds were collected. For analysis, organ numbers were categorized as normal (five for sepals, stamens, and petals, three for carpels), more than normal, or fewer than normal, and each of these categories was expressed as a proportion of the total flowers on a plant. Nearly all abnormally-petaled flowers consisted of either four or six petals; therefore, the “more than normal” category is referred to as *six-petaled flowers* and the “fewer than normal” category as *four-petaled flowers*. Variation in number for the other three floral organs were categorized similarly.

Using seed from this first generation, a second generation of *P. drummondii* was planted in November 2011 to perform a selection experiment. I artificially selected for three groups: a higher proportion of six-petaled flowers (selection up), a higher proportion of four-petaled

flowers (selection down), and a higher proportion of five-petaled flowers (constancy). That is, out of the 60 original plants in the parental generation, the five with the highest proportion of six-petaled flowers were used as parents of the selection up regime and the five with the highest proportion of four-petaled flowers were used to start selection down regime. Finally, five of the plants with no variation in petal number (38% of the parental generation) were chosen at random and were used to start the constancy regime. Seed from each of these 15 self-pollinated plants from the parental generation was planted, forming 15 maternal families. Seeds from the original wild-collected *P. drummondii* stock were also planted as an unselected control regime. Interestingly, plants with high proportions of four-petaled flowers also tended to have relatively high proportions of six-petaled flowers. One plant was eligible for both selection regimes, and because four-petaled flowers were much rarer, it was assigned to the selection down regime. Seeds were sown in seedling flats and four seedlings from each of the five maternal families per selection regime (20 total plants per regime) were randomly selected and transplanted to four-inch pots. Twenty seedlings were also selected from the control. Methods then followed those of the parental generation, with the exception that the length of a petal on each flower (corolla radius) was measured as a floral size covariate to determine if flower size was related to petal number.

A third generation was planted in October 2014 using seed from the maternal families with the largest selection responses in each selection regime and following the methods from the second generation. In this generation, instead of measuring floral size, positional effects were assessed, and flowers scored according to where on the plant they originated: i.e., the terminal cluster, a basal branch, or a numbered progression of axillary branches from below the terminal

cluster to the base of the plant. Data from this generation was only used to determine positional effects on petal number and not used for selection analyses.

Statistical Analyses

All analyses were conducted using R (R Core Team 2015). General data manipulation was carried out using the following packages: dplyr (Wickham & Francois 2015), plyr (Wickham 2011), and tidyr (Wickham 2015). Figures were generated using the cowplot (Wilke 2015) and ggplot2 (Wickham 2009) packages.

Correlations between floral organ numbers - Pearson correlation coefficients among floral organ numbers were calculated. To account for intercorrelation between organ numbers, partial correlations were calculated using the ggm package (Marchetti et al., 2015). Finally, to further study patterns of intercorrelation, a principal components analysis and a hierarchical k-means clustering analysis on the principal components was conducted using the FactoMineR package (Husson et al., 2015), using the four floral organ numbers as traits.

Heritability - Realized heritability (H^2) was estimated with the breeder's equation (Falconer & Mackay 1996) by calculating the ratio between the selection response (R) and the selection differential (S). Selection responses were calculated by subtracting the per-individual parental population mean proportion of four-, five-, or six-petaled flowers from the per-individual means of each selection regime after selection. Selection differentials were calculated by subtracting the per-individual parental means from the per-individual mean of the five individuals that were the progenitors of each selection regime.

Statistical significance of selection responses - Separate sets of binomial logistic mixed models were constructed to quantitatively test for selection responses in the selection up and down regimes respectively using the lme4 package (Bates et al., 2015). A binary representation

of petal number (e.g., flowers coded as $< \text{five petals}$ or $\geq \text{five petals}$ for selection down models) was used as the response variable. To account for variation among plants, these models always contained the plant identity as a random term. Selection regime, days since onset of flowering, number of flowers, and corolla radius were considered as fixed factors, with the control selection regime as the reference factor. No interactions among fixed factors were apparent during exploratory analysis, and thus they were excluded. Model selection followed the methods of Zuur *et al.* (2009), starting with a full model and removing fixed terms in a stepwise fashion to minimize AICc scores calculated using the *bbmle* package (Bolker & R Development Core Team 2014). Attention was given to model weights, and fixed factors were kept if removing them did not improve AICc scores by ~ 2 or if model weights of models including them were sizeable (Zuur *et al.*, 2009). Additionally, a cumulative link mixed ordinal model was constructed to consider all of the selection regimes and petal numbers in the same framework using the *ordinal* package (Christensen 2015).

Correlated selection response - To test for correlated selection on other floral organ numbers when selecting for petal number, the selection response for the five maternal families within each selection regime was calculated by subtracting the per-individual mean proportion of four- or six-petaled flowers in the parental generation from that of the maternal family after selection. The selection responses for sepals, stamens, and carpels for the same maternal family were then regressed against the petal selection response.

RESULTS

Parental Generation

In the parental generation, 6674 *P. drummondii* flowers from 60 plants were scored for all four floral organs. Plants averaged 2.5% abnormally-petaled flowers (range: 0–22%) and 10.3% abnormal flowers for at least one floral organ (range: 0–44%). Six-petaled flowers were much more common than four-petaled (Figure 1.1), and although petal number ranged from two to eight, four- and six-petaled flowers were by far the most common abnormal flowers. The frequency distribution for the percentage of six-petaled flowers per plant did not match an expected Poisson distribution based on the per-plant mean percentage. Plants with no six-petaled flowers or a high percentage of six-petaled flowers were overrepresented (Figure 1.1), suggesting genetic differences among plants. The percentage of four-petaled flowers per plant conformed well to a Poisson distribution (Figure 1.1).

Deviations from the normal merosity among all four floral organ numbers were correlated. Correlations between carpel number and the numbers of other organs were low ($r < 0.33$), while correlations between sepals, petals and stamens were high ($r > 0.73$ for all) (Figure 1.2, Table 1.1). Partial correlations between organ numbers were lower, with only those between petals and sepals and petals and stamens still strong (Table 1.1), indicating a high degree of intercorrelation. Therefore, correlation between petals and sepals and between petals and stamens (adjacent organs in both cases) were the main drivers of these correlational patterns and carpels were almost completely decoupled from the system. The principal components analysis on floral organs explained 89% of the variation in organ number in the first two axes, with sepals, petals, and stamens loading strongly on the first axis and carpels loading on the second axis (Figure 1.3; Table 1.S1). Again, carpels were nearly completely decoupled from the

other floral organs (Figure 1.3). Nearly every combination of floral formulae within the numerical range of organ number variation in each organ was present (Figures 1.S1-1.S3). The most common abnormal floral formula was the addition of a carpel, but the second and third most common abnormal formulae consisted of synchronized changes in several floral organs (from 5553 to 6664 or 6663; Figure 1.S3), suggesting that coordinated changes were more likely. This variation in floral formulae was present within plants, with one third of the plants exhibiting five or more different formulae (Figure 1.S4).

The per-plant percentage of six-petaled flowers decreased over time ($p = 0.02$; Figure 1.4), but there were no temporal changes to the percentage of four-petaled flowers (Figure 1.4). There was no relationship between either four- or six-petaled flowers and the total number of flowers a plant produced either before or after selection (Figures 1.S5, 1.S6).

Selection Response

Both the selection up and selection down regimes registered positive selection responses (Table 1.2). Per-individual percentages of both four- and six-petaled flowers increased roughly four-fold in their respective selection regimes over one generation (Figures 1.5, 1.6). Realized heritability was high for selection on six-petaled flowers ($H^2 = 0.7$), and lower for selection on four-petaled flowers ($H^2 = 0.5$) (Table 1.2). The proportion of six-petaled flowers also increased slightly in the selection down regime (Figure 1.5). Selection for constancy was not successful. Proportions of four- and six-petaled flowers both appeared to increase in this selection regime instead of decreasing (Figures 1.5, 1.6). Neither of these increases was statistically different from the control, though, combined, they suggest a negative selection response in the opposite direction of the selection differential. Thus, there is no evidence for heritable variation for

increased petal number constancy (Table 1.2). The control group had proportions of four- and six-petaled flowers similar to those in the parental generation (Figures 1.5, 1.6).

In both the selection up and selection down binomial model sets, the inclusion of plant as a random factor greatly improved the model with ΔAICc scores of -189 (selection up) and -17 (selection down) indicating considerable variance explained by differences among plants. The best model for the selection up model set contained selection regime and day since onset of flowering as fixed factors (Table 1.3). A second model containing only day since onset of flowering also garnered support with 25% of the model weight (Table 1.3). Effects of both selection regime and day were statistically significant in the best selection up model (Table 1.4). Parameter estimates mirrored qualitative results (Figures 1.4, 1.5), with selection regime (selection up) increasing the proportion of six-petaled flowers, and day since onset of flowering decreasing it (Table 1.4).

Binomial models for selection down again showed two models with some support (Table 1.5). The best model contained only selection regime, but a model with selection regime and corolla radius comprised 35% of the model weight (Table 1.5). In contrast to the selection up model (Table 1.4), the effect of selection regime was not statistically significant for selection down (Table 1.6). The effect of corolla radius in the weaker supported selection down model was negative: smaller flowers were more likely to be four-petaled, but this was not statistically significant (not shown).

Ordinal model results were similar to those of the binomial models. The best ordinal models were the same as in the selection up binomial models, and these models showed selection up and day as important factors, but not selection down. (Tables 1.S2, 1.S3). However ordinal models did not capture any effect of corolla radius.

Correlated Selection Responses

When selecting for a higher proportion of six-petaled flowers in the selection up regime, the petal number selection response was mirrored by positive correlated responses in sepal and stamen number (Figure 1.7). In both cases, the correlation was very strong (Table 1.7), and even held for maternal families that expressed negative selection responses. The slope of the correlated response for sepal number was very close to one (Table 1.7), while the correlated response for stamen number had a lower slope (Table 1.7). While the slope for carpel number was positive (Figure 1.7), neither the slope nor the correlation between petal number and carpel number response was statistically supported (Table 1.7); therefore, there was no evidence of a correlated response in carpel number.

In contrast, selection for a higher proportion of four-petaled flowers in the selection down regime did not lead to correlated responses in any other floral organ (Figure 1.8). Correlations were all less than 0.4 and not statistically significant (Table 1.7), and slopes, though all positive, were low and also not statistically significant (Table 1.7). There were changes in the proportion of flowers with fewer sepals, stamens, and carpels that were on the same order as changes in the proportion of flowers with fewer petals, but these changes occurred in both directions and independently of petals (Figure 1.8). Additionally, the magnitude of proportion changes for all four floral organ numbers was much lower than those in the selection up regime (Figure 1.7).

DISCUSSION

Selection Response

Similar to what others have found in different species (Huether 1968; Vlot et al., 1992; Monniaux et al., 2015; Pieper et al., 2015), this study shows that variation in the proportion of abnormal four- and six-petaled flowers on a plant in *P. drummondii* is highly heritable and

standing genetic variation can be selected on. In both cases, selection provoked a four-fold response in the direction of selection. This study shows that it is easier to select for variation in one direction (more petals) than in the direction of fewer petals due to higher heritability for the proportion of six-petaled flowers. This same conclusion was also reached by Vlot *et al.* with pappus number (modified sepals) in *Microseris douglasii* (Asteraceae) (1992), though in their case they were more successful in selecting for fewer pappus parts.

Selection for constancy could not be achieved. This selection for constancy can be thought of as selection for canalization or developmental robustness, and in this case, there does not appear to be any genetic variation to increase canalization on petal number. Lack of statistical support for a response to selection increasing the proportion of four-petaled flowers was likely due to low sample sizes of four-petaled flowers, even after selection. However, selection for four-petaled flowers is clearly more difficult than for six-petaled flowers. Because maternal lines used for the selection down regime also contained high proportions of six-petaled flowers, selection for fewer petals may also have been confounded by selection for an overall increase in petal number variation. After selection, the proportion of six-petaled flowers in the selection down regime did increase slightly (Figure 1.5), though this increase was not statistically different from the control. While selection for increased variability rather than decreased petal number remains a possibility, any effects of this additional selection are, in general, small relative to the response in the four-petaled direction (Figures 1.5, 1.6).

Correlated Responses & Pleiotropy

Studies that have looked for correlations between floral organ numbers (including this one) have found them (Roy 1963; Ellstrand 1983; Lehmann 1987; Byerley 2006), suggesting that this is a common pattern in Pentapetalae. Floral organ correlations must arise from some

combination of shared genetic control, shared resource base, shared functional role, or correlated response to microenvironmental variability (Schlichting 1989a; Schlichting 1989b), and phenotypic integration among floral organs is in keeping with our understanding of flowers as integrated structures (Smith 2016). As might be expected, correlational effects on organ number were highest between adjacent whorls (Table 1.1). Flowers exhibited many different abnormal floral formulae (Figures 1.S1, 1.S3), thus correlation between organs was clearly not a strong constraint; however, some of the most common floral formulae consisted of synchronized changes in several floral organs (Figure 1.S3). With this in mind, if floral organ numbers share underlying genetic mechanisms, then selection for changes in petal number ought to provoke pleiotropic effects in the form of correlated responses in other organs, especially in sepals and stamens whose whorls lie adjacent to petals.

That strong correlated responses in sepals and stamens are seen in selection for a higher proportion of six-petaled flowers (Figure 1.7) supports the pleiotropic effects hypothesis and indicates that shared genetic control is likely, at least among sepals, petals, and stamens. The slope between the selection response in petals and the correlated response in sepals is very close to one (Table 1.7), suggesting genetic control of number in these organs is completely shared. The slope for the correlated response in stamens is lower than one, indicating only partial shared genetic control for number with petals. Stebbins (1974) mentions unpublished research showing evidence of putatively maladaptive pleiotropy linking petal number changes to non-floral traits in Huether's work on *Leptosiphon androsaceus*, stating that plants in the selection up line were weak and slow-growing even before flowering. However, no obvious pleiotropic effects were observed in this study beyond those on the numbers of other organs, and the number of flowers on a plant or flower size did not change after selection.

In contrast to the correlated responses observed with selection for six-petaled flowers, there were no correlated responses in sepal, stamen, or carpel number when selecting for a higher proportion of four-petaled flowers (Figure 1.8). Therefore, unlike the evidence presented for the increase of floral organ numbers above, the decrease of organ numbers does not seem to be under shared genetic control. I propose two hypotheses that may explain this phenomenon. First, since selection down may be confounded by selection for more petal number variation, selection could manifest in higher levels of random variation in the other floral organs. Second, selection for four-petaled flowers could act to break any existing genetic correlations. Regardless of the reason, it seems clear that petal loss occurs via different mechanisms than petal gain, and has different effects on adjacent floral organs.

Shared genetic control does not rule out the effect of environment in contributing to the correlation between floral organs. Because the variation in floral organ number occurs within individuals as well as between individuals, microenvironmental effects at the level of the developing floral meristem may provoke correlated shifts in organ number in some of a plant's flowers. However, environmental effects cannot explain the correlated responses recorded for the selection up regime. Though environmental factors have been suggested (Huether 1969; Ellstrand & Mitchell 1988; Byerley 2006) or shown (Huether 1969) to exert some influence on floral organ numbers, the selection responses observed are unlikely to be strongly influenced by differences in greenhouse environment between the generations. Plants were grown at the same time of year in the same greenhouse for each generation, and the proportions of four- and six-petaled flowers in the control group grown along with the selection regimes in the second generation were no different than those observed in the parental generation.

Parental Generation

In the parental generation, the amount of variation in floral organ number (10.3% overall, 2.5% for the corolla), with more increases than decreases in petal number, was similar to that found in previous greenhouse studies on *P. drummondii* (Lehmann 1987; Byerley 2006). Other species in the Polemoniaceae such as *Ipomopsis aggregata* and *P. longifolia* exhibit percentages of abnormal flowers as high as 16% (Ellstrand 1983; Byerley 2006). Though increases in petal number were more prevalent than decreases in this study, the opposite trend has also been described in other species (Huether 1969; Byerley 2006). More general patterns in the amount and direction of intraspecific meristic variation among species have not been quantified, especially outside of the Polemoniaceae. The variation in the propensity of plants in the parental generation to produce six-petaled flowers (Figure 1.1), offers further evidence of genetic regulation of the six-petaled phenotype. However, this trend was reduced or absent for four-petaled flowers, suggesting either less genetic variation or weaker genetic regulation than that for six-petaled flowers.

Correlations between the numbers of floral organs in this study mirrored those of other studies in the Polemoniaceae (Ellstrand 1983; Lehmann 1987; Byerley 2006). It is not surprising that carpel number was decoupled from the number of other floral organs. First, in both the Polemoniaceae and most Pentapetalae (Ronse de Craene 2016), carpels do not subscribe to the pentamerous phenotype seen in the other three whorls. In the Polemoniaceae, carpels are trimerous rather than pentamerous. Some changes in genetic mechanism may be necessary to produce a different number of carpels in the carpel whorl. Second, because carpels are located in the center of the flower, they are more likely to be limited by space on the floral meristem (Ronse de Craene 2016). Finally, since each carpel produces one seed in *P. drummondii*, the

number of carpels is directly related to seed set and changes in carpel number may have fitness consequences (Stebbins 1967; Byerley 2006).

Temporal trends in petal number variation within an individual such as those observed in this study (Figure 1.4) have been observed in various species (Roy 1963; Huether 1969; Ellstrand & Mitchell 1988; Byerley 2006; Monniaux et al., 2015), though the type of trend varies. In some species, the proportion of six-petaled flowers increases over time (Byerley 2006), and in others the proportion of four-petaled flowers increases (Huether 1969; Byerley 2006). Some studies reported changes in the total variation over time (Roy 1963; Ellstrand & Mitchell 1988), though it is unclear whether it is the proportion of four-petaled flowers, six-petaled flowers, or both that have changed. While these temporal trends have been attributed to environmental stress as the flowering season progresses (Huether 1968; Huether 1969; Ellstrand & Mitchell 1988; Byerley 2006), I observed them within a greenhouse with relatively constant environment over the flowering period; therefore, the argument for attribution to environment is weak.

These observed temporal trends are confounded by positional effects, which may be the ultimate cause. Floral initiation in *P. drummondii* proceeds basipetally, with the earliest flowers emerging from the terminal meristem, and subsequent flowers emerging from axillary branches progressively further down the plant from the terminal meristem (Figure 1.9A). Flowers closest to the terminal meristem (which are also the first flowers) had the highest proportion of six-petaled flowers, and this proportion decreased with distance from the terminal meristem (Figure 1.9B). Positional effects on merosity have been noted before (Matzke 1932; Bancroft 1935; Charlton & Posluszny 1991; Vlot et al., 1992; Byerley 2006; Zhao et al., 2010; Ronse de Craene 2016) and seem to apply broadly to different species. Given that auxin concentrations

within a plant are similarly highest at the terminal meristem and decrease with distance (Went 1935), it is plausible that auxin may play a role in the developmental mechanisms responsible for petal number.

The Genetics of Petal Number Variation

The genetic architecture of petal number and organ number in general is thought to be polygenic, with associated quantitative trait loci identified in several systems. *Cardamine hirsuta* (Brassicaceae) is a species that does exhibit variation in the otherwise invariant tetramerous flowers that characterize most other species of the Brassicaceae (Hay et al., 2014). In *C. hirsuta*, multiple quantitative trait loci have effects on petal number, and some are temporally isolated, exerting their effects at different points during the course of the flowering period (Monniaux et al., 2015; Pieper et al., 2015). In *M. douglasii*, pappus number is reduced in ray florets (mode of 3) compared to most species in the Asteraceae (mode of 5). Variation in pappus number is also polygenically inherited (Vlot & Bachmann 1991). Both *M. douglasii* and *M. biglovii* have variation for pappus number, and, when crossed, the results suggest a dominant gene of large effect which acts to reduce pappus number, with only one or a few additive modifiers (Vlot et al., 1992). Vlot *et al.* (1992) suggest that this gene of large effect is a canalization gene acting in similar fashion to SCUTE in *Drosophila* (Rendel et al., 1965), and that in its recessive form, the gene releases hidden polygenic variation previously buffered from expression. Notably, *C. hirsuta* is a species that exhibits primarily a reduction in petal number, and the apparent polygenic control of petal number in this species could also have resulted from a breakdown of canalization. Thus, the lower petal numbers and their variability in these systems may both be due to breakdowns in canalization, that in both cases only act to lower petal number.

Following, I enumerate the several lines of evidence I have found that point to the gain of a petal being different genetically from the loss of a petal: 1) four-petaled flowers are much rarer in general than six-petaled flowers, yet more common in plants with generally higher levels of petal number variation; 2) four-petaled flowers have a lower heritability than six-petaled flowers; 3) four-petaled flowers are not temporally or positionally mediated and may instead be correlated with smaller flower size; and 4) selection for four-petaled flowers does not evoke a correlated response in sepals and stamens. The view that gain and loss of petals had a different genetic basis was also held by Vlot *et al.* (1992), based on the canalization gene of large effect in *M. douglasii* only affecting variation in the direction of loss of petals. Monniaux *et al.* (2015) point out that a trait such as petal number that is held at a particular stable number must be buffered against changes in *both directions*. There exists a normal range of developmental pathway activity in which the trait is canalized against environmental perturbations, and changes in petal number occur when perturbations occur above or below the zone of buffering. Therefore, it is not difficult to envision a system in which *different* mechanisms are required to buffer the two extremes. These two mechanisms could easily have different patterns of pleiotropic effects, effectiveness of buffering, heritability, and responses to environmental effects. A model separately buffered against both increases and decreases in petal number provides a good explanation for my results showing differences between gains and losses of petals.

Selection upwards and downwards for a particular trait in other systems, such as the oil and protein content in corn (Dudley 2007) and the vibrissa number in tabby mutant mice (Dun & Fraser 1958), have shown that the heritability can differ between the two directions of selection, and that absolute constraints can be reached in one direction, but not the other (Dun & Fraser 1958; Dudley 2007). This might indicate that constraints are more difficult to circumvent for

selection decreasing petal number than for increasing it. In contrast to studies showing successful selection in both directions, selection for increased and decreased ovule number in *Spergularia marina* (Caryophyllaceae) was only successful in the direction of more ovules (Mazer et al., 1999). Because of this asymmetrical response to selection, there was no correlated negative response in anther number to selection for fewer ovules in a flower, or in ovule number to selection for more anthers (Mazer et al., 1999). However, the reverse was true: selection for more ovules led to fewer anthers, and selection for fewer anthers led to more ovules (Mazer et al., 1999). Therefore, lower heritability for selection of four-petaled flowers may prevent correlated responses in other organs.

Conclusions

Variation in petal number in *P. drummondii* has a genetic basis and responds to selection, especially for increases in petal number, indicating that standing genetic variation exists. Hypotheses for petal number stasis involving pollinator-mediated or tradeoff-mediated stabilizing selection remain plausible, and within-individual petal number variation is not due solely to developmental noise. Unlike shifts in petal number, it has not been possible, at least in this system, to select for increased constancy. This lack of heritable variation for increased constancy suggests there is no potential for the evolution of increased canalization of the system. Correlations between sepal, petal, and stamen number are strong, though some of this correlation structure is driven by intercorrelation and correlations between adjacent organs carry more weight. There is the potential for selection on the number of one organ driving the evolution of other organ numbers through correlated responses, though this potential is not universal and does not occur in both directions of changes in organ number. Notably, carpels are decoupled and their merosity is not part of the integration observed between the other three organs, a conclusion

supported in other studies. No obvious correlated changes in floral size or flowers per plant with changes in petal number were observed, and anecdotally, vegetative traits and seed set were the same among selection regimes. Thus, there is no strong evidence for pleiotropic effects aside from the numbers of other organs providing selective constraints on merosity of flowers.

There is evidence that the genetic or developmental mechanism controlling the loss of a petal is not the same as that controlling the gain of a petal. Therefore, future research should examine floral organ number variation in both directions, especially when seeking to uncover the underlying genetic or developmental mechanisms governing petal number. The genetic and evolutionary implications of floral merosity, particularly exploring the hypotheses seeking to explain stasis in petal number and eliciting the genetic and developmental basis governing floral organ number, require more research. Our knowledge of these areas is woefully lacking, and they are critical to addressing questions of why or how so many taxa have five-petaled flowers.

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Over the course of several years, many undergraduates helped count floral organs on *Phlox* flowers in the UConn greenhouse. Counting floral organs is very labor intensive and it would have been difficult to get similar sample sizes without their help; they were fantastic! Lauren Abbott and Ellen Deering worked on the first generation after selection, while Matthew Benedict, Connor Hill, Emilia Mason, Genevieve Nuttall, Darren Thorne, Jeffrey Hammond, Hillary Holt, Max Engel, Jenny Yung, and Thomas Jordan helped count the second generation. My sister, Julia Mickley, and Dr. Johana Goyes-Vallejos also helped with data collection in the greenhouse for several days.

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TABLES

Table 1.1: Full and partial correlation coefficients between floral organ numbers. Partial correlations are consistently much lower than full correlations, indicating an appreciable level of intercorrelation among floral organ numbers.

Significance codes: *** 0.001, ** 0.01.

Correlation Type		Petals		Sepals		Stamens	
Full correlations							
	Sepals	0.83	***				
	Stamens	0.79	***	0.73	***		
	Carpels	0.31	***	0.29	***	0.32	***
Partial correlations							
	Sepals	0.59	***				
	Stamens	0.47	***	0.21	***		
	Carpels	0.05	***	0.03	**	0.13	***

Table 1.2: Selection differential, selection response, and realized heritability calculated for each selection regime. Differentials and responses are expressed as changes in per-individual percentages of flowers with six, four, and five petals respectively. Because the selection response for constancy is in the opposite direction of the differential, no heritability is calculated.

Selection Regime	Selection Differential	Selection Response	Heritability (H^2)
Up	9.05	6.33	0.70
Down	1.62	0.82	0.51
Constancy	2.23	-1.61	N/A

Table 1.3: Binomial models for the selection up regime. The best model was selection regime + days since onset of flowering, and a model with only days since onset of flowering was supported to a lesser extent by model weights.

Model terms and reported values: selection regime = selection up and control; day = day since onset of flowering; flowers = number of flowers per plant; radius = corolla radius; rand(plant) = identity of the plant included as a random factor; AICc = AICc score; Δ AICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight.

Model	AICc	Δ AICc	df	weight
selection regime + day + rand(plant)	1117.5	0.0	4	0.65
day + rand(plant)	1119.4	1.9	3	0.25
selection regime + day + flowers + radius + rand(plant)	1121.2	3.7	6	0.10
null model	1390.9	273.4	1	0.00

Table 1.4: Binomial model coefficients for the best selection up model from Table 1.3. Both fixed effects (selection regime and days since onset of flowering) were statistically supported. The reference selection group is the control.

Model terms: selection regime = selection up and control; day = day since onset of flowering. Significance codes: *** 0.001, ** 0.01, * 0.05.

Factor	Estimate	Std. Error	z-value	Pr(> z)	
(Intercept)	-4.27	0.49	-8.78	< 0.001	***
selection regime Up	1.23	0.59	2.07	0.038	*
day	-0.03	0.01	-2.68	0.007	**

Table 1.5: Binomial models for the selection down regime. The best model contained only selection regime as a fixed factor. A model with selection regime and corolla radius was also supported to a lesser extent by model weights.

Model terms and reported values: selection regime = selection down and control; day = day since onset of flowering; flowers = number of flowers per plant; radius = corolla radius; rand(plant) = identity of the plant included as a random factor; AICc = AICc score; Δ AICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight.

Model	AICc	Δ AICc	df	weight
selection regime + rand(plant)	294.5	0.0	3	0.60
selection regime + radius + rand(plant)	295.6	1.1	4	0.35
selection regime + day + flowers + radius + rand(plant)	299.2	4.7	6	0.05
null model	329.8	35.3	1	0.00

Table 1.6: Binomial model coefficients for the best selection down model from Table 1.5. The effect of selection regime was not statistically significant, though marginally so. The reference selection group is the control.

Model terms: selection regime = selection down and control.

Significance code: *** 0.001.

Factor	Estimate	Std. Error	z-value	Pr(> z)	
(Intercept)	-6.96	0.82	-8.47	< 0.001	***
selection regime Down	1.38	0.83	1.66	0.097	

Table 1.7: Slopes (β coefficients) and correlations between the response to selection for petal number and the response of the other three other floral organs. Sepals and stamens show strong correlated responses with the selection up regime, but not for the selection down regime. Carpels show no correlated responses. The slope of the correlated response for stamens is less than that of sepals.

Significance code: *** 0.001.

Selection Regime	Floral Organ	Slope (β)		Correlation (r)	
Selection Up					
	Sepals	1.08	***	0.99	***
	Stamens	0.67	***	0.99	***
	Carpels	0.61		0.60	
Selection Down					
	Sepals	0.10		0.13	
	Stamens	0.24		0.37	
	Carpels	0.04		0.39	

FIGURES

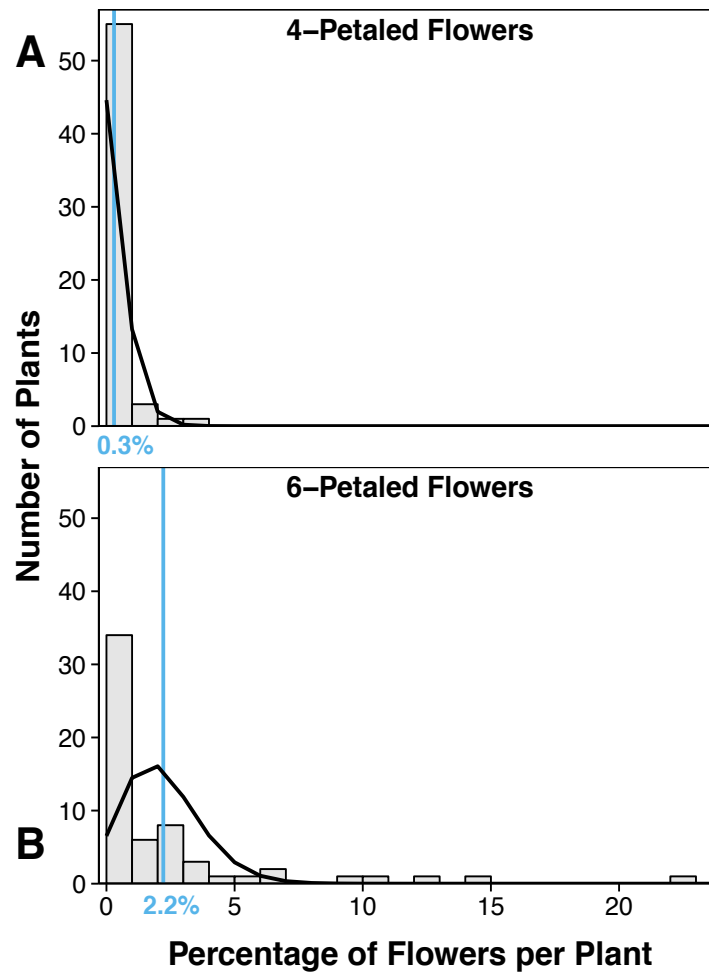


Figure 1.1: Frequency distribution of the percentage of A) four- and B) six-petaled flowers by plant in the parental generation. Per-individual means for four- and six-petaled percentages are shown with blue lines, and a Poisson distribution based on those means is plotted in black. More plants have high percentages of six-petaled flowers than four-petaled flowers. The per-individual distribution of six-petaled flowers does not fit the random Poisson expectation.

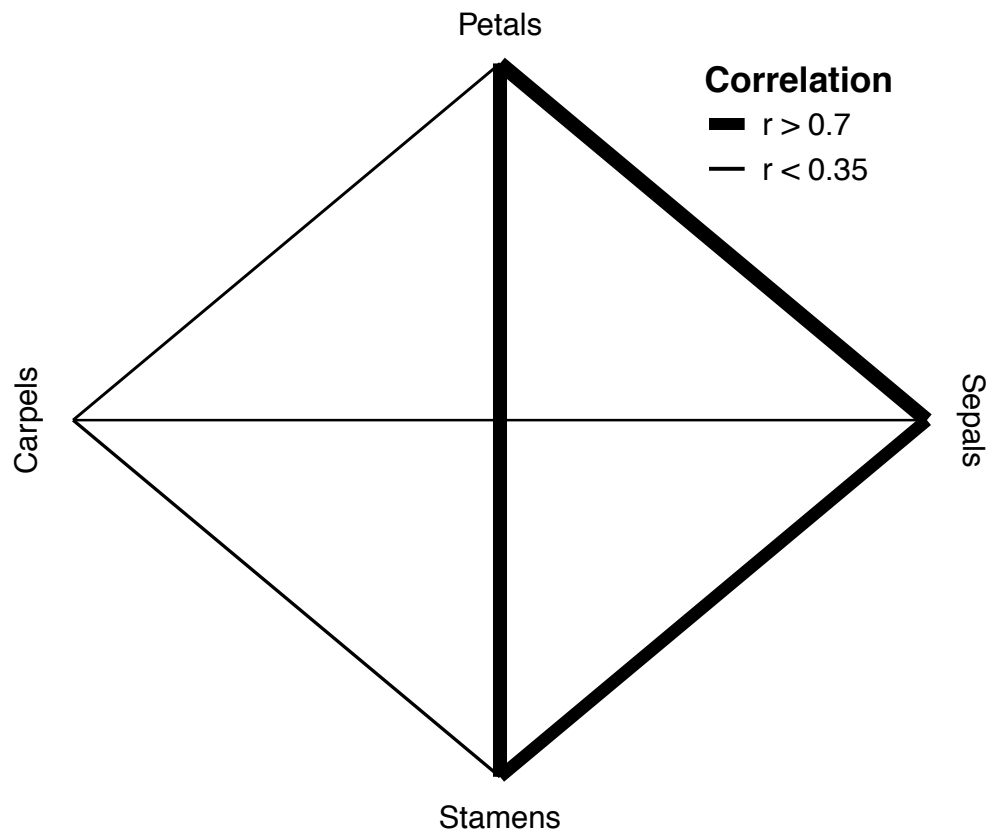


Figure 1.2: Correlation network of correlations between floral organ numbers in the parental generation. The thickness of the line denotes the strength of correlation. Correlations are high among sepals, petals, and stamens, but much lower for carpels.

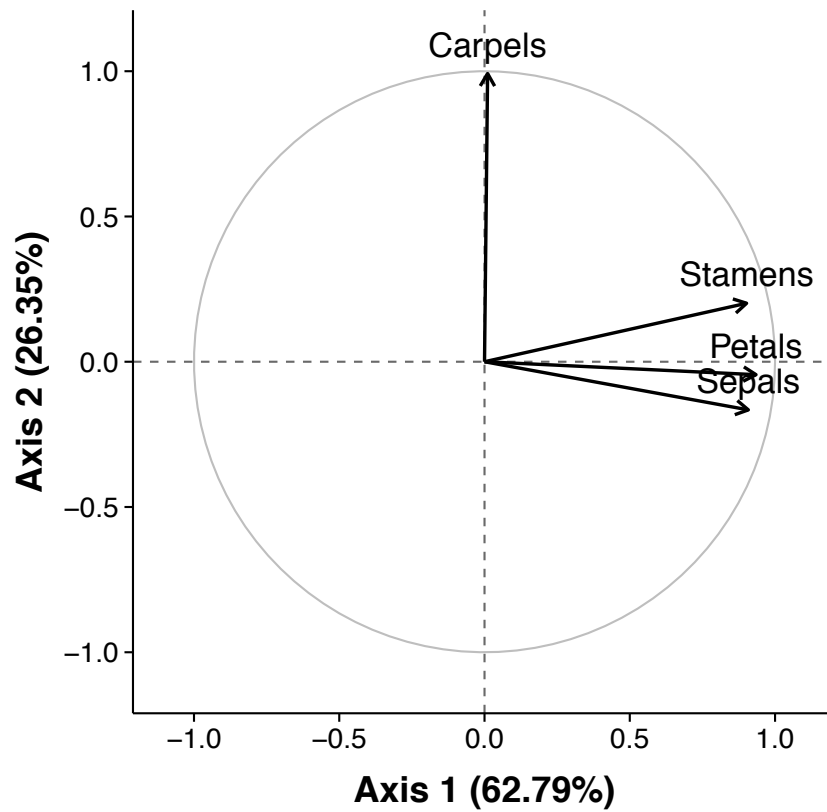


Figure 1.3: A loadings plot for the principal components analysis of floral organ numbers from the parental generation. The direction and length of arrows shows how much each floral organ contributes to the two major principal component axes. Numbers in parentheses in axis labels show the percentage of variation explained by each axis. Sepal, petal, and stamen number all load on the first axis, while carpel number loads exclusively on the second axis.

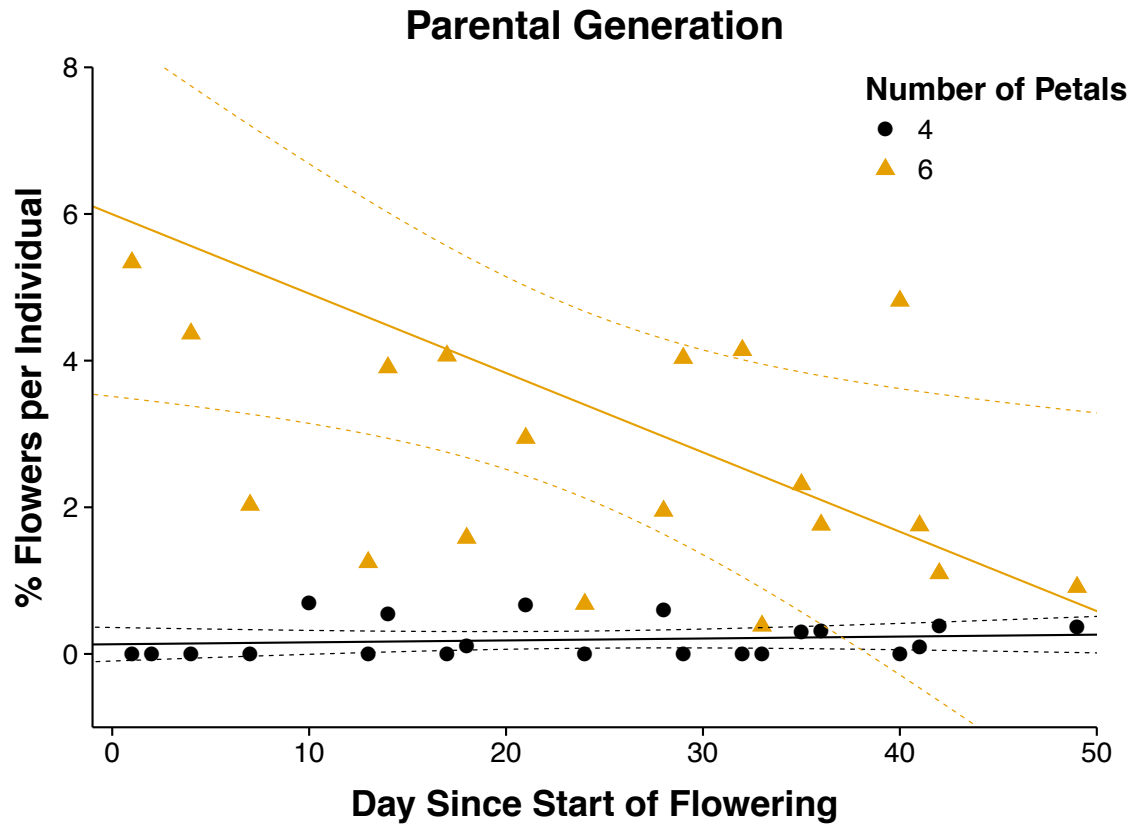


Figure 1.4: The per-individual percentage of four-petaled flowers (black) and six-petaled flowers (orange) plotted against day since onset of flowering. Here, the percentage of four-petaled flowers is independent of time since start of flowering. However, the percentage of six-petaled flowers decreases over the course of flowering, with early flowers more likely to be six-petaled.

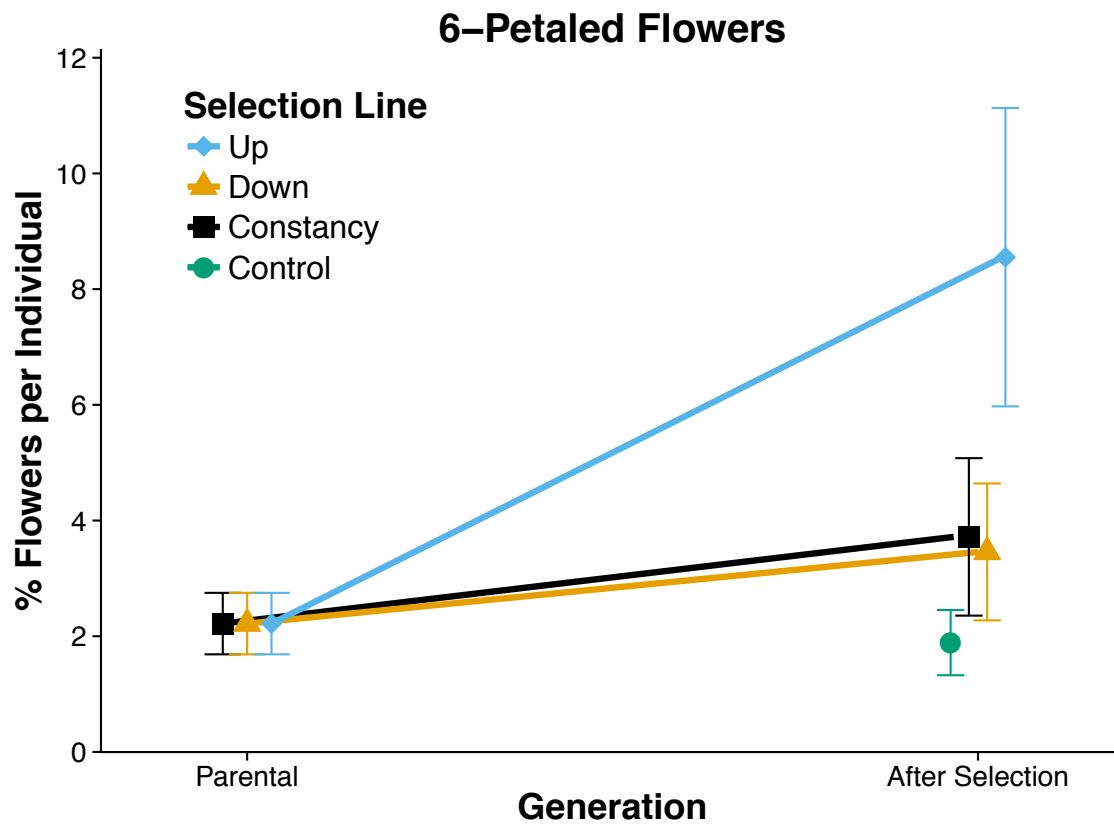


Figure 1.5: Change in the per-individual percentage of six-petaled flowers in each selection regime (up, down, constancy, and control) after one generation of selection. Selection resulted in a four-fold increase in the percentage of six-petaled flowers in the selection up regime, but no decrease in the constancy regime.

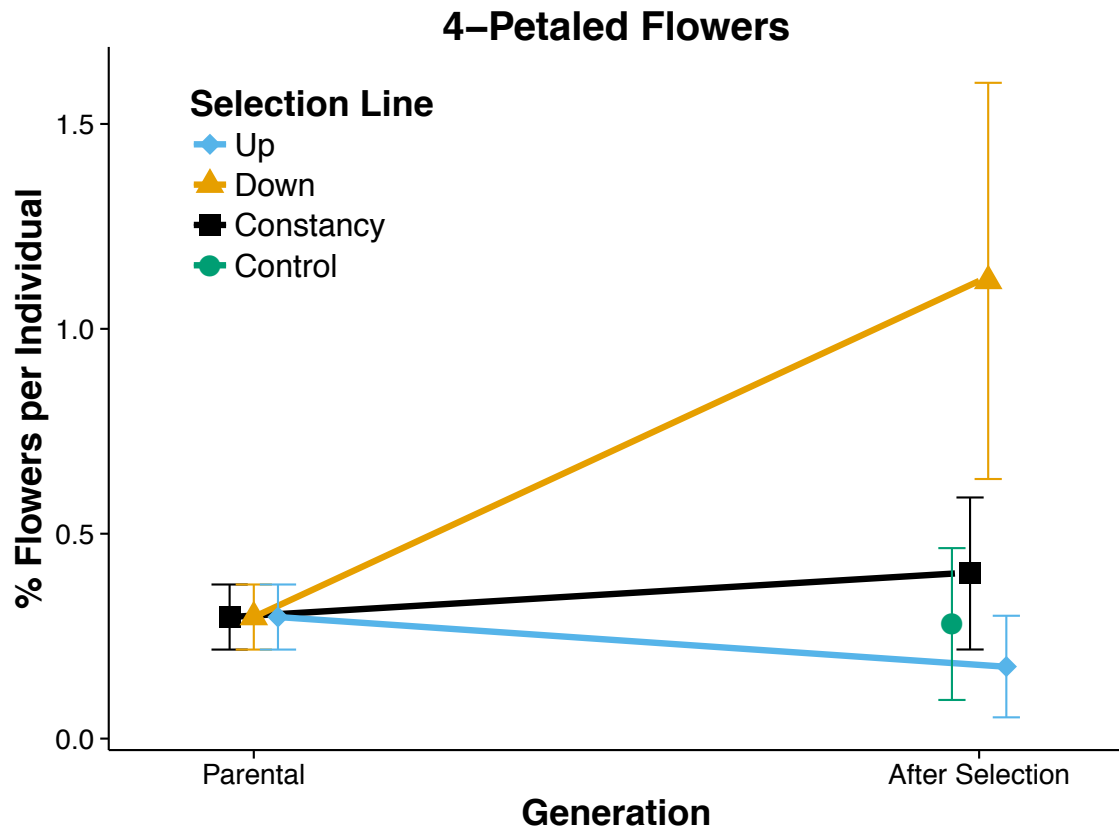


Figure 1.6: Change in the per-individual percentage of four-petaled flowers in each selection regime (up, down, constancy, and control) after one generation of selection. Note the change in scale compared to Figure 1.5; four-petaled flowers were less common. Selection resulted in a four-fold increase in the percentage of four-petaled flowers in the selection down regime, but no decrease in the constancy regime.

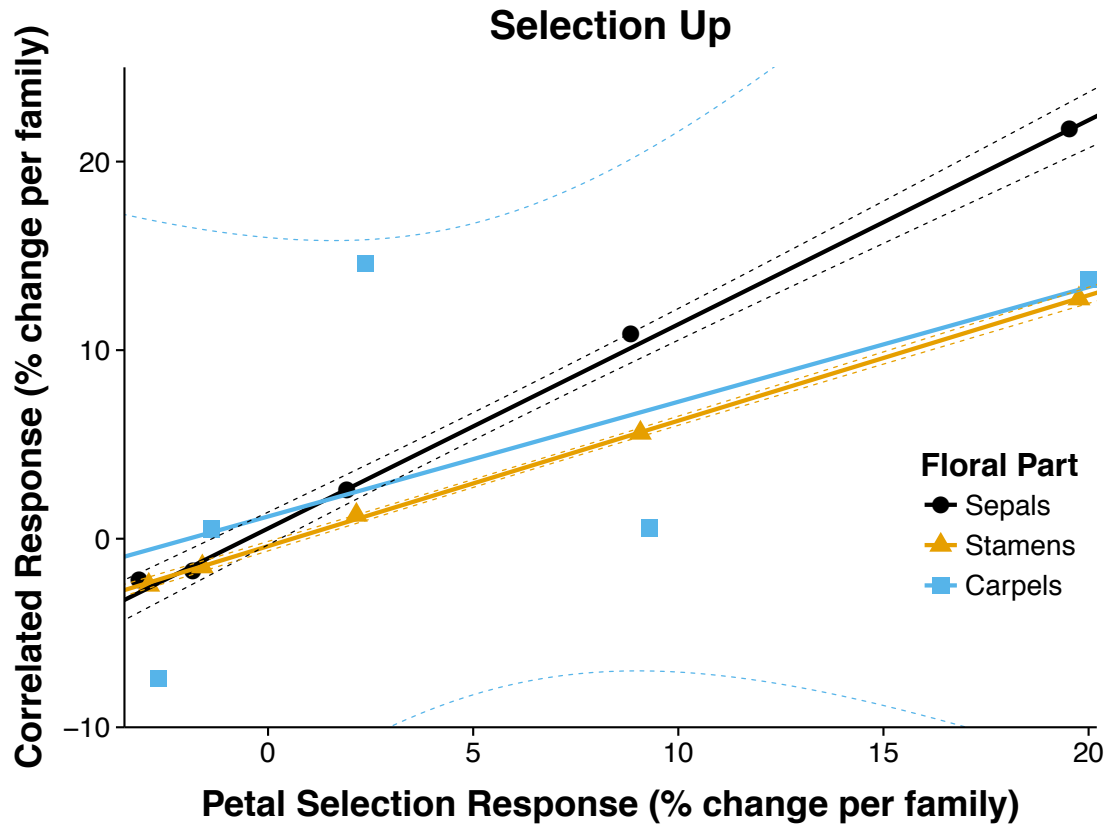


Figure 1.7: Correlated responses by maternal family when selecting for a higher proportion of six-petaled flowers (selection up). Solid lines show the slope of the response and dashed lines denote 95% confidence intervals around the slope. Sepals and stamens show correlated responses to selection for more petals. Carpels exhibit no statistically significant response; the slope is indistinguishable from zero due to wide confidence intervals.

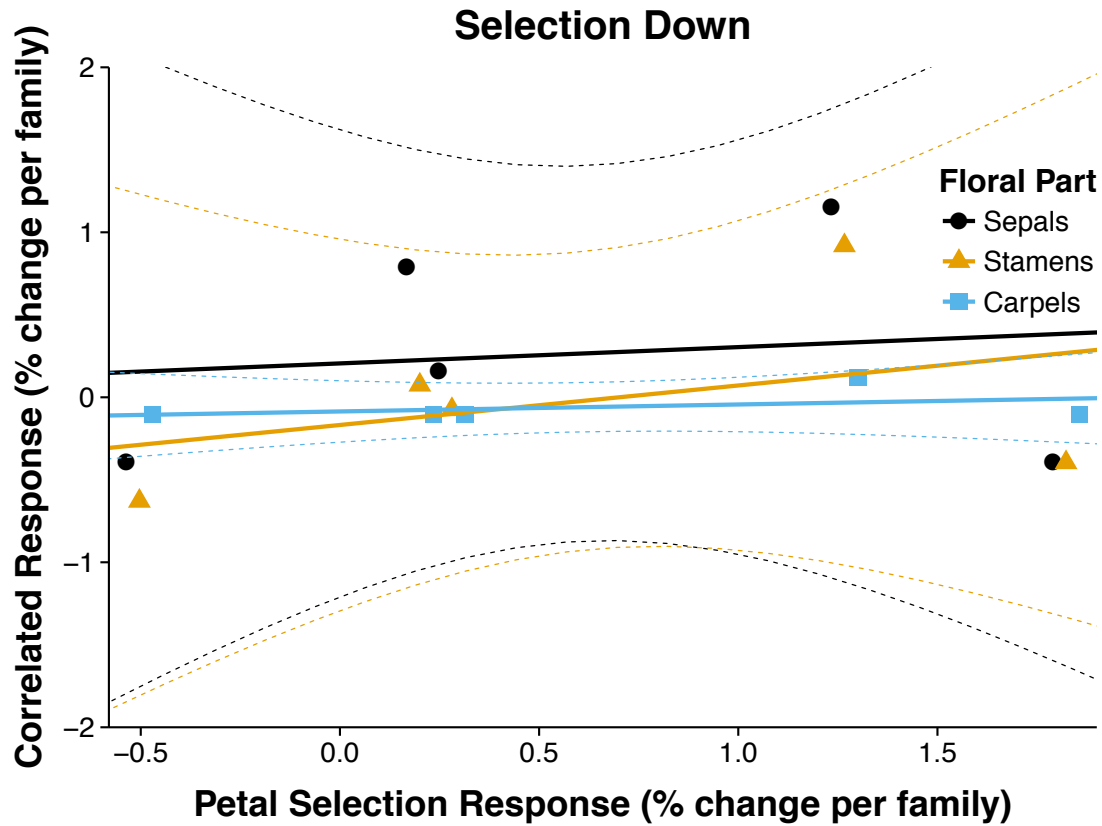


Figure 1.8: Correlated responses by maternal family when selecting for a higher proportion of four-petaled flowers (selection down). Solid lines show the slope of the response and dashed lines denote 95% confidence intervals around the slope. Sepals, stamens, and carpels show no correlated responses to selection for fewer petals.

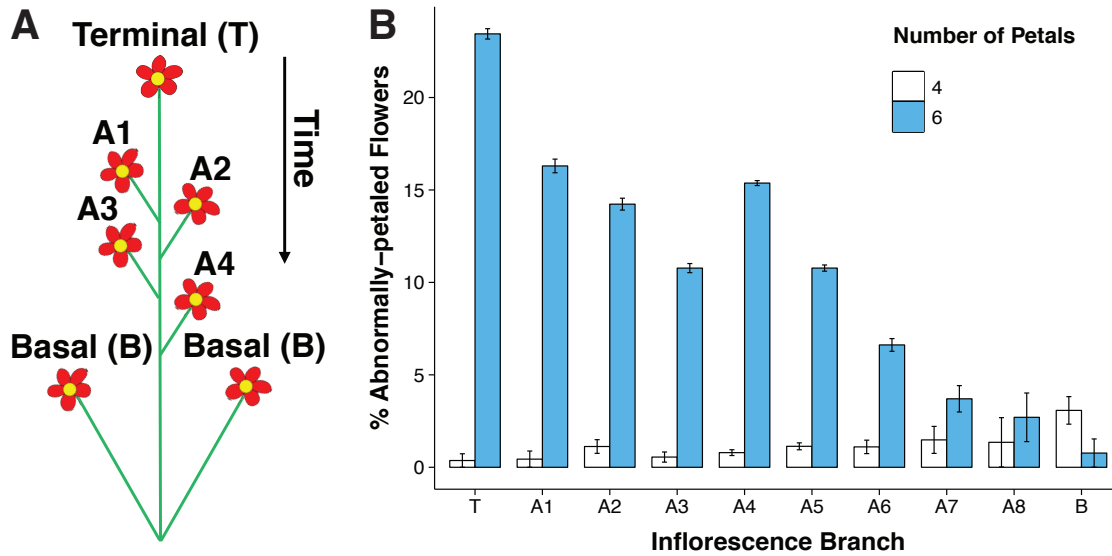


Figure 1.9: Plant positional effects on petal number. A) *Phlox drummondii* flowering proceeds basipetally, starting at the top with the terminal inflorescence branch (T) and proceeding downwards over time through axillary branches increasingly farther from the terminal branch (A1-A4) to the basal branches (B). B) The percentage of six-petaled flowers decreases with distance from the terminal inflorescence branch.

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CHAPTER 1 SUPPLEMENT

Table 1.S1: Principal component analysis results for floral organ numbers in the parental generation. Most of the variance (89%) is contained within the first two axes, and additional axes are not warranted as eigenvalues are < 1 . Loadings show the correlations between a floral organ number and the principal component axis. The number of petals, sepals, and stamens are strongly positively loaded on Axis 1, while carpel number is loaded on Axis 2. Petal, sepal, and stamen numbers contribute equal percentages to Axis 1, and only small percentages to Axis 2.

	Axis 1	Axis 2
Eigenvalues		
Eigenvalue	2.51	1.05
Variance		
% Var.	62.79	26.35
Cum. % Var.	62.79	89.14
Loadings		
Petals	0.93	-0.04
Sepals	0.91	-0.17
Stamens	0.90	0.20
Carpels	0.01	0.99
Contributions		
Petals	34.77	0.19
Sepals	32.83	2.61
Stamens	32.40	3.85
Carpels	0.00	93.36

Table 1.S2: Ordinal models with ΔAICc scores, degrees of freedom and model weights for the two best models compared to the full and null models. The best model is one with selection regime and day since onset of flowering, though a model with only days since onset of flowering was supported to a lesser extent by model weights.

Model terms and reported values: selection regime = selection up, selection down, constancy, and control; day = day since onset of flowering; flowers = number of flowers per plant; radius = corolla radius; rand(plant) = identity of the plant included as a random factor; AICc = AICc score; ΔAICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight.

Model	AICc	ΔAICc	df	weight
selection regime + day + rand(plant)	2992.3	0.0	7	0.56
day	2993.4	1.1	4	0.33
selection regime + flowers + day + radius + rand(plant)	2995.6	3.3	9	0.11
null model	3204.9	212.6	6	0.00

Table 1.S3: Fixed factor coefficients and significance levels for the best ordinal model in Table 1.S2. The reference selection group is the control.

Model terms: selection regime = selection up, selection down, constancy, and control; day = day since onset of flowering.

Significance codes: *** 0.001, * 0.05.

Fixed Factor	Estimate	Std. Error	z-value	Pr(> z)	
selection regime Constancy	0.197	0.411	0.478	0.632	
selection regime Down	-0.001	0.408	-0.002	0.998	
selection regime Up	0.947	0.408	2.320	0.020	*
day	-0.025	0.007	-3.428	0.001	***

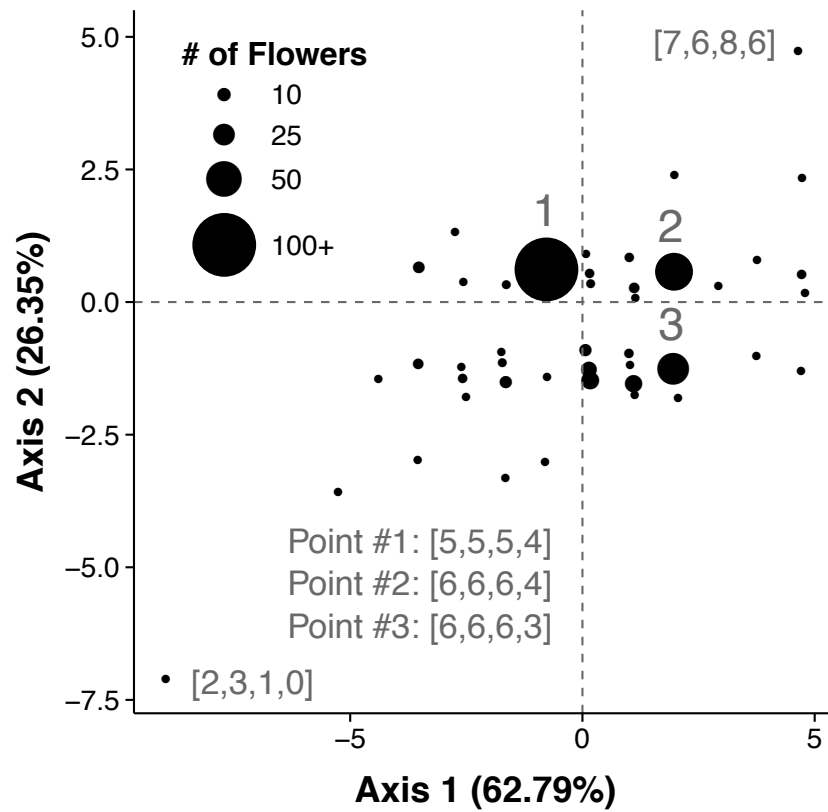


Figure 1.S1: Plot of all combinations of abnormal floral formulae from the parental generation in principal component space. The size of the circles denotes the number of flowers represented by each formula. Extremes and common formulae are labeled.

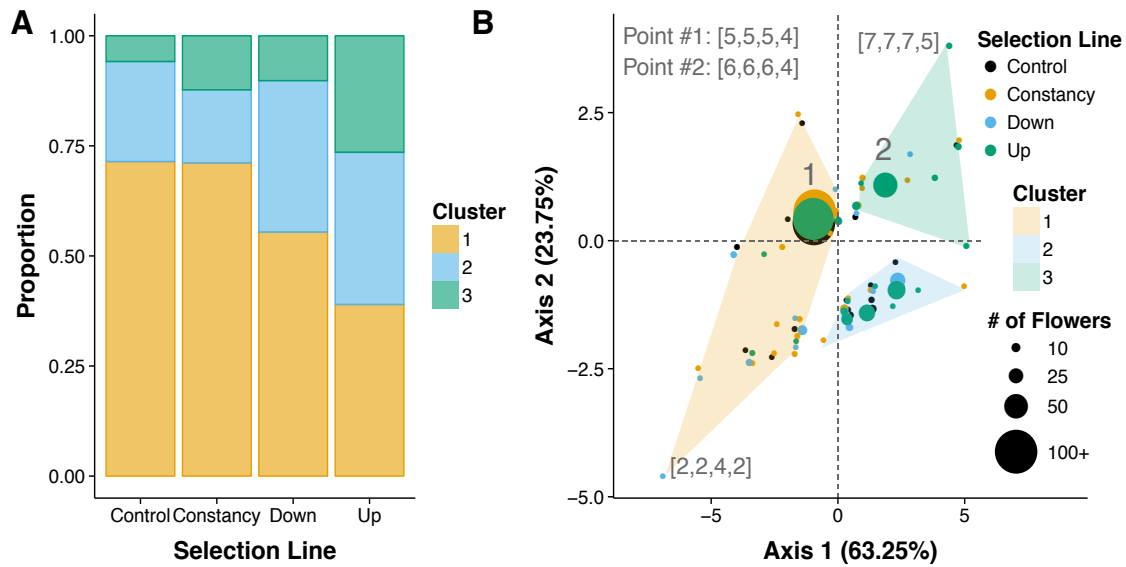


Figure 1.S2: Principal component analysis after one generation of selection. A) The contribution of the individual selection regimes to each of three clusters in floral formula principal component space. Clustering on the principal components was conducted using hierarchical k-means clustering. B) Plot of all combinations of floral formulae in principal component space. Points are offset slightly to improve readability. The size of the circles denotes the number of flowers represented by each formula while the color matches each selection regime. Convex hulls of the three clusters are shaded. Extremes and common formulae are labeled.

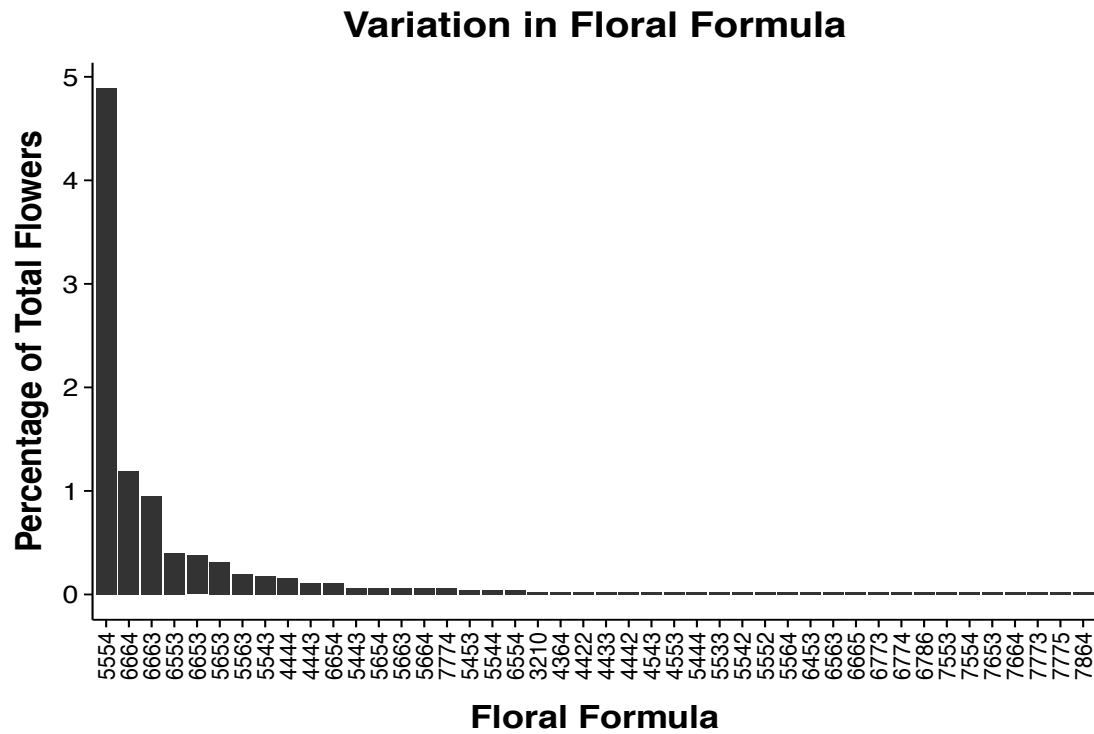


Figure 1.S3: All abnormal floral formulae observed in the parental generation shown as a percentage of total flowers. Nearly every combination of organ number within the numerical range for each individual organ is present; however, only a few abnormal formulae are common.

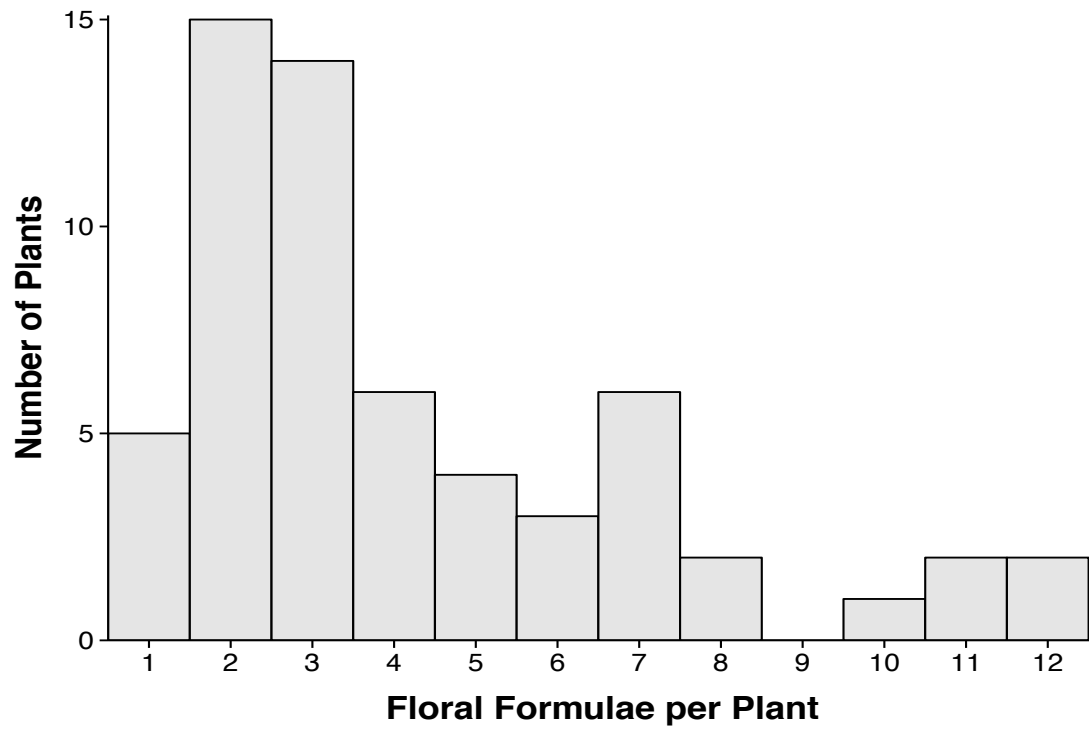


Figure 1.S4: The distribution of the number of different floral formulae present in a plant. One third of the plants bore flowers with five or more different floral formulae.

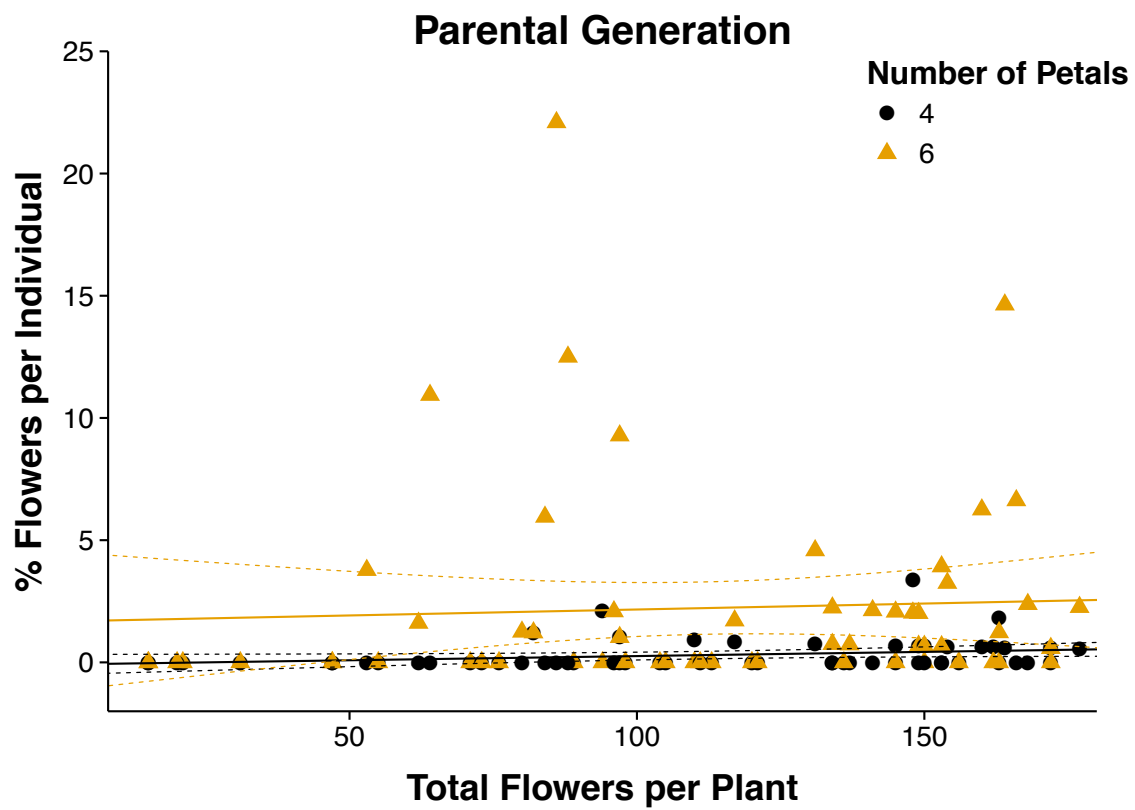


Figure 1.S5: Percentage of flowers per plant with four (black) and six petals (orange) as a function of total flowers in the parental generation. Plants with higher percentages of four- or six-petaled flowers did not exhibit any difference in total flower number.

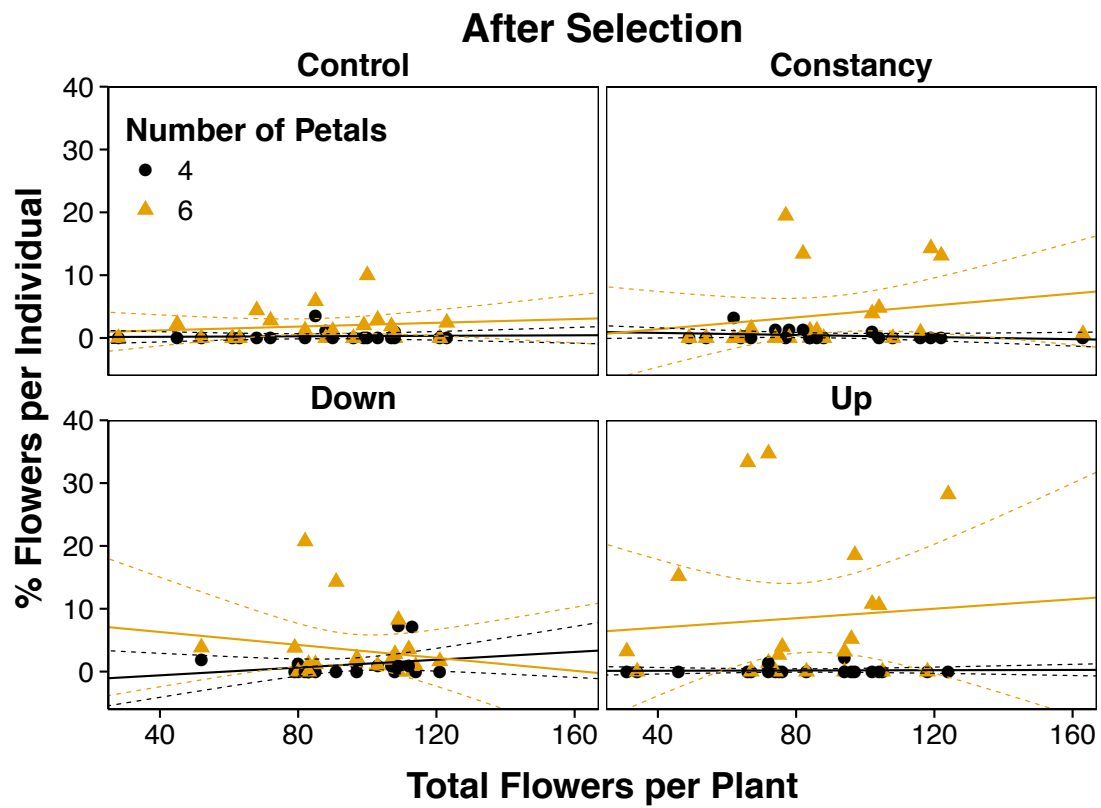


Figure 1.S6: Percentage of flowers per plant with four (black) and six petals (orange) as a function of total flowers for each of the four selection regimes (up, down, constancy, and control). The number of flowers per plant did not change the incidence of abnormal flowers in any selection regime.

CHAPTER 2:

Testing for Pollinator-mediated Stabilizing Selection on Petal Number: Comparisons of Petal Number Variation between Autogamous and Outcrossing Species

ABSTRACT

Petal number within and among early angiosperm species is variable. However, as angiosperms diversified, this variability decreased and petal number became fixed for five-petaled flowers in much of the massive eudicot clade Pentapetalae. This stasis is unexplained, though it has been presumed to be adaptive and maintained by stabilizing selection, especially via pollinator preferences. Some species, particularly in the Polemoniaceae, have low levels of natural, genetic, within-individual variation for petal number, though this has only been quantified in outcrossing species. Autogamous species are not under selection pressure from pollinators, and would be expected to have higher levels of variation in petal number than outcrossers if pollinators impose stabilizing selection. Furthermore, if stabilizing selection acts via a tradeoff between petal costs and pollination, autogamous species might be expected to shift towards fewer petals to reduce costs. To test for differences in petal number variation between autogamous and outcrossing species, three sets of closely-related species within the Polemoniaceae with similar ranges and habitats were sampled for petal number variation across multiple sites per species. For each set, at least one of the species was autogamous and one was an outcrosser. There was no evidence for a relaxation of stabilizing selection on petal number in autogamous species; most autogamous species had less petal number variation than outcrossers. Overall, autogamous species had no tendency towards more four-petaled flowers or fewer six-petaled flowers, refuting the tradeoffs hypothesis. This study provides the most comprehensive survey to date of differences in petal number variation among species and populations within

species. These differences among species were large relative to differences between mating systems. This comparison of mating systems suggests that pollinators play no role in mediating stabilizing selection on petal number, and that research into what drives differences in petal number variation among species may prove more fruitful in explaining stasis.

INTRODUCTION

Though there is tremendous diversity in petal number within angiosperms ranging from zero to many petals (Ronse de Craene 2016), this character is remarkably constant within many clades (Cronquist 1981; Givnish 2002; Herrera 2009). Petal number in early angiosperms was labile, but evolutionary trends toward decreased lability and, to some extent, an overall reduction in petal number have long been noted (Schoute 1932; Breder 1955; Leppik 1957; Stebbins 1974; Stebbins 1967; Soltis et al., 2003). In particular, a constant five petals or *pentamery* is the predominant *merosity* (number of organs) in much of the aptly named Pentapetalae, which includes some 175 plant families and a significant portion of angiosperm diversity (Breder 1955; Soltis et al., 2003; Cantino et al., 2007; Ronse de Craene 2016). Pentapetalae are predominantly five-petaled with relatively few shifts in petal number (Breder 1955; Stebbins 1967), although some lineages (e.g., Brassicaceae) are fixed for other petal numbers (reviewed in Ronse de Craene 2016). This raises an interesting question: why has there been stasis for petal number in a large portion of the angiosperms?

Stasis in petal number has been posited to be adaptive and maintained by stabilizing selection (Leppik 1953; Leppik 1956; Stebbins 1974), presumably by pollinator preferences for certain petal numbers (e.g., pentamery) (Leppik 1953; Leppik 1955a; Leppik 1956; Lehrer et al., 1995; Ronse de Craene 2016; Monniaux et al., 2015), or pollinator avoidance of abnormality (Herrera 2009). Many studies have shown that despite the larger pattern of constancy, some

species exhibit low levels of natural within-individual variation in petal number or other floral organs (Stark 1918; Lowndes 1931; Roy 1963; Huether 1969; Ellstrand 1983; Lehmann 1987; Ellstrand & Mitchell 1988), and that this variation is heritable (Huether 1968; Vlot & Bachmann 1991; Vlot et al., 1992; Byerley 2006; Monniaux et al., 2015; Pieper et al., 2015; Mickley 2017, Chapter 1). Therefore, stasis is unlikely to be maintained by a lack of genetic variation (Stebbins 1974; cf. Cresswell 1998), and stabilizing selection on some aspect of petal number must inhibit divergence. One issue with the hypothesis of pollinators as a mediator of stabilizing selection is the lack of an adaptive scenario for pollinator preference. Leppik suggests that the ability to distinguish petal number might give pollinators an advantage in distinguishing among flower types (1955a), though this is unhelpful where stasis predominates within a clade and many taxa have the same petal number. Lehrer (1995), in a study that tested preferences for two, four, and six petals, suggested that preferences for hexamery might reflect better vision by hexagonal-eyed bees for hexamerous patterns, but this is untested and, of course, does not explain the much larger proportion of taxa expressing stasis for pentamerous flowers. Finally, evidence from floral symmetry shows that pollinators select against asymmetrical flowers as an indicator of nectar quality (amount and concentration; Møller & Eriksson 1994; Møller & Eriksson 1995; Møller & Sorci 1998), and similar trends might be extended to abnormal petal numbers (Herrera 2009). This would explain selection for constancy, but not for stasis on pentamery.

Much of the evidence for petal number preferences in pollinators comes from the work of Leppik (1953; 1954; 1955a; 1955b; 1956; 1957), who tested whether pollinators were able to detect differences in petal number by manipulating flowers. He established that bees (Leppik 1953; Leppik 1956) and butterflies (Leppik 1954; Leppik 1955b) could distinguish between different petal numbers, and flies, beetles, and weevils could not, and noted that bees had the

highest preference for pentamery (without showing data: Leppik 1953). Similar work with bees by Lehrer (1995) came to the same conclusion that bees could discriminate petal numbers, though pentamery was not tested and there was a slight preference for hexamery. Zhao and Huang measured selection on petal number in *Trollius ranunculoides* (Ranunculaceae) and found that at high altitudes there was selection for increased petal number by nectarivorous flies (Zhao & Huang 2013). However, in this species, sepals are the attractive organ and petals are anther-like and nectariferous; thus, petal number is directly related to the amount of nectar (Zhao & Huang 2013), a case that makes generalization to petal number stasis in other angiosperms inapplicable. Finally, Stebbins cites unpublished work by Huether, in which flies did not discriminate against flowers with abnormal petal numbers (Stebbins 1974). Despite several early calls to test pollinator preference for and stabilizing selection on petal number more thoroughly (Breder 1955; Stebbins 1970; Stebbins 1974), this has never been done.

Alternative hypotheses for stasis exist that do not involve pollinators: 1) a fixed petal number may be a precondition for predictable development of more phenotypically complex flowers (Breder 1955; Endress 2001b; Ronsch De Craene 2010); 2) pleiotropic effects and phenotypic integration of floral organs within the flower may stabilize petal number (Stebbins 1974; Byerley 2006; Herrera 2009; Diggle 2014; Smith 2016); and 3) other pleiotropic effects may produce selection on petal number (e.g., petals function in opening the flower bud, Monniaux et al., 2015). These hypotheses are not mutually exclusive, and none have been thoroughly tested.

Removing petals from flowers lowers rates of visitation and seed set (Johnson et al., 1995; Kudoh & Whigham 1998). Petals are known to be costly, contributing as much as 50% to a plant's water loss and consuming up to 40% of the nitrogen associated with reproduction

(Ashman 1994; Galen 1999; Lambrecht 2013). Petals also often attract herbivores as well as pollinators (Strauss & Whittall 2007), such that increased petal number might increase herbivory. Thus, as petal number increases to attract pollinators, these costs might also increase, resulting in a tradeoff between petal costs and pollinator attraction. No one has examined the possibility of costs and tradeoffs with increased petal number. These costs are a plausible mechanism for a one-sided constraint on petal number, preventing an increase in petals especially in dry or nutrient-poor environments. In addition, the hypotheses involving predictable development and phenotypic integration described above do not explain stasis for the number of ray florets present in some members of Asteraceae (often 4, 5, or 6 ray florets), since they are individual flowers as Leppik points out (1956). Thus, by hypothesis exclusion, this lends credence to hypotheses that involve pollinators or selection directly on numbers of petals (or ray florets).

All previous research on natural variation in petal number has focused on outcrossing species (Stark 1918; Lowndes 1931; Roy 1963; Huether 1969; Ellstrand 1983; Lehmann 1987; Ellstrand & Mitchell 1988; Byerley 2006). Comparing natural variation in petal number between outcrossers and autogamous congeneric species poses an interesting test for selection on petal number mediated by pollinators. If pollinators are mediators of selection, then autogamous species should be free from pollinator-imposed selection, potentially allowing divergence in petal numbers or increased variation in petal number via genetic drift. Furthermore, if costs or tradeoffs exist for petal number, then selection to reduce expenditures of water or other resources via petals, especially in environments where resources are costly, could favor a reduction in petal number or a shift in the direction of variation towards fewer petals under autogamy, where there is no tradeoff in pollination efficiency. Absent any selection, autogamous species might be

expected to have less variation in petal number than outcrossers, because of lower overall levels of genetic variation due to inbreeding.

Here, I test these two hypotheses: 1) if pollinator-mediated stabilizing selection on petal number occurs, then autogamous species will have *more variation* in petal number than outcrossers (stabilizing selection hypothesis); and 2) if *costs* are higher with increased petal number, autogamous species should be under selection to register a shift towards *fewer petals* (i.e., a change in absolute number) to reduce those costs (costs hypothesis). To test these hypotheses, I compare petal number variation in natural populations in three genera displaying variation in mating system (outcrossing and autogamous species in *Phlox*, *Gilia*, and *Saltugilia*) within the Polemoniaceae. The Polemoniaceae are good candidates to test these hypotheses because of high pollinator diversity within the family (Grant & Grant 1965), flowers that are typically pentamerous but with documented natural variation in merosity, including petal number (Grant 1959; Huether 1969; Ellstrand 1983; Lehmann 1987; Ellstrand & Mitchell 1988; Byerley 2006), and a wealth of pollinator and mating system data for most of the species in the family (Grant & Grant 1965). This work is the first to compare petal number variation in an assemblage of related species, and it is the most comprehensive survey to date of natural variation in petal number. The goal is that these approaches will enable the detection of species- and site-specific differences in petal number. In addition, this is the first test of differences in petal number variation between mating systems and pollinators. Comparative tests between mating systems, presents a new approach (differing from that of Leppik 1953) to the broader question of pollinator-mediated selection on petal number, thus testing for causal mechanisms of stabilizing selection (cf. Cresswell 1998) that may lead to evolutionary stasis—and pentamery in this group.

METHODS

Study System

Flowers of eight species and two additional subspecies from three genera in the Polemoniaceae (*Phlox*, *Gilia*, and *Saltugilia*) were sampled for petal number (Table 2.1) across 23 field sites in Texas and California (Table 2.2). For each of the genera sampled, an autogamous and an outcrossing species were represented. *Phlox drummondii* and *P. cuspidata* are closely-related species of annual *Phlox* native to eastern and central Texas. Their ranges overlap, though they have distinct soil type and moisture preferences and generally do not co-occur (Ruane & Donohue 2007). Since hybrid incompatibility might play a role in increasing petal number variation, it is notable that these two *Phlox* species form hybrid zones in areas of overlap (Levin & Kerster 1967; Ferguson et al., 1999). Putative hybrids are noticeably intermediate between the parents in a number of traits including flower color and size, leaf size and shape, and pubescence. *Phlox drummondii* is predominantly an outcrosser pollinated by lepidopteran species, primarily *Battus philenor* and *Hyles lineata* (Grant & Grant 1965), while *P. cuspidata* is generally autogamous, though neither is obligate (Levin 1978; Ruane 2008).

Gilia cana and *G. sinuata* belong to a poorly resolved clade (Section *Arachnion*) within *Gilia* (Porter & Johnson 2000; Grant 2004). Both are desert annuals native to southern California. *Gilia sinuata* is a small-flowered autogamous species, while *G. cana* is an outcrosser pollinated mostly by bombyliid flies such as *Oligodranes* (Grant & Grant 1965).

Saltugilia is the sister taxon to *Gilia*, and contains four species: *S. australis*, *S. latimeri*, *S. caruifolia*, and *S. splendens* (Porter & Johnson 2000). Saltugilias are also desert annuals native to southern California (Grant & Grant 1954). The genus is remarkable in that four pollination modes are represented among the four species: bee, beefly, hummingbird, and autogamy (Grant

& Grant 1965; Weese & Johnson 2001). *Saltugilia australis* and *S. latimeri* are autogamous. *Saltugilia splendens* ssp. *splendens* is pollinated by cyrtid and bombyliid flies (mostly *Oligodranes*, *Eulonchus smaragdinus*, and *Bombylius lancifer*) and some halictid bees (Grant & Grant 1965), while *S. splendens* ssp. *grantii* is hummingbird-pollinated. *Saltugilia caruifolia* is pollinated predominantly by bees. Because of the pollinator diversity within the genus, *Saltugilia*s represent an ideal system in which to study constraints on petal number under different pollinators.

Field Surveys

Four natural populations each of *P. drummondii* and *P. cuspidata* were sampled for petal number along roadsides in southeastern Texas in March 2014. In addition, both species and their putative hybrids were sampled in two sites with hybrid zones. Populations were chosen that were sufficiently dense to allow for sampling several hundred plants, and that were minimally several miles from other sampled populations, and with no sightings of the other species in the vicinity (except for the hybrid sites). All plants along a 1 m wide transect were sampled; sampling was done in 0.5 m quadrats until the total number of flowers sampled along the transect was at least 700 (136–392 individual plants, 2.0–5.6 flowers/plant). The number of petals on every flower of each plant was recorded. To test for plant density effects on petal number, the number of plants per quadrat was recorded. Voucher specimens were collected for both species from each site where they were present and these specimens were deposited in the herbarium at the University of Connecticut (CONN; Mickley 001–030, CONN00191711–CONN00191739).

In April 2015, similar sampling was performed on three populations each of *G. sinuata*, *S. splendens* ssp. *splendens*, *S. australis*, *S. latimeri*, and *G. cana* in southern California at various sites in the Mojave National Preserve, the Transverse Ranges, and the Peninsular Ranges

(Table 2.2). Because *Gilia* and *Saltugilia* occur in sparser populations than *Phlox*, plants were sampled along mountain washes or in wide transects until the petal number for at least 500 flowers had been recorded (140–434 plants, 1.2–4.8 flowers/plant); measures of plant density were not recorded. Additional smaller populations with fewer than 500 flowers were sampled for both *S. australis* and *G. cana* ssp. *speciformis* (Table 2.2). Few *S. caruifolia* or *S. splendens* ssp. *grantii* were in bloom in April, but ~70 flowers on 12 *S. caruifolia* plants (at unusually low elevation) were sampled and all were five-petaled. *Saltugilia caruifolia* was therefore excluded from our analysis, though plants grown in the greenhouse from wild-collected seed exhibited petal number variation on par with that of other species (0.4% 4-petaled flowers, 1.6% 6-petaled flowers, $n = 754$ flowers). Voucher specimens were collected for all the species sampled at each site and deposited in the herbarium at the University of Connecticut (CONN; Mickley 033–095, CONN00200743–CONN00200833).

At four sites, both an outcrossing species and its autogamous congener were present: two sites with both *P. drummondii* and *P. cuspidata* (as well as their hybrids), and two sites with *S. latimeri* and *S. splendens*. Coordinated differences in both species between sites would suggest some environmental role: either directly (plasticity) or as a shared selection pressure eliciting local adaptation in both species. Hybrid plants in the *Phlox* sites were sampled to examine any evidence that hybridization might upset a genic balance of canalization of petal number (cf. Møller & Eriksson 1995).

Statistical Analyses

Analyses were done using R (R Core Team 2015). The packages dplyr (Wickham & Francois 2015), plyr (Wickham 2011), and tidyr (Wickham 2015) were used for general data

manipulation. All figures were generated in R using the cowplot (Wilke 2015) and ggplot2 (Wickham 2009) packages.

Only 0.1% of flowers characterized (20) had a petal number more extreme than four or six; therefore, these were grouped with the four- and six-petaled flower groups, hereafter referred to as *four-petaled flowers* and *six-petaled flowers*. Data were expressed as means of per-individual percentages of abnormally-petaled, four-, and six-petaled flowers within a species, to avoid bias due to individuals with many flowers or high percentages of abnormal flowers. Within-genus differences between autogamous and outcrossing species were calculated by finding the difference between per-individual species means; 95% confidence intervals were computed based on the standard error of the difference between those means. Tukey post-hoc tests were performed using linear models in the built-in stats package (R Core Team 2015) and the multcomp package (Hothorn et al., 2008).

RESULTS

Differences Between Species

Natural variation for petal number varied by species (Figure 2.1, Table 2.S1), with *Gilia cana* and particularly *Saltugilia latimeri* having more variation than most species, and *Saltugilia australis* having slightly less. Differences in overall petal number variation were driven largely by these species' propensity to produce six-petaled flowers (Figure 2.2): there was a 50-fold difference in the percentage of six-petaled flowers between the species with the highest and lowest percentages. The percentage of four-petaled flowers was less variable across species, though a four-fold difference among species still existed. The most notable variation in petal number was manifest in *S. latimeri*. This species bore far more six-petaled flowers (3.85%) than any other species in this study. Within *S. latimeri*, flowers with seven or more petals were more

common, and as many as 12 petals were recorded on a flower, both conditions extremely unusual compared to other species sampled in this study, or other studies of natural variation in petal number (Stark 1918; Lowndes 1931; Roy 1963; Huether 1969; Ellstrand 1983; Lehmann 1987; Ellstrand & Mitchell 1988; Byerley 2006).

Variation Between Mating Systems

When all species were combined irrespective genus, there was no difference in the amount of petal number variation between outcrossing and autogamous species (Figure 2.3). However, individual genera showed varying responses: *Saltugilia* (three species, two autogamous) had significantly more variation in autogamous species (a 107% increase) consistent with relaxed stabilizing selection (stabilizing selection hypothesis). However, *Gilia* (two species) had significantly less variation in the autogamous species (a 71% reduction in variation) and *Phlox* (two species) showed no differences, both rejecting the stabilizing selection hypothesis.

Genera were more in agreement on the direction of the difference in variation between outcrossers and autogamous species when *S. latimeri* was removed from the analysis (Figure 2.4), leaving the comparison in *Saltugilia* to *S. australis* versus *S. splendens*. Instead of the increase in variation in autogamous species expected with a relaxation of selection, *Saltugilia* showed less variation, though the difference was not statistically supported. With *S. latimeri* removed, all three genera had less variation in the autogamous species, and the combined trend was for significantly less variation in autogamous species (Figure 2.4), with no genera supporting the stabilizing selection hypothesis.

Shifts in Petal Number Between Mating Systems

There was no evidence of any differences in the percentage of four-petaled flowers between outcrossers and autogamous species, either overall or within the three genera (Figure 2.5). However, autogamous species had significantly fewer six-petaled flowers in *Gilia* as expected by the costs hypothesis, but significantly more in *Saltugilia*, and with no differences in *Phlox* (Figure 2.5). Once again, the removal of *S. latimeri* from the analysis changed the results, leading to the conclusion that *Saltugilia* has significantly fewer instead of more six-petaled flowers in the autogamous species, similar to *Gilia* (Figure 2.6). Thus, with *S. latimeri* excluded, there were fewer six-petaled flowers in the autogamous species in two of the three genera (*Gilia* and *Saltugilia*), with only *Phlox* showing no support for the costs hypothesis (Figure 2.6).

Site-specific Differences

At shared sites, *P. drummondii* and *P. cuspidata* had similar percentages of four-petaled flowers and similar percentages of six-petaled flowers (Figure 2.7) between the two species at a site. However, those percentages were site-specific and the relative percentage of four- and six-petaled flowers differed between sites. At site H153, the proportions of four- and six-petaled flowers were roughly equal within both species and also between species (Figure 2.7). The other site (HCirc) had twice as many four-petaled flowers in both species compared to H153, and fewer six-petaled flowers in both species (none in *P. cuspidata*). Sites with both *S. latimeri* and *S. splendens* did not show a marked site effect. The percentage of four- and six-petaled flowers in *Saltugilia splendens* hardly differed between sites, while *S. latimeri* had more four- and six-petaled flowers at one site (Black Rock) than the other (Figure 2.8). At Black Rock, *S. latimeri*

also had more variation than *S. splendens*, but not at the other site (Figure 2.8). Therefore, there appeared to be a *species* × *site* effect, with only one species (*S. latimeri*) showing site effects.

Hybrids

Putative hybrids at the two shared *Phlox* sites had a higher proportion of six-petaled flowers, and a considerably higher proportion of four-petaled flowers, than the parents (Figure 2.9). However, sample sizes of hybrids were too small (46 hybrid individuals with 198 total flowers) to test for statistical significance; therefore, there is no evidence that this increased variation in hybrids represents a real trend. Putative hybridization between *S. latimeri* and *S. splendens* was observed at one site Figure 2.S1, though differences among plants were too slight to allow for clear categorization.

DISCUSSION

Comparing the amount of natural variation and examining any shifts in petal number between outcrossing and autogamous congeners represent a new way to test for pollinator-mediated selection on petal number. If pollinator-mediated stabilizing selection is responsible for five-petaled stasis in flowers, then selection should be relaxed or absent in autogamous species leading to higher levels of natural *variation* in petal number. Alternatively, if a higher petal number imposes a *cost*, despite any attractive benefits to outcrossing species, then autogamous species should be under selection to shift towards fewer petals.

Pollinator-mediated Stabilizing Selection

I found no evidence for a relaxation of stabilizing selection on petal number in autogamous species (the stabilizing selection hypothesis) when compared to their outcrossing congeners in any of the three genera examined (Figure 2.3). In fact, there is some evidence of

less variation in petal number in three of the four autogamous species examined relative to their outcrossing congener (Figure 2.4, i.e., with the exception of *S. latimeri*). Less variation in petal number may simply be due to less genetic variation for the underlying genes due to inbreeding, though it could also be caused by stronger stabilizing selection. Given a lack of clear hypotheses for mechanistic reasons why a pollinator might prefer a *particular* number of petals, failure to find evidence that stabilizing selection is canalizing petal number in outcrossing species should not be surprising—it may not be.

Yet the problem remains: why does heritable genetic variation for petal number or other floral organ number persist, when it exists in populations (Huether 1968; Vlot & Bachmann 1991; Vlot et al., 1992; Byerley 2006; Monniaux et al., 2015; Pieper et al., 2015; Mickley 2017, Chapter 1), without leading to at least some changes to the normal number or organs? Because differences in the amount and direction of variation exist among species (Figures 2.1, 2.2), there have likely been low levels of divergence, yet these are not related to the mating system (Figure 2.3) and do not appear to have been affected by pollinator-mediated selection.

Furthermore, if pollinators are not the agents of selection, what can be made of evidence that pollinators prefer certain petal numbers in other systems (Leppik 1953; Leppik 1954; Lehrer et al., 1995; Zhao & Huang 2013)? Two factors may account for this. First, pollinators represent a wide diversity of taxa and therefore have variation in sensory systems for perceiving flowers and may use different phenotypic components as cues (Fægri & Pijl 1980; Willmer 2011). My study considered only species pollinated by Lepidoptera and Bombyliidae (Diptera). Selective regimes may be different for plants with other pollinators, particularly bees, for which most of evidence for preference of certain petal numbers exists. However, when grown in a greenhouse, the bee-pollinated *S. caruifolia* had levels of petal number variation similar to other species such

as *S. splendens*. Second, all studies that have evaluated a pollinator's petal number preference have been done with non-naïve individuals that had already foraged in the wild. Detection of a preference may thus conflate innate preference with learned search images. Therefore, it is unclear whether the displayed preference would translate to a selective force for a particular petal number, or merely represents preference for the norm. More research is needed to determine whether other pollinator taxa have a clear capability to count or have preferences for certain petal numbers, and all research of this nature should ideally be conducted with naïve individuals and measure selection on both male and female plant reproductive success (Bell 1985; Johnson et al., 1995; Kudoh & Whigham 1998).

Costs of Petal Number

If the number of petals required to attract pollinators imposes a cost, then there should be a shift towards fewer petals in autogamous species. The cost hypothesis is not supported by this study: there was no evidence for more four-petaled flowers in any autogamous species (Figure 2.5), and evidence for fewer six-petaled flowers in only two of the four autogamous species (*S. australis* and *G. sinuata*, Figure 2.6). *Phlox cuspidata*, which did not show any shifts in petal number, is not obligately autogamous, with outcrossing rates of ~25% (Levin 1978; Bixby & Levin 1996; Ruane 2008). Facultative selfing could buffer any cost-mediated selection pressure imposed by autogamy, leading to less difference between the two *Phlox* species compared to species with more obligate autogamy. Outcrossing rates in autogamous *Saltugilia* and *Gilia* species are not known. Autogamous species in these genera do occasionally get floral visitors, although changes in style length and positioning (shorter and retracted inside the floral tube) may render these visits ineffective for pollination. Petal costs may also be mitigated by changing the size of the petal itself, rather than the petal number (cf. Stebbins 1967), at least to

the extent that petal size is unconstrained. Preliminary observations (J. Mickley, pers. obs.), indicate that smaller petals do characterize autogamous species in *Gilia* and *Saltugilia*, potentially lowering costs to the extent that they might not constrain petal number.

Alternative Explanations

Despite a lack of support for the stabilizing selection and the petal costs hypotheses in these results, differences among species in the amount and direction of petal number variation merit further research. The results from *S. latimeri* are especially interesting, since this species had a high proportion of petal number variation and also of six-petaled flowers, supporting the stabilizing selection hypothesis, and refuting the costs hypothesis. Relative to the other species studied, *S. latimeri* has a highly restricted geographic range, and small, disjunct populations. It is plausible that genetic drift may have played a larger role in promoting petal number variation in *S. latimeri*

The increase in both four- and six-petaled flowers in *Phlox* hybrids (Figure 2.9) suggests that petal number is normally canalized in the parent species and that hybrid incompatibility upsets the genic balance of this canalization. That would in turn release hidden genetic variation for organ number (cf. Møller & Eriksson 1995; Vlot et al., 1992), leading to more phenotypic variation. Hybrid incompatibility in these two species of *Phlox* generally decreases fitness, and selection against hybrids is strong enough drive character displacement based on flower color (Levin 1985; Hopkins & Rausher 2014). Therefore, hybrid incompatibility upsetting petal number canalization is plausible.

Conclusions

My results show no evidence for higher levels of variation in petal number in autogamous species, a conclusion that would indicate a release from pollinator-mediated stabilizing selection

on petal number. This suggests that pollinators of these species have no preferences for either five-petaled flowers or constancy. Furthermore, only two of the four autogamous species show a small shift in petal number towards fewer petals, consistent with a petal costs hypothesis. Even if more species were sampled, it seems unlikely that a tradeoff between attraction and costs plays a large role in constraining petal number. Alternative hypotheses to explain petal number stasis in Pentapetalae, such as a requirement to maintain predictable development of complex flowers (Breder 1955; Endress 2001b; Ronse De Craene 2010), or pleiotropic effects of changes in petal number on other floral traits (Stebbins 1974; Byerley 2006; Herrera 2009) remain to be tested. Tests of these hypotheses are currently hampered by a lack of understanding of the underlying genetic control of petal number, though some progress is being made (e.g., Monniaux et al., 2015; Pieper et al., 2015). If stabilizing selection is acting to maintain an adaptive petal number as Stebbins suggested (1974), the agent of selection remains unknown and does not seem to be differentially affected by mating system.

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TABLES

Table 2.1: List of the species sampled, along with their mating system and primary pollinators mostly as described by Grant and Grant (1965).

Species	Mating System	Pollinators	Populations	Plants Sampled	Flowers Sampled
<i>Phlox cuspidata</i> Scheele	Autogamous		6	1756	4311
<i>Phlox drummondii</i> ssp. <i>drummondii</i> Hook.	Outcrosser	Lepidoptera	4	1066	3082
<i>Phlox drummondii</i> ssp. <i>mcallisteri</i> (Whitehouse) Wherry	Outcrosser	Lepidoptera	2	422	1460
<i>Phlox drummondii</i> ssp. <i>drummondii</i> x <i>Phlox cuspidata</i> (Hybrid)			2	46	198
<i>Gilia sinuata</i> Douglas ex Benth.	Autogamous		3	1062	1592
<i>Gilia cana</i> ssp. <i>speciformis</i> A.D. Grant & V.E. Grant	Outcrosser	Bombyliidae	1	155	731
<i>Gilia cana</i> ssp. <i>bernardina</i> A.D. Grant & V.E. Grant	Outcrosser	Bombyliidae	2	792	1524
<i>Saltugilia latimeri</i> T.L. Weese & L.A. Johnson	Autogamous		3	1074	1662
<i>Saltugilia australis</i> (H. Mason & A.D. Grant) L.A. Johnson	Autogamous		4	1236	1809
<i>Saltugilia splendens</i> ssp. <i>splendens</i> (Douglas ex H. Mason & A.D. Grant) L.A. Johnson	Outcrosser	Bombyliidae	3	834	1563
<i>Saltugilia caruifolia</i> (Abrams) L.A. Johnson	Outcrosser	Bees	1	12	78
Total			32	8455	18010

Table 2.2: A list of the field site locations where species were sampled. Note that multiple species were sampled at several sites.

Site	Species	State	Latitude	Longitude
D2091	<i>P. drummondii</i> ssp. <i>mcallisteri</i>	TX	29.55512	-97.57046
D290	<i>P. drummondii</i> ssp. <i>mcallisteri</i>	TX	30.30574	-97.29814
D95	<i>P. drummondii</i> ssp. <i>drummondii</i>	TX	30.03553	-97.16402
D304	<i>P. drummondii</i> ssp. <i>drummondii</i>	TX	29.68885	-97.41715
C214	<i>P. cuspidata</i>	TX	30.07110	-96.92975
C3011	<i>P. cuspidata</i>	TX	30.03976	-96.87029
C71	<i>P. cuspidata</i>	TX	30.11089	-97.40288
C448	<i>P. cuspidata</i>	TX	30.13699	-96.96793
HCirc	<i>P. cuspidata</i> , <i>P. drummondii</i> ssp. <i>drummondii</i> , Hybrids	TX	30.07052	-97.08694
H153	<i>P. cuspidata</i> , <i>P. drummondii</i> ssp. <i>drummondii</i> , Hybrids	TX	30.00441	-96.97653
Granite Cove	<i>G. sinuata</i>	CA	34.78238	-115.65548
Black Rock	<i>G. sinuata</i> , <i>S. latimeri</i> , <i>S. splendens</i> ssp. <i>splendens</i>	CA	34.06982	-116.39351
Burns Crossroad	<i>G. sinuata</i>	CA	34.22112	-116.62119
Kelbaker ¹	<i>G. cana</i> ssp. <i>speciformis</i>	CA	35.20488	-115.87035
Aiken Mine	<i>G. cana</i> ssp. <i>speciformis</i>	CA	35.18528	-115.76691
Rattlesnake Canyon	<i>G. cana</i> ssp. <i>bernardina</i>	CA	34.23017	-116.65197
Smarts Quarry	<i>G. cana</i> ssp. <i>bernardina</i>	CA	34.30404	-116.79989
Elata Ave.	<i>S. latimeri</i> , <i>S. splendens</i> ssp. <i>splendens</i>	CA	34.07416	-116.41512
Elk Trail	<i>S. latimeri</i>	CA	34.07486	-116.43531
Burns Spring	<i>S. splendens</i> ssp. <i>splendens</i>	CA	34.20462	-116.57495
HWY 243 ²	<i>S. australis</i>	CA	33.89241	-116.85896
S22 PCT 0.4S	<i>S. australis</i>	CA	33.21182	-116.58227
S22 PCT 1.5S	<i>S. australis</i>	CA	33.20837	-116.57798
S22 PCT 2.4S	<i>S. australis</i>	CA	33.20389	-116.56817
Vallecito ³	<i>S. caruifolia</i>	CA	32.93511	-116.41384

¹Small population of 53 flowers

²Small population of 124 flowers

³Small population of 78 flowers

FIGURES

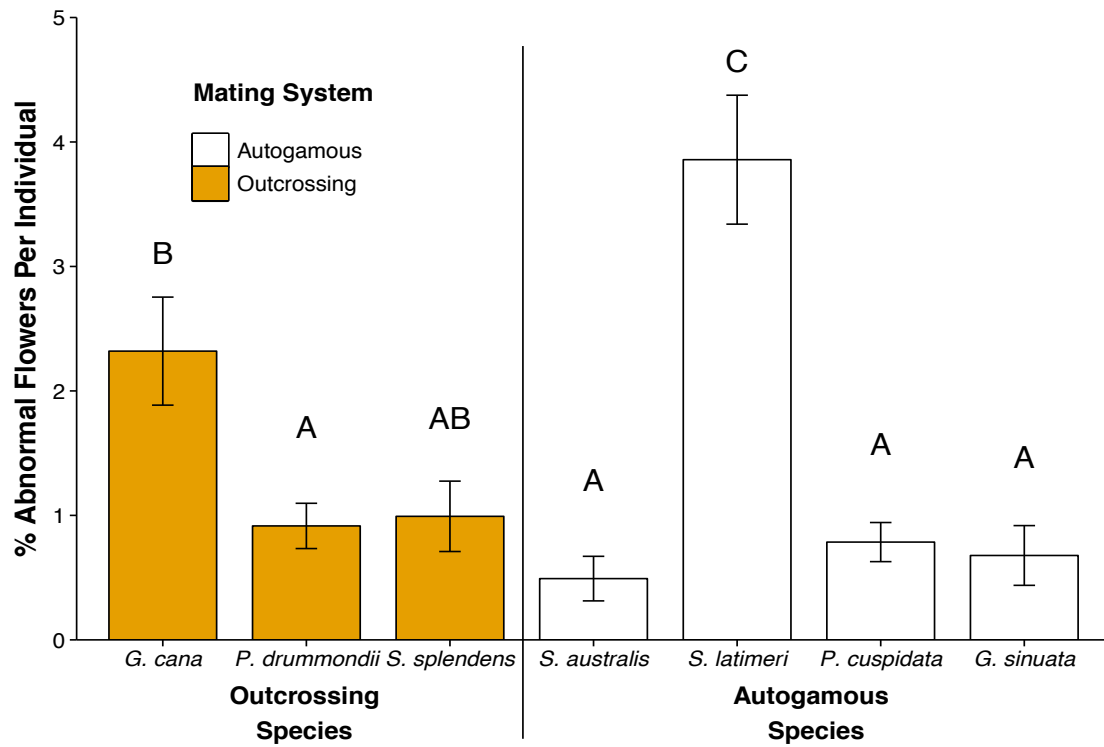


Figure 2.1: Comparison of the percentage of abnormal flowers between species. Error bars are standard errors of the per-individual mean percentage of abnormal flowers and letters denote groupings based on Tukey post-hoc comparisons. With the exception of *S. latimeri*, autogamous species have somewhat less petal number variation than outcrossers.

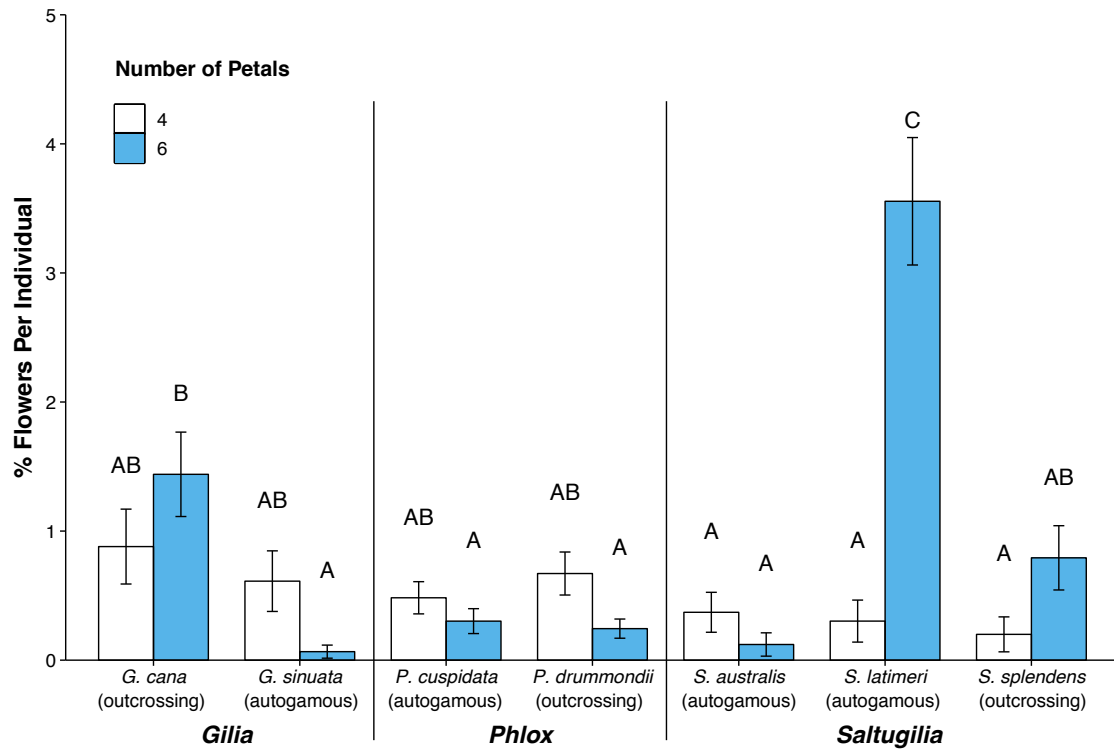


Figure 2.2: Comparison of the percentage of four- and six-petaled flowers by species. Error bars are standard errors of the per-individual mean percentage of abnormal flowers and letters denote groupings based on Tukey post-hoc comparisons. Most species have similar levels of variation; however, *S. latimeri* has far more 6-petaled flowers than other species, driving the high levels of overall petal number variation seen in Figure 2.1

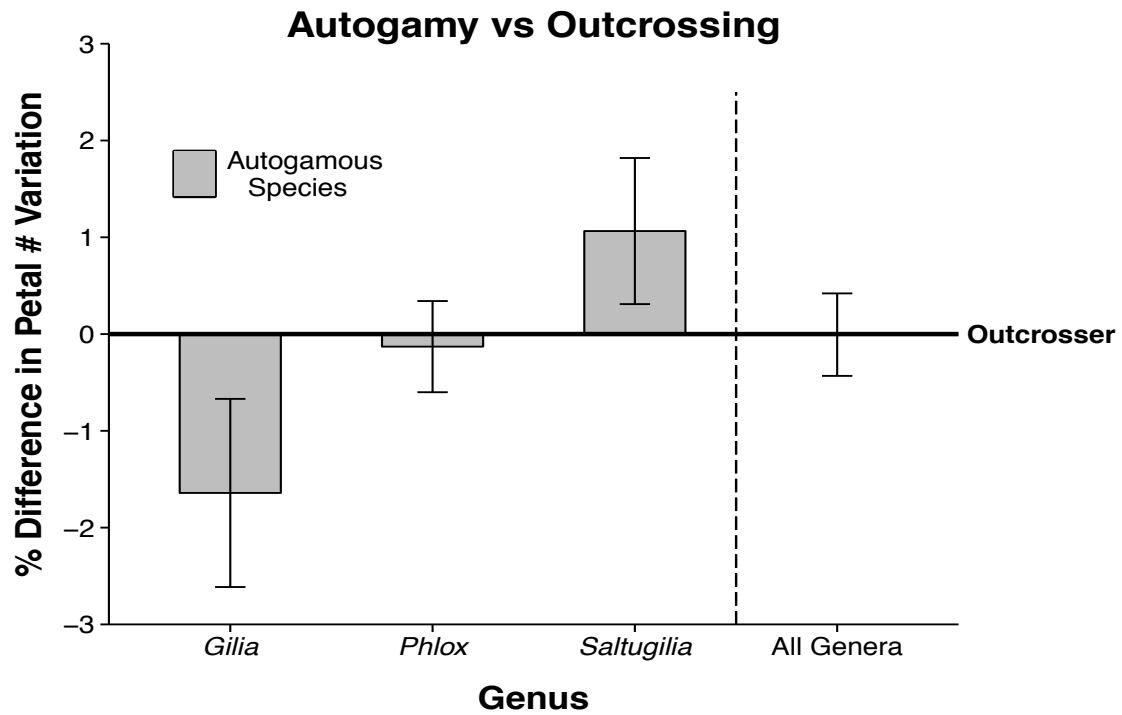


Figure 2.3: The difference in petal number variation for autogamous species relative to that of outcrossers within each genus. Error bars represent 95% confidence intervals around the difference between per-individual mean percentages of the autogamous species relative to the outcrosser. In *Gilia* there is significantly less variation in the autogamous species, while in *Saltugilia* the trend is reversed, and there is no difference within *Phlox* or among all genera combined.

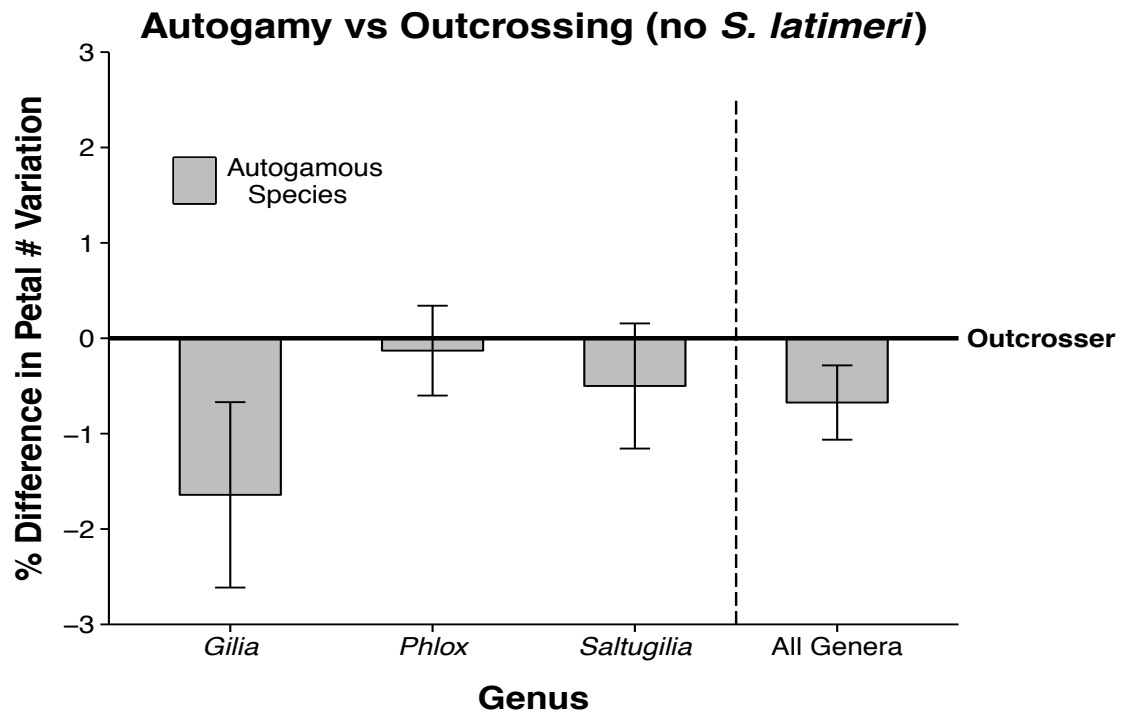


Figure 2.4: The difference in petal number variation for autogamous species relative to that of outcrossers within each genus, excluding *S. latimeri*. As in Figure 2.3, error bars represent 95% confidence intervals around the difference between per-individual mean percentages of each mating system.

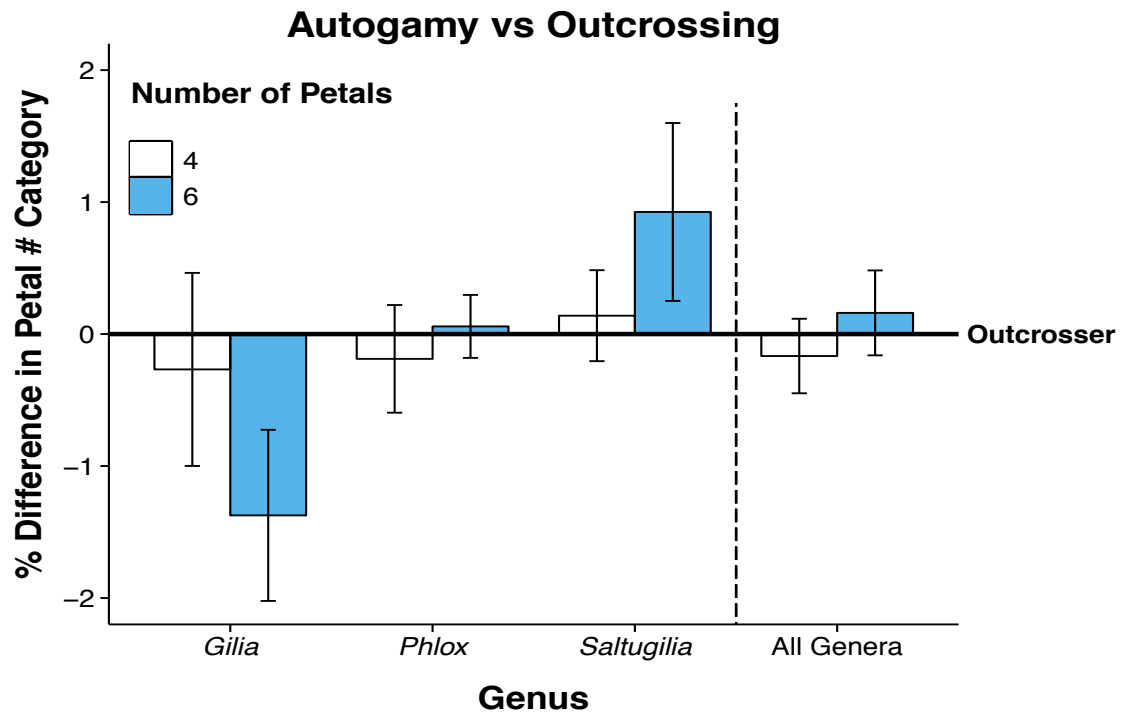


Figure 2.5: The difference in the percentage of four- and six-petaled flowers for autogamous species relative to that of outcrossers within each genus. Error bars represent 95% confidence intervals around the difference between per-individual mean percentages of the autogamous species relative to the outcrosser. In *Gilia* there are significantly fewer six-petaled flowers in the autogamous species, while there are significantly more in *Saltugilia*. No genera show differences in the percentage of four-petaled flowers between mating systems.

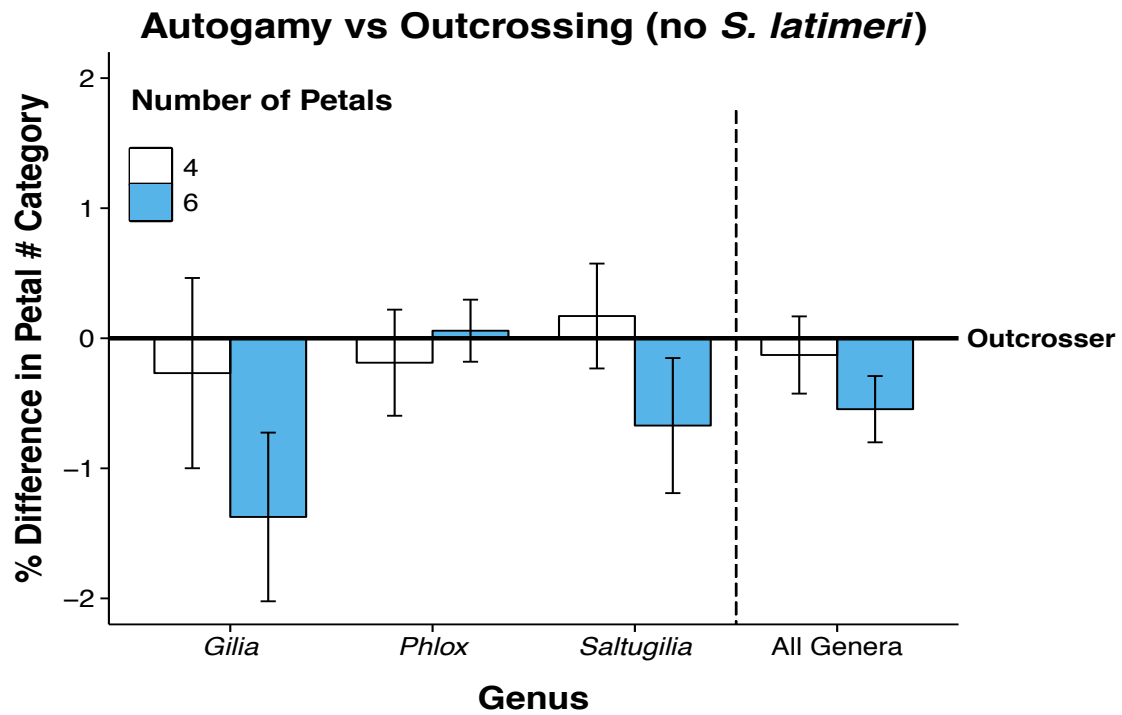


Figure 2.6: The difference in the percentage of four- and six-petaled flowers for autogamous species relative to that of outcrossers within each genus with *S. latimeri* excluded. As in Figure 2.5, error bars represent 95% confidence intervals around the difference between per-individual mean percentages of each mating system.

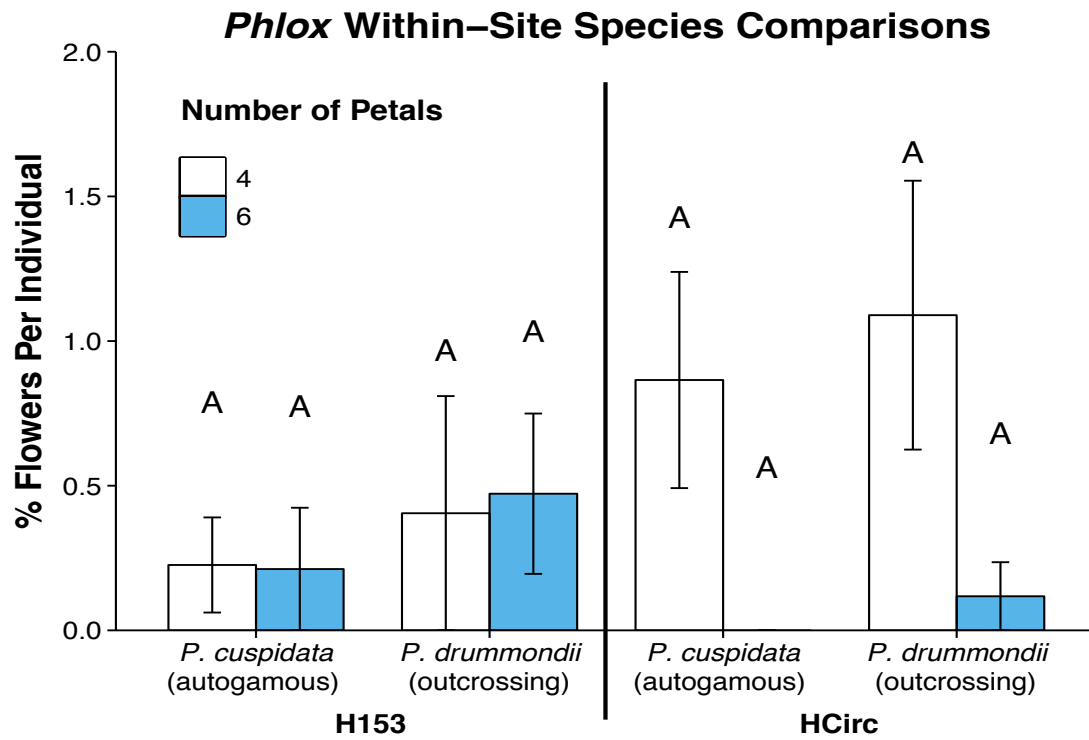


Figure 2.7: Comparison of petal number variation between co-occurring *P. drummondii* and *P. cuspidata*. At H153, both species have similar percentages of four- and six-petaled flowers; however, at HCirc both species have more four-petaled flowers and few six-petaled flowers.

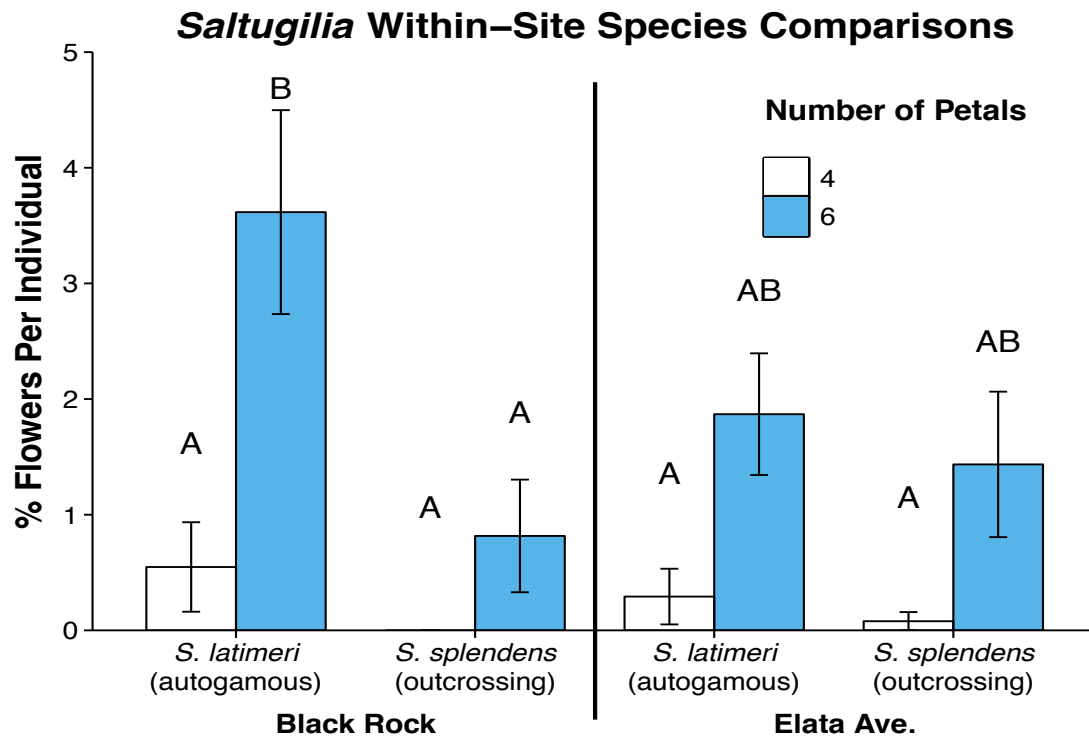


Figure 2.8: Comparison of petal number variation between co-occurring *S. latimeri* and *S. splendens*. The percentage of both four- and six-petaled flowers for *S. latimeri* is higher at Black Rock than at Elata. Ave.; however, the opposite is true for *S. splendens*.

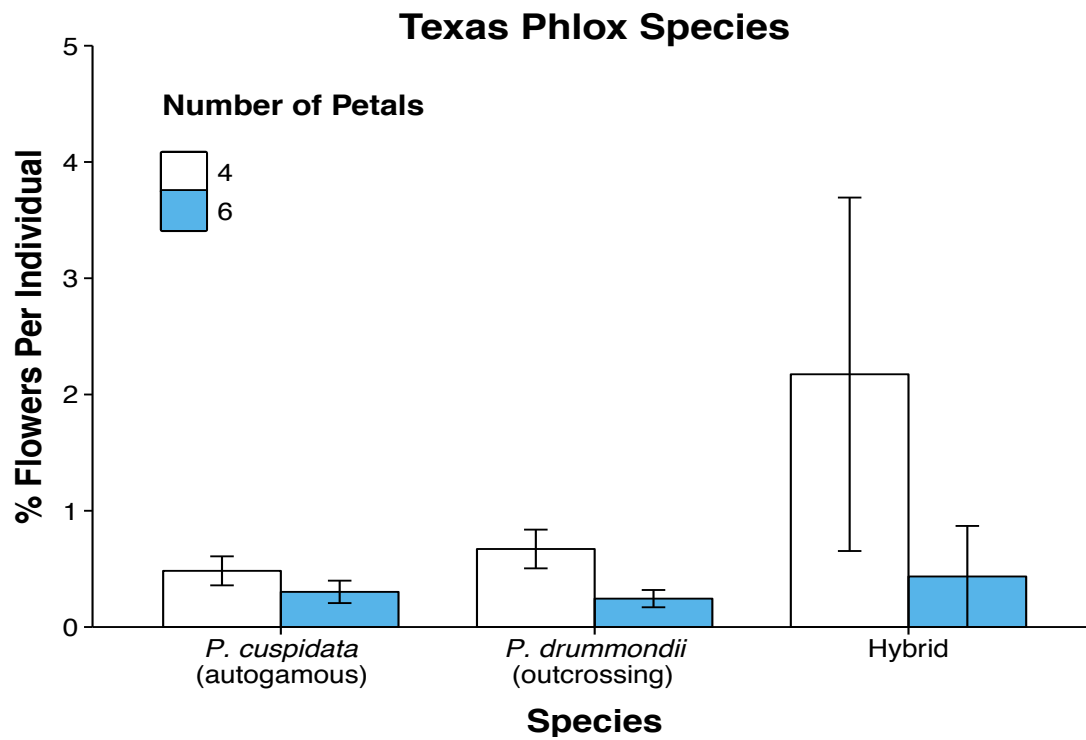


Figure 2.9: *Phlox* hybrids exhibited higher levels of four- and six-petaled flowers than both of their parents, though the sample size was small (Table 2.1).

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CHAPTER 2 SUPPLEMENT

Table 2.S1: A binomial logistic mixed model of the proportion of abnormally-petaled flowers attributable to differences among plants, sites, and species. All three explanatory variables were included as random factors. The best model by AICc score was one with the identity of the species and the individual plant included; this model explained 97% of the variance. Much of this variance was explained by differences among plants, but differences among species were also important. Differences among sites contributed little to the model. For all models, the marginal R^2 ($R^2_{\text{glmm(m)}}$) was zero, since there were no fixed effects, and the conditional R^2 ($R^2_{\text{glmm(c)}}$) denotes the proportion of variance explained by the random factors.

Model reported values: Model = the random terms included in the model; AICc = AICc score; ΔAICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(c)}}$ = conditional R^2 (random factors).

Model	AICc	dAICc	df	weight	$R^2_{\text{glmm(c)}}$
(1 species) + (1 plant)	1809.48	0.00	3	0.80	0.974
(1 species) + (1 site) + (1 plant)	1812.27	2.79	4	0.20	0.970
(1 plant)	1923.19	113.71	2	0.00	0.969
(1 site) + (1 plant)	1925.15	115.67	3	0.00	0.965
(1 species) + (1 site)	2424.82	615.34	3	0.00	0.169
(1 species)	2431.77	622.29	2	0.00	0.131
(1 site)	2457.46	647.98	2	0.00	0.130
null model	2526.81	717.33	1	0.00	0



Figure 2.S1: Putative hybridization between *S. latimeri* and *S. splendens* ssp. *splendens*. The photos show the (A) dorsal view and (B) lateral view of the same flowers from plants at the Black Rock site. The left is a typical *S. splendens* ssp. *splendens* individual, the right *S. latimeri*, and the middle is an intermediate. The middle individual has larger flowers and a wider throat consistent with *S. splendens* ssp. *splendens*, but acute corolla lobes and a glandular calyx consistent with *S. latimeri*, and a corolla tube only partially exerted from the calyx and intermediate between the two species.

CHAPTER 3:

Testing Pollinator Preferences for Petal Number in *Phlox drummondii*

ABSTRACT

Petal number is diverse among angiosperm flowers. However, a large proportion of species have five petaled flowers, with little or no petal number diversity within species. This stasis for petal number has not been explained, though a popular hypothesis is that pollinators impose stabilizing selection on five petals through petal number preferences. Though most pollinator groups can differentiate between various petal numbers, little evidence exists to support a preference and causal explanations for putative preferences are lacking. Though normally five-petaled, many species in the Polemoniaceae exhibit low level within-individual natural variation for petal number. This variation can be increased via selection, and a prior experiment provided a selection line of *Phlox drummondii* (Polemoniaceae) with 30–50% six-petaled flowers. Pollinator visitation by lepidopteran pollinators in the field was compared among plants of this six-petaled line, plants with predominantly five-petaled flowers, and plants on which a petal was removed from each flower. Visitation was quantified for each plant using visits per day and the number of flowers visited per plant during each visit. Plant height, inflorescence width, and the number of flowers on a plant were also measured as covariates and visitation was modeled in a multivariate context. There was no evidence of a preference by lepidopterans for five-petaled flowers. Instead, the visits per day a plant received increased with the proportion of six-petaled flowers, and petal number had no effect on flowers visited per plant. Covariates explained most of the variation in visitation, and apparent preferences disappeared or changed when covariates were accounted for, highlighting the importance of measuring multiple floral attractive traits in addition to the focal trait. As the first explicit test of

pollinator preferences for petal number, these results cast doubt on pollinator-mediated stabilizing selection as a mechanism for stasis.

INTRODUCTION

There is considerable diversity in petal number among angiosperm groups (Ronse de Craene 2016), though this character is often constant within specific lineages (Cronquist 1981; Givnish 2002; Herrera 2009). In particular, five-petaled or *pentamerous* flowers are highly conserved within the aptly-named Pentapetalae, marking a shift from lability in petal number in many phylogenetically earlier-diverging angiosperms that has long been noted (Schoute 1932; Breder 1955; Leppik 1957; Stebbins 1974; Stebbins 1967; Soltis et al., 2003; Soltis & Soltis 2013; Chanderbali et al., 2017). Pentapetalae is a large group, comprising some 175 plant families and 70% of all angiosperm diversity (Cantino et al., 2007; Chanderbali et al., 2017). Though some lineages within Pentapetalae (e.g. Brassicaceae) are fixed on different petal numbers, the five-petaled phenotype remains remarkably prevalent. The reasons for this relative stasis in petal number in such a large and diverse clade are an unsolved and understudied mystery.

Stasis in petal number has often been presumed to be adaptive (Leppik 1953; Leppik 1956; Stebbins 1974), driven by pollinator-mediated stabilizing selection for five-petaled flowers (Leppik 1953; Leppik 1955a; Leppik 1956; Lehrer et al., 1995; Ronse de Craene 2016; Monniaux et al., 2015), or simply pollinator avoidance of abnormally-petaled flowers (Herrera 2009). Such a pollinator preference would imply that at least some pollinator species can differentiate between different petal numbers on flowers. However, pollinators comprise a wide array of disjunct taxa, possessing different visual and cognitive systems (Giurfa & Lehrer 2004; Weiss 2004; Willmer 2011). Despite their diversity, some pollinators have been shown to be able

to differentiate between flowers by petal number. By manipulating the number of ray flowers of *Tagetes* (Asteraceae), Leppik established that bees could distinguish between symmetrical “figure numerals” of one, two, three, four, five, six, eight, ten, and twelve petals (1953; 1956), but flies, beetles, and weevils could not (Leppik 1953). In a separate experiment Leppik showed that butterflies could distinguish between symmetrical flowers of few, three, five, and many petals (Leppik 1954; Leppik 1955b). Similarly, Lehrer *et al.* (1995) showed that bees could distinguish between artificial floral patterns of two, four, and six petals. Despite Leppik’s results showing that flies were unable to differentiate among different petal numbers (1953), Zhao and Huang (2013) found directional selection for increased petal number in *Trollius ranunculoides* (Ranunculaceae) by nectarivorous flies. In this species, petals are not attractive organs, but anther-like and nectariferous and petal number is directly related to the amount of nectar (Zhao & Huang 2013). Nevertheless, this suggests that flies can differentiate between flowers based on the number of floral organs. No known data exists on the ability of bird or bat pollinators to differentiate between petal numbers.

The ability to differentiate between flowers with different petal numbers is only a precondition for pollinator preference, and evidence for extending differentiation to pollinator preference is much weaker. Without showing data, Leppik (1953) noted that bees had the highest preference for five-petaled flowers, with no comment on the relative petal number preferences of the other animal pollinators he studied. In artificial flowers, Lehrer *et al.* (1995) found that bees preferred six-petaled artificial flowers over those with two or four petals, but five-petaled artificial flowers were not included in the study. Citing unpublished data from Huether’s (1968) studies of petal number in *Leptosiphon androsaceus*, (Polemoniaceae) Stebbins mentioned that bombyliid and syrphid flies did not discriminate against flowers with abnormal petal numbers

(Stebbins 1974). Despite several historical calls for more scrutiny (Breder 1955; Stebbins 1970; Stebbins 1974), overall, only one report supports the assertion that pollinators prefer five-petaled flowers, and only bees and flies have been studied.

While it has been shown that pollinators can exert stabilizing selection on other floral traits (e.g., size, color, symmetry; Cresswell 1998), and are generally able to differentiate between different petal numbers, the pollinator-mediated stabilizing selection hypothesis lacks a clear underlying causal explanation for petal number stasis. Leppik (1955a) suggested that pollinators that were able to distinguish between different petal numbers might have an advantage in distinguishing between flower types, thus improving foraging efficiency. However, obviously, the strength of this advantage is diminished if most flowers are five-petaled. Lehrer *et al.* (1995) posited that hexagonal-eyed bees might have better vision for hexamorous patterns; this hypothesis remains untested and also would not explain stasis for pentamery. Finally, pollinators do select against asymmetrical flowers, as an indicator of nectar quality (amount and concentration), leading to stabilizing selection in plants for increased floral symmetry (Møller & Eriksson 1994; Møller & Eriksson 1995; Møller & Sorci 1998). While this selection for indicators of nectar quality might extend to abnormal petal numbers (Herrera 2009) and explain constancy within a species, it would not select for stasis among species. With little evidence for the pollinator-mediated stabilizing selection and no convincing causal explanation for pollinator preference for five petals, perhaps this explanation of stasis is itself unlikely. Nevertheless, more research is still needed to determine whether a broad range of pollinator taxa exhibit any preference for specific petal numbers.

Despite broad patterns of constancy in flower petal number within species, some species show within-individual natural variation in petal number (Stark 1918; Lowndes 1931; Roy

1963), although abnormal flowers are typically rare. This natural variation has been shown to be particularly prevalent in the Polemoniaceae (Huether 1969; Ellstrand 1983; Lehmann 1987; Ellstrand & Mitchell 1988; Byerley 2006; Mickley 2017, Chapter 2). Within-individual variation in petal number has been shown to be heritable in Brassicaceae (Monniaux et al., 2015; Pieper et al., 2015), Polemoniaceae (Huether 1968; Mickley 2017, Chapter 1), and in the pappus organs (modified sepals) of Asteraceae (Vlot & Bachmann 1991; Vlot et al., 1992). The proportion of abnormal flowers in these groups can be greatly increased via selection (up to 30–50% or more of the flowers on a plant: Huether 1968; Mickley 2017, Chapter 1). Thus, at least for the species studied in these groups with known heritability for petal number variation, stasis is not maintained by a lack of genetic variation (Stebbins 1974; cf. Cresswell 1998). Therefore, stabilizing selection or canalization directly on petal number, or on a trait that is genetically correlated with petal number, must inhibit divergence.

Two alternative hypotheses might explain stasis in petal number without involving pollinators. First, stasis in petal number in Pentapetalae corresponds to stasis in several other floral traits: whorled phyllotaxis, alternation of organs in adjacent whorls, and single whorls of sepals and petals (Chanderbali et al., 2017). A fixed petal number or a specifically five petaled arrangement may be necessary for predictable and synchronous development of this shared suite of traits in more complex flowers (Breder 1955; Stebbins 1974; Endress 2001b; Ronse De Craene 2010). This hypothesis remains untested, though flowers with natural variation in petal number do not exhibit gross abnormalities. Second, changes in petal number are accompanied by correlated changes in the number of other organs, particularly stamens and sepals (Ellstrand 1983; Byerley 2006; Mickley 2017, Chapter 1), though no other obvious changes in other traits. This leads to the explanation that pleiotropic effects and phenotypic integration within the flower

constrain petal number indirectly via constraints on other floral organs or traits (Stebbins 1974; Byerley 2006; Herrera 2009; Diggle 2014; Smith 2016). This pleiotropy hypothesis is supported only by anecdotal evidence mentioned by Stebbins (1974): that plants selected for increased petal number were weak and slow-growing. These two alternative hypotheses are not mutually exclusive, nor do they exclude those hypotheses positing pollinator-mediated stabilizing selection. Each hypothesis merits further investigation.

A prior experiment with *Phlox drummondii* (Polemoniaceae), a normally five-petaled species pollinated by lepidopterans (Grant & Grant 1965), increased the proportion of six-petaled flowers on plants to 30–50% via selection (Mickley 2017, Chapter 1). Having plants with high proportions of a different petal number provides a useful system to explicitly test for pollinator preferences. These plants can be compared to those with five-petaled flowers, and to those for which the flowers have been artificially manipulated to be four-petaled to determine which are preferred by pollinators. When presented with various choices, a pollinator's preferred petal number should receive a higher proportion of that pollinator's attention. If that preference is for the predominant petal number in the population, this could impose stabilizing selection against abnormality. However, if preferences were for a petal number other than the predominant one, then pollinator-mediated selection would be directional, and stasis could only be explained by counteracting selective forces.

Here, I use field experiments involving selected and controlled lines of *P. drummondii* ask the following questions: 1) Do lepidopterans exhibit any preferences for a particular petal number? 2) Do plants with five-petaled flowers receive more visits by pollinators? 3). Do pollinators spend more time on plants with five-petaled flowers, visiting more of the flowers on the plant? Lepidopterans can distinguish between different petal numbers (Leppik 1954; Leppik

1955b), but whether they exhibit any preference for petal number is unknown. A lack of preference by lepidopterans or other pollinators would argue against any selective effect on petal number, stabilizing or otherwise. Therefore, a lack of evidence for differences in visitation would provide evidence against pollinator-mediated stabilizing selection as a driver of stasis, at least for lepidopterans and any other common pollinators of *P. drummondii*. Furthermore, previous studies focused on whether a pollinator could differentiate between petal numbers (Leppik 1953; Lehrer et al., 1995), and any noted preference for a particular number was a secondary byproduct of their primary focus pollinator counting ability, with little supporting data.

METHODS

Study System

Phlox drummondii Hook. is an annual herb in the Polemoniaceae native to eastern and central Texas (Grant 1959; Erbe & Turner 1962; Turner 1998). The species is easy to grow and has frequently been grown in the greenhouse (Schlichting & Levin 1988; Lehmann 1987; Byerley 2006) and field (Hopkins & Rausher 2014). *Phlox drummondii* petals are fused into a tube 2–3 mm wide and 10–15 mm long with stamens attached to and inside the tube, and petal lobes arranged perpendicular to the end of the tube. Concentrated nectar is produced from a ring nectary at the base of the tube. *Phlox drummondii* is predominantly a facultative outcrosser (Levin 1975; Levin 1978) pollinated in its native range by *Battus philenor*, *Hyles lineata*, and other lepidopterans (Grant & Grant 1965), including skippers (family Hesperidae, Hopkins & Rausher 2014).

Generally, *P. drummondii* flowers have five sepals, five petals, five stamens, and a tricarpellate gynoecium with three stigma lobes, as do most species in Polemoniaceae (Grant 1959; Lehmann 1987; Byerley 2006). However, *P. drummondii* has natural intra-individual

variation in number for all floral organs (Ellstrand 1983; Lehmann 1987; Byerley 2006), which seems to be widespread in the Polemoniaceae (Huether 1968; Huether 1969; Ellstrand 1983; Ellstrand et al., 1984; Ellstrand & Mitchell 1988). In most cases, this variation consists of an increase or decrease by one in the number of a particular organ, though more extreme patterns occur. In natural populations, abnormally-petaled flowers make up 1–2% of the flowers (Lehmann 1987; Mickley 2017, Chapter 2). The proportion of six-petaled flowers on a plant has been shown to be heritable and can be increased considerably by selection (Mickley 2017, Chapter 1). The heritability is lower for the proportion of four-petaled flowers, and while they can be increased via selection, it is to a much lesser extent (Mickley 2017, Chapter 1). Selection to increase the proportion of six-petaled flowers also provokes correlated and positive selection responses on the number of sepals and stamens (Mickley 2017, Chapter 1).

Experimental Design

Phlox drummondii seeds were sown in the greenhouse in May 2016 in 36-section seedling flats containing a peat and perlite growing mix (Fafard #2, Sungro Horticulture, Agawam, MA). Seedlings were randomly thinned to one per section after their first true leaves emerged, for a total of 48 plants in three groups: a group with a high proportion of six-petaled flowers, a group whose flowers would be manipulated to be four-petaled, and a primarily five-petaled control. The 16 plants in the six-petaled group were descendants of a selection experiment in which the proportion of six-petaled flowers had been increased to 30–50% per plant (Mickley 2017, Chapter 1). The remaining 32 plants in the four- and five-petaled groups were grown from a combination of unselected greenhouse-grown plants and wild-collected seed, primarily of the same origin. After five weeks in the greenhouse, plants were moved outside for a week to harden off. In late June, plants were transplanted to an experimental plot at the UConn

Experimental Farm in Storrs, CT (41.798674, -72.227327; elevation: 180 m). Plants were spaced 1 m apart in a 6x8 grid in random order. To facilitate easy observation of pollinator visitation patterns, 1 m high stakes with numbered signs (1–48) were placed beside the plants. The plot on the UConn Experimental Farm was in the corner of an open and recently plowed field with grass borders. Beyond the grass borders was forest ~15 m away and overgrown fields 15 m and 40 m away. The latter overgrown field contained a diverse assemblage of feral plants in bloom and very high lepidopteran activity throughout the experiment.

Plants began flowering in early July (9 weeks after sowing), though pollinator visitation was sparse until the plants were more fully in flower and plants in the surrounding field had grown, obscuring open soil. Pollinator observations were conducted during late July and early August in 1-6.5 hour blocks at various times of day spanning early morning to dusk on sunny or partly cloudy days (29 total hours). Previous observations had determined that pollinator activity was minimal on cloudy or rainy days; therefore, these were avoided.

For each floral visitor, the order of plants visited and the number of flowers probed on each plant were recorded. Each day, the number of flowers on each plant was counted and the height and width of the inflorescence were measured, because previous studies showed that these traits strongly affected visitation rates (Eckhart 1991; Conner & Rush 1996; Totland & Matthews 1998; Thompson 2001). For the 32 plants in the five- and six-petaled groups, the proportion of four- and six-petaled flowers were recorded each day, because these proportions varied by plant. All flowers in these two groups were left on the plants, un-manipulated. Each day, all newly-opened flowers on plants in the four-petaled group were manipulated to be four-petaled. To control for any petal removal effects (e.g., odor from the cut surface), on four of the plants one

petal was bent down and held in place with a small section of clear twist-tie; the remaining plants had a petal removed from each flower with dissection scissors.

Statistical Analyses

Analyses were conducted using R (R Core Team 2015). Data manipulation in R used the dplyr (Wickham & Francois 2015), plyr (Wickham 2011), and tidyr (Wickham 2015) packages. Figures were constructed using the cowplot (Wilke 2015) and ggplot2 (Wickham 2009) packages. During exploratory data analysis, edge and spatial effects and petal removal effects were tested for, ruled out, and subsequently excluded from further analysis.

Differences among visitation rates were tested using Chi-squared tests comparing the three petal number groups and generalized linear mixed models using the lme4 package (Bates et al., 2015), with the number of visits a plant received each day and the number of flowers per visit as response variables. As counts, both of these responses approximated a Poisson distribution; therefore, a Poisson model family with a log link was used in constructing the models (Zuur et al., 2009). The identity of the plant was added as a random effect. Fixed factors consisted of the per-plant percentage of four- and six-petaled flowers, as well as the covariates with likely effects on visitation (Conner & Rush 1996): number of flowers per plant, width of the inflorescence, and height of the plant. Plants from the manipulated four-petaled group were coded as having 100% four-petaled flowers. Using per-plant percentages of four- and six-petaled flowers as fixed factors was deemed better than using petal number group as a fixed factor because the plants in the six-petaled group varied in their percentage of six-petaled flowers and abnormally-petaled flowers were also occasionally present in the control group.

Model selection followed methods from Zuur *et al.* (2009), starting with a full model (including first-order interactions) and removing fixed terms to minimize AICc scores calculated

using the *bbmle* package (Bolker & R Development Core Team 2014). Fixed factors were kept if removing them did not improve AICc scores by ~ 2 (Zuur et al., 2009), or if they were involved in an interaction term. Marginal and conditional R^2 s were calculated with the *piecewiseSEM* package (Lefcheck 2016) using methods from Nakagawa and Schielzeth (Nakagawa & Schielzeth 2013) that provide an R^2 estimate for generalized linear mixed models. The marginal R^2 ($R^2_{\text{glmm(m)}}$) denotes the proportion of variance explained by fixed factors alone, while the conditional R^2 ($R^2_{\text{glmm(c)}}$) denotes the proportion of variance explained by both fixed and random factors (Nakagawa & Schielzeth 2013).

RESULTS

A total of 141 individual pollinators were observed visiting the plants (Table 3.1). These individuals made a cumulative total of 1,702 visits to plants, probing 8,350 individual flowers. Overall, most of the visitors were lepidopterans. Hawkmoths (*Hemaris diffinis*, *Hyles lineata*, *Hemaris thysbe*) were the most common visitors (Table 3.1). They also probed the most plants per visit and visited more flowers per plant than most species (Table 3.1). The nymphalid *Phyciodes tharos* was also a regular visitor, probing only a few flowers during each visit. The swallowtails *Papilio polyxenes* and *Papilio glaucus* (Papilionidae) also visited, but only rarely and, when they did visit, only briefly. Several species of Hesperidae occasionally probed flowers, and brief visits from *Bombus* and other members of the Apoid bee clade Anthophila were recorded (Table 3.1), though it is unclear if they effected pollination.

Exploratory analysis showed that edge (Figure 3.S1) and spatial (Figures 3.S2, 3.S3) effects on visitation were minimal; therefore, these factors were excluded from analysis. Furthermore, there was no apparent effect of petal removal, so the two methods of decreasing petal number were pooled (data not shown). The percentage of six-petaled flowers on a plant in

the six-petaled group for a given day varied widely from 0–100%. However, the peak density or modal value in the distribution was around 48%, with few plants experiencing less than 25% or more than 75% six-petaled flowers (Figure 3.1). High proportions of four- or six-petaled flowers were nonexistent in the five-petaled group, and four-petaled flowers were rare in the six-petaled group (Figure 3.1). Data from the four-petaled group are not shown because all flowers on those plants were manipulated to be four-petaled. However, prior to manipulation, four- or six-petaled flowers were uncommon.

Plants in the five-petaled group garnered the most flowers visited per visit ($\chi^2 = 262.4$; $p < 0.001$; Figure 3.2A), and the most visits per plant ($\chi^2 = 8.2$; $p < 0.02$; Figure 3.2B), though differences were mostly small. Plants in the four- and six-petaled group experienced reductions in the number of visits per plant that were similar to one another (Figure 3.2B). However, the reduction in flowers visited per visit compared to the five-petaled group was more pronounced in the six-petaled group (Figure 3.2A). The six-petaled group showed 37% fewer flowers visited per visit than the five-petaled group, while the decline was minimal in the four-petaled group (Figure 3.2A). All three covariates (plant height, inflorescence width, and the number of flowers per plant) positively influenced both the number of visits and the number of flowers per visit, though the extent of their influence (regression slopes) varied by petal number group (Figure 3.3A-F). Plant height and inflorescence width were correlated; correlations among covariates were otherwise low and not statistically supported (Table 3.2). The number of visits per plant for the six-petaled group was more sensitive (higher slope) to changes in inflorescence width and flowers per plant than for the other groups (Figure 3.3E-F). Furthermore, plants in the six-petaled group tended to have fewer flowers and narrower inflorescences (Figure 3.4B-C). Overall, the number of flowers per plant had the largest effect on both visits and number of

flowers per visit. Despite shifts in the distribution of flowers per plant and inflorescence width between the six-petaled group and other groups, within the six-petaled group, these covariates were not noticeably affected by the percentage of six-petaled flowers (Figure 3.5).

The set of generalized linear mixed models with the number of visits a plant received each day as the response variable yielded a best model with all fixed factors included: the percentage of four-petaled flowers, percentage of six-petaled flowers, plant height, width of the inflorescence, number of flowers per plant, and three interactions: inflorescence width×number of flowers, inflorescence width×percentage of four-petaled flowers, and percentage of six-petaled flowers×number of flowers (Tables 3.3, 3.4). This best model comprised 79% of the model weight, and its fixed factors explained 57% of the variation in visits per day ($R^2_{\text{glmm(m)}}$; Table 3.3), with the random factor plant explaining an additional 29%. Combined, the whole model explained 86% of the variation ($R^2_{\text{glmm(c)}}$; Table 3.3), indicating a relatively informative model. The next best model, with the percentage of four-petaled flowers and the interaction inflorescence width×percentage of four-petaled flowers removed, comprised only 13% of the model weight (Table 3.3).

The effects of the number of flowers per plant and the inflorescence height on visits per day were highly statistically supported model terms, with less (though significant) support for the percentage of six-petaled flowers (Table 3.4). All three had a positive effect on visits per day, while inflorescence width and the percentage of four-petaled flowers had no statistically significant effect (Table 3.4), but remained in the model because they were part of an interaction.

Higher percentages of six-petaled flowers increased the number of visits per day. Visits increased by 1.7 times the standard deviation of the percentage of 6-petaled flowers ($e^{2.080 + 0.189 \cdot \text{SD}}$ - $e^{2.080}$; Table 3.4). Prior to centering, the mean and standard deviation for the percentage of six-

petaled flowers were 14.2% and 23.1%. Therefore, a plant with 48% six-petaled flowers (the modal percentage; 2.1 standard deviations above 0%), would be expected to receive 3.5 more visits per day (i.e., 10.5 visits/day) than a plant with no six-petaled flowers (7.0 visits per day), a 50% increase. By comparison, the number of flowers per plant (the strongest effect) increased visits per day by a factor of 7.5 times the standard deviation in the number of flowers per plant.

Results differed for the set of models built with the number of flowers per visit as the response variable. While the best model included the same primary fixed factors (percentage of four- and six-petaled flowers, number of flowers per plant, inflorescence width and height), there were different interaction terms (Tables 3.5, 3.6). The best model was nearly equivalent to one without the percentage of six-petaled flowers (model weight of 0.56 vs 0.38), and the models including plant as a random factor explained only 48% of the variation (Table 3.5). While the factor coefficient for the percentage of six-petaled flowers was negative, as suggested in the preliminary data (Figure 3.2A), it was small and had no statistically significant effect on the flowers per visit (Table 3.6). The percentage of four-petaled flowers also had no statistically significant effect (Table 3.6). As with visits per plant, the number of flowers per plant had the largest effect on the number of flowers visited (Table 3.5).

DISCUSSION

Although this experiment found some initial support for higher pollinator visitation rates at plants with five-petaled flowers (Figure 3.2), subsequent statistical modeling suggested that this visitation rate effect was due to the attractive potential of other plant and floral traits (Figure 3.3). When plant height, inflorescence width, and the number of flowers were accounted for, no evidence suggested that pollinators visited plants with five-petaled flowers more often or spent more time probing their flowers (Tables 3.4, 3.6). In fact, when other traits were accounted

for, the number of visits a plant received increased with its percentage of six-petaled flowers (Table 3.4), suggestive of directional, de-stabilizing selection for more petals. Therefore, the only way that stasis could be maintained here is if the preference for six petals was offset by some other trade-off favoring fewer petals. Increased visitation to taller plants with wider inflorescences and more flowers dominated pollinator preference relative to the effects of petal number (Tables 3.4, 3.6), indicating that petal number was not a primary determinant of pollinator preference in this system.

The fact that plants from the six-petaled group tended to have narrower inflorescences and fewer flowers than the other groups (Figure 3.4B-C) warrants further attention. It is suggestive of selection for a higher proportion of six-petaled flowers in these *P. drummondii* plants decreasing fitness via energetic or other pleiotropic constraints. Writing about Huether's work on *Leptosiphon androsaceus* (1968), Stebbins (1974) noted similar observations from unpublished data that were suggestive of pleiotropy (plants selected for increased petal number were "weak and slow-growing"). The existence of pleiotropic effects associated with changes in petal number could explain stasis in petal number by providing a stabilizing selective force, though pleiotropic constraints of this nature may not be strong (Beldade et al., 2002). However, in this study, none of the traits measured increased or decreased with the proportion of six-petaled flowers in the plants derived from the six-petaled selection line (Figure 3.5). Therefore, to the extent that the variance in the percentage of six-petaled flowers observed in the plants in this study is genetically determined (Mickley 2017, Chapter 1), this would seem to rule out pleiotropy. One plausible alternative is that the reductions in number of flowers and inflorescence width seen in the six-petaled group were due to inbreeding. *Phlox drummondii* is known to suffer from inbreeding depression (Levin 1989). During the selection experiment,

plants were self-pollinated (Mickley 2017, Chapter 1). Thus, inbreeding could result in decreased fitness in the six-petaled group without pleiotropic effects due to selection, and without explaining petal number stasis.

This study was not conducted in the native range of *P. drummondii*, or with its native pollinator assemblage. Nevertheless, its primary native pollinators are also members of the same families (Papilionidae, Hesperidae, and Sphingidae), as were seven of the top eight visitors in this experiment (Table 3.1), and *Hyles lineata*, a visitor in this study, is also a common pollinator of *P. drummondii* in its native range (Erbe & Turner 1962; Grant & Grant 1965). These pollinators share similar visual systems and are likely to have similar preferences, especially because none are specialist pollinators on *P. drummondii*. It is therefore unlikely that drastically different preferences for petal number exist in pollinators from the native range. One possibility though, is that without *P. drummondii* present in the surrounding landscape, pollinators had not developed a search image for normal five-petaled *P. drummondii* flowers, leading to a lack of discrimination. While pollinator search images for normal flowers resulting in a preference has been proposed as an explanation for constancy in petal number within a species (Herrera 2009), it is not a convincing explanation for supra-specific evolutionary stasis: different species could fix on different petal numbers.

Conclusions

No evidence was found for the hypothesis that pollinator-mediated stabilizing selection favors five-petaled flowers over other petal numbers, acting as the mechanism for maintaining stasis for five-petaled flowers (Leppik 1953; Leppik 1956; Stebbins 1974). Preferences for petal number based on visitation rates were masked by confounding traits (such as inflorescence characteristics, and overall flower number per plant), for which pollinators had a higher

preference. These results are in agreement with prior work comparing levels of variation between outcrossed and autogamous congeners to determine if autogamous species experienced relaxed stabilizing selection (Mickley 2017, Chapter 2). Furthermore, these results call into question previous published results indicating insect preferences for particular petal numbers, or lack thereof (Leppik 1953; Stebbins 1974). These authors simply looked for numerical preferences, without considering other attractive traits in a multivariate context, a still-persistent problem in pollinator visitation studies (Conner & Rush 1996). As the results of this study show, an apparent preference for a certain petal number can arise as a byproduct of preferences for other traits, if those traits vary non-randomly among plants. Pollinators make decisions on which plants or flowers to visit based on a whole suite of traits, not just one (Willmer 2011). Therefore, when conducting experiments measuring pollinator preference for a given trait of interest, it is critical to measure or control for variation in other traits that might affect visitation (Conner & Rush 1996).

Several avenues merit further research in explaining petal number stasis. 1) A more thorough examination of pleiotropic effects of changing petal number in either direction is needed. 2) That visits per plant increased with the percentage of six-petaled flowers suggests directional selection, but this needs to be measured in a fitness context, and a concomitant tradeoff from some other selective force against increasing petal number would be required to enforce stasis. 3) Since much of the prior research on pollinator discriminatory capacity to petal number was conducted with bees (Leppik 1953; Lehrer et al., 1995), it would be informative to conduct controlled experiments in a primarily bee-pollinated species. Relative to lepidopterans and other pollinators, bees have especially well-developed visual systems (Giurfa & Lehrer 2004; Weiss 2004; Willmer 2011), and may be more sensitive to differences in petal number. It

remains possible that some pollinator taxa have preferences for petal number while others do not. These other research avenues to explain petal number stasis may prove more fruitful than extensive work testing for pollinator-mediated stabilizing selection.

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TABLES

Table 3.1: A list of pollinators that visited *P. drummondii* identified to the lowest taxonomic rank possible, along with their higher taxon, number of plot visits, total plant visits, and number of flowers visited per plant. Only 33 visits were from non-lepidopteran pollinators.

Species/Taxon	Higher Taxon	Plot Visits	Plant Visits	Flowers Visited/Plant
<i>Hemaris diffinis</i>	Lepidoptera	38	1452	5.2
<i>Phyciodes tharos</i>	Lepidoptera	37	85	1.7
Hesperiidae spp.	Lepidoptera	24	31	2.1
<i>Papilio glaucus</i>	Lepidoptera	5	28	5.0
<i>Hyles lineata</i>	Lepidoptera	4	27	4.0
<i>Hemaris thysbe</i>	Lepidoptera	2	16	8.7
<i>Bombus impatiens</i>	Anthophila	7	15	1.6
<i>Papilio polyxenes</i>	Lepidoptera	3	10	7.8
Papilionoidea spp.	Lepidoptera	5	10	1.2
<i>Archilochus colubris</i>	Aves	4	8	1.6
<i>Polites peckius</i>	Lepidoptera	2	7	1.1
<i>Apis mellifera</i>	Anthophila	3	5	3.2
Anthophila sp.	Anthophila	4	4	2.8
Lepidoptera spp.	Lepidoptera	1	2	1.5
Diptera sp.	Diptera	1	1	1.0
Pieridae sp.	Lepidoptera	1	1	1.0
Total		141	1702	4.9

Table 3.2: Pearson correlations between floral traits used as covariates.
Significance code: *** 0.001.

	Inflorescence Width	Plant Height
Plant Height	0.10	
Number of Flowers	0.63 ***	0.11

Table 3.3: The best set of models with visits per plant per day as the response variable. All five of these models received some model weight, though the best two accounted for most of the weight. The best model contained all fixed factors and several interactions, with fixed factors explaining 57% of the variance. The combination of fixed factors and plant as a random factor explained 86% of the variance.

Model terms and reported values: height = plant height; width = inflorescence width; flowers = number of flowers per plant; pct4pet = percentage of four-petaled flowers; pct6pet = percentage of six-petaled flowers; Model = the fixed factor terms included in the model (all include plant as a random factor); AICc = AICc score; Δ AICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(m)}}$ = marginal R^2 (fixed factors); $R^2_{\text{glmm(c)}}$ = conditional R^2 (fixed + random factors).

Model	AICc	Δ AICc	df	weight	$R^2_{\text{glmm(m)}}$	$R^2_{\text{glmm(c)}}$
pct4pet + pct6pet + height + width + flowers + width×flowers + pct6pet×flowers + pct4pet×width	1305.49	0.00	10	0.79	0.574	0.858
- pct4pet - pct4pet×width	1309.17	3.68	8	0.13	0.579	0.861
- pct4pet×width	1311.13	5.64	9	0.05	0.581	0.861
- width×flowers	1312.46	6.98	9	0.02	0.554	0.843
- pct6pet - pct6pet×flowers	1314.516	9.03	8	0.01	0.561	0.872

Table 3.4: The fixed factor coefficients of the best model in Table 3.3. Number of flowers per plant had the largest effect, followed by plant height. The percentage of six-petaled flowers had a positive effect on the number of visits per day.

Model terms: height = plant height; width = inflorescence width; flowers = number of flowers per plant; pct4pet = percentage of four-petaled flowers; pct6pet = percentage of six-petaled flowers.

Significance codes: *** 0.001, ** 0.01, * 0.05.

Factor	Estimate	Std. Error	z-value	Pr(> z)	
Intercept	2.080	0.081	25.53	< 0.001	***
pct4pet	-0.051	0.084	-0.61	0.544	
pct6pet	0.189	0.084	2.26	0.024	*
height	0.349	0.051	6.80	< 0.001	***
width	0.094	0.079	1.19	0.236	
flowers	0.661	0.070	9.49	< 0.001	***
width×flowers	-0.107	0.036	-3.00	0.003	**
pct6pet×flowers	0.190	0.056	3.41	< 0.001	***
pct4pet×width	0.200	0.072	2.78	0.006	**

Table 3.5: The best set of models with flowers per visit as the response variable. As in the visit models (Table 3.3), the best model contained all fixed factors and several interactions. However, fixed factors only explained 39% of the variance and the combination of the fixed factors and plant as a random factor explained 48%. A model without the percentage of six-petaled flowers received nearly as much model weight as the best model.

Model terms and reported values: height = plant height; width = inflorescence width; flowers = number of flowers per plant; pct4pet = percentage of four-petaled flowers; pct6pet = percentage of six-petaled flowers; Model = the fixed factor terms included in the model (all include plant as a random factor); AICc = AICc score; Δ AICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(m)}}$ = marginal R^2 (fixed factors); $R^2_{\text{glmm(c)}}$ = conditional R^2 (fixed + random factors).

Model	AICc	Δ AICc	df	weight	$R^2_{\text{glmm(m)}}$	$R^2_{\text{glmm(c)}}$
pct4pet + pct6pet + height + width + flowers + pct4pet×flowers + height×flowers	10478.22	0.00	9	0.555	0.387	0.476
- pct6pet	10479.19	0.97	8	0.342	0.382	0.481
- height×flowers	10482.54	4.32	8	0.064	0.378	0.478
- pct6pet - height×flowers	10483.57	5.35	7	0.038	0.372	0.481

Table 3.6: The fixed factor coefficients of the best flowers per visit model in Table 3.5. Once again, the number of flowers per plant (flowers) had the largest effect (Table 3.4), followed by inflorescence width (width). Inflorescence height (height) also positively influenced the number of flowers visited per visit, but to a smaller extent. The percentage of four- and six-petaled flowers had no statistically significant effects.

Model terms: height = plant height; width = inflorescence width; flowers = number of flowers per plant; pct4pet = percentage of four-petaled flowers; pct6pet = percentage of six-petaled flowers.

Significance codes: *** 0.001, * 0.05.

Factor	Estimate	Std. Error	z-value	Pr(> z)
pct4pet	0.049	0.033	1.48	0.139
pct6pet	-0.060	0.033	-1.77	0.077
height	0.099	0.021	4.73	< 0.001 ***
width	0.193	0.035	5.47	< 0.001 ***
flowers	0.210	0.027	7.66	< 0.001 ***
pct4pet×flowers	0.091	0.022	4.08	< 0.001 ***
height×flowers	-0.055	0.022	-2.55	0.011 *

FIGURES

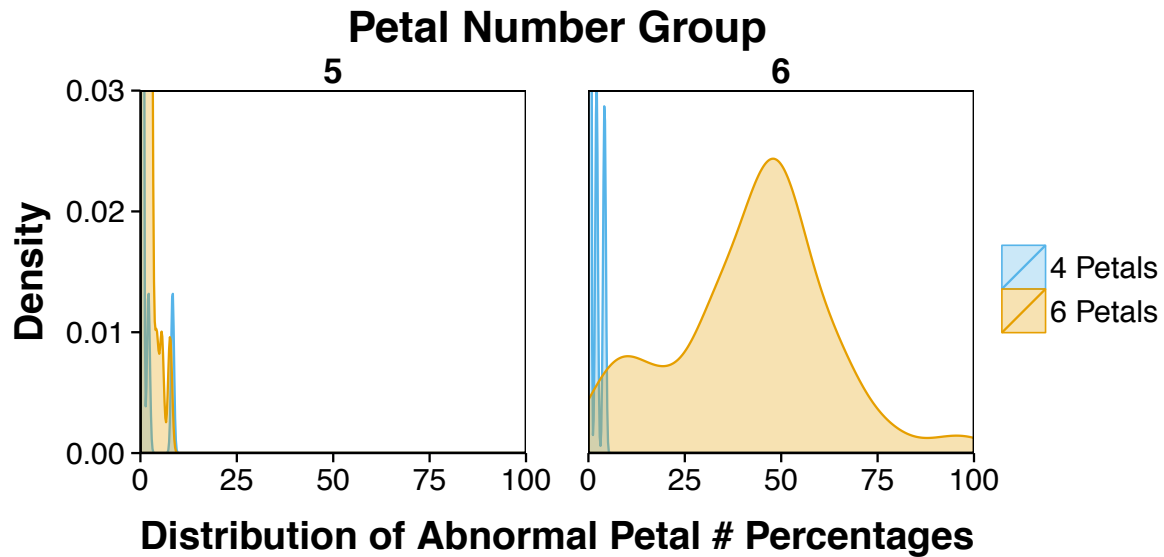


Figure 3.1: Density plot of the distribution of four- and six-petaled flower percentages in the five-petaled and six-petaled groups. High percentages of abnormal flowers are nonexistent in the five-petaled group, and likewise with four-petaled flowers in the six-petaled group. The percentage of six-petaled flowers in the six-petaled group was much higher, with a modal value of ~48%. Because all flowers on plants from the four-petaled group were manipulated, the distribution of original percentages of four- and six-petaled flowers is not shown.

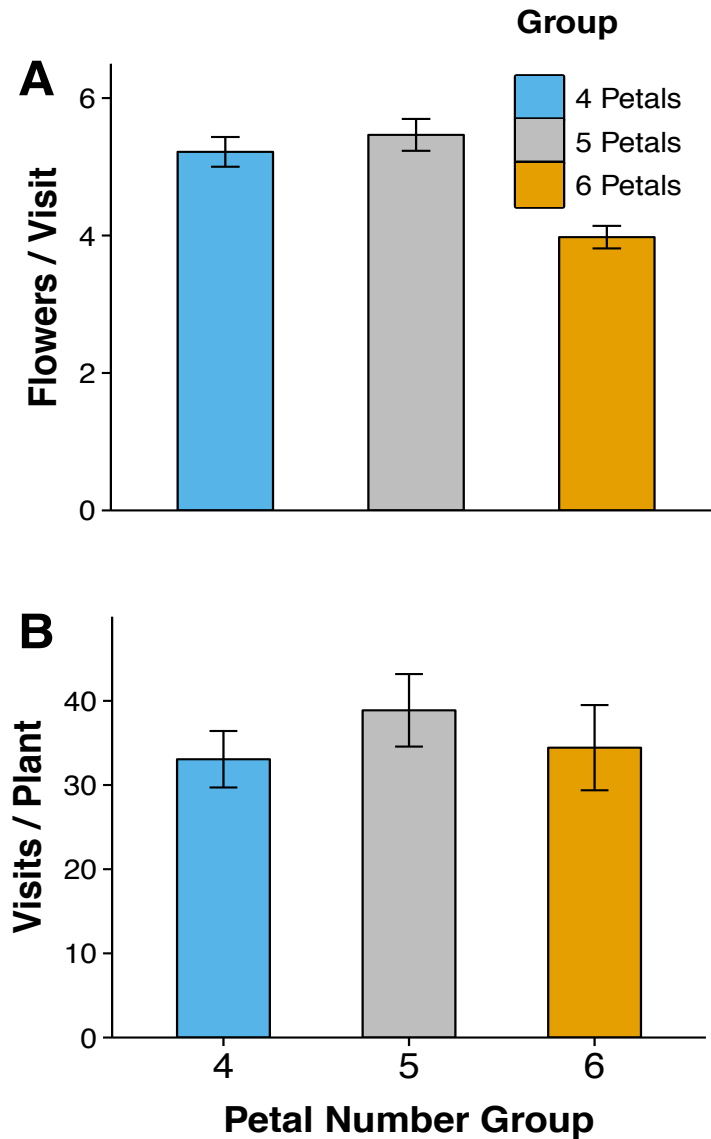


Figure 3.2: The number of flowers visited per visit (A), and the number of visits per plant (B) in each petal number group. In both cases, the five-petaled group received significantly more pollinator attention. Pollinators visited ~1.5 fewer flowers per visit in the six-petaled group (A). While both the four- and six-petaled group received fewer visits per plant (B), the differences were slight.

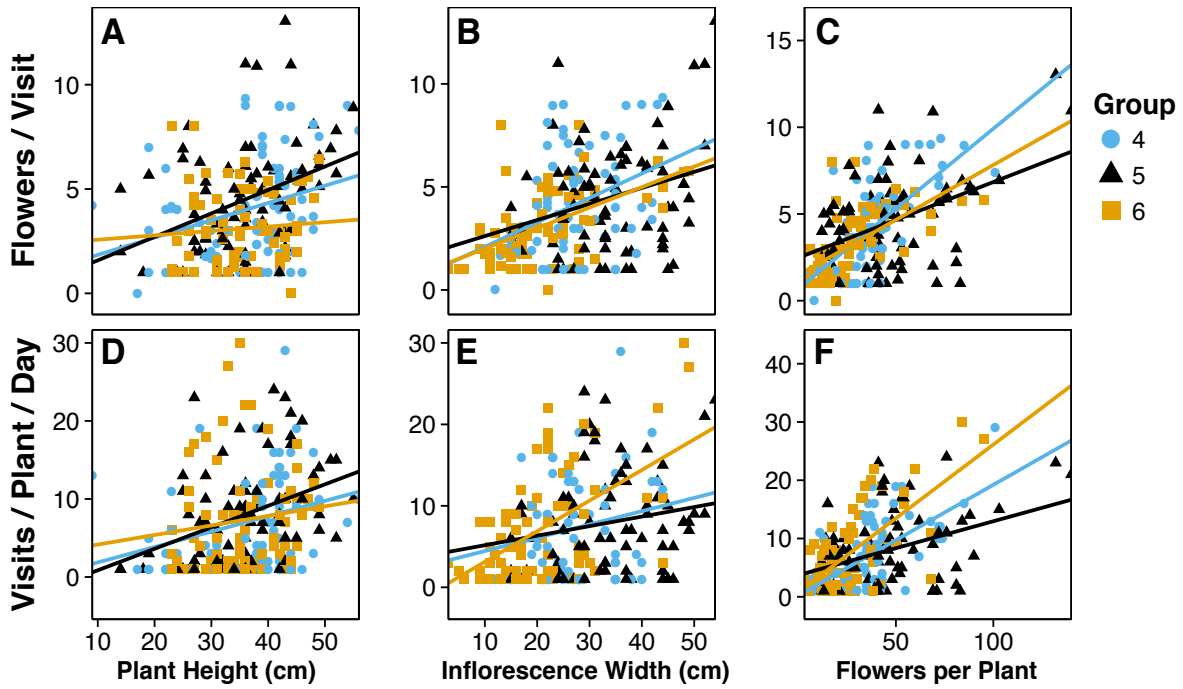


Figure 3.3: Effects of covariates on flowers visited per visit (A-C), and visits per plant per day (D-F). All three covariates had positive effects on pollinator visitation, though the extent varied by covariate and also among petal number groups, suggesting interactions. Total flowers per plant had the strongest effect on both visits (F), and the number of flowers visited (C).

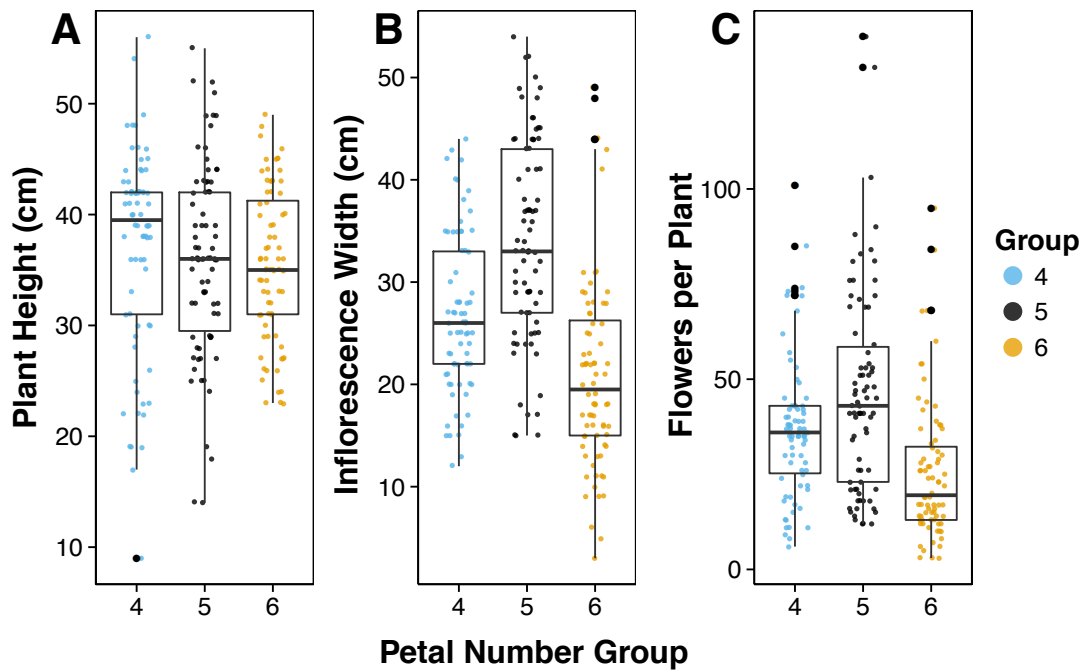


Figure 3.4: Distribution of covariates for each petal number group (A-C). The distribution of inflorescence width (B) and number of flowers per plant (C) varied among petal number groups, with minimal changes in plant height among groups (A). In particular, the six-petaled group had fewer flowers and smaller inflorescences (C).

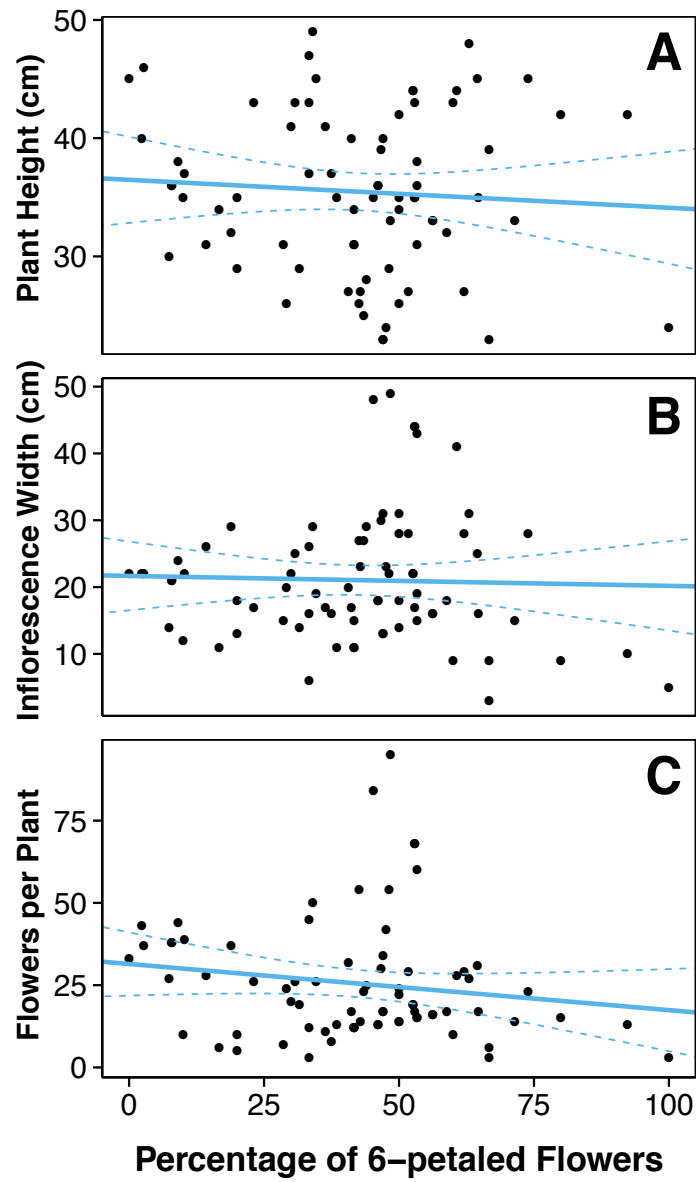


Figure 3.5: The effect of six-petaled flower percentage on covariates within the six-petaled group (A-C). Higher percentages of six-petaled flowers had no effect on any of the three covariates.

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CHAPTER 3 SUPPLEMENT

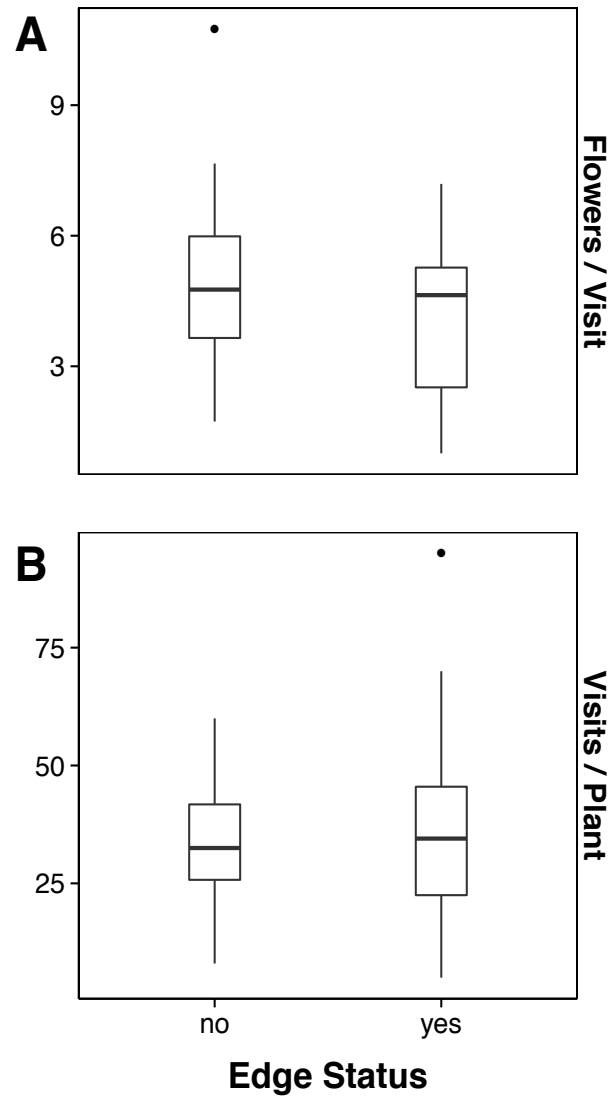


Figure 3.S1: Comparison of flowers per visit and visits per plant between edge and non-edge plants. There was no evidence of edge effects.

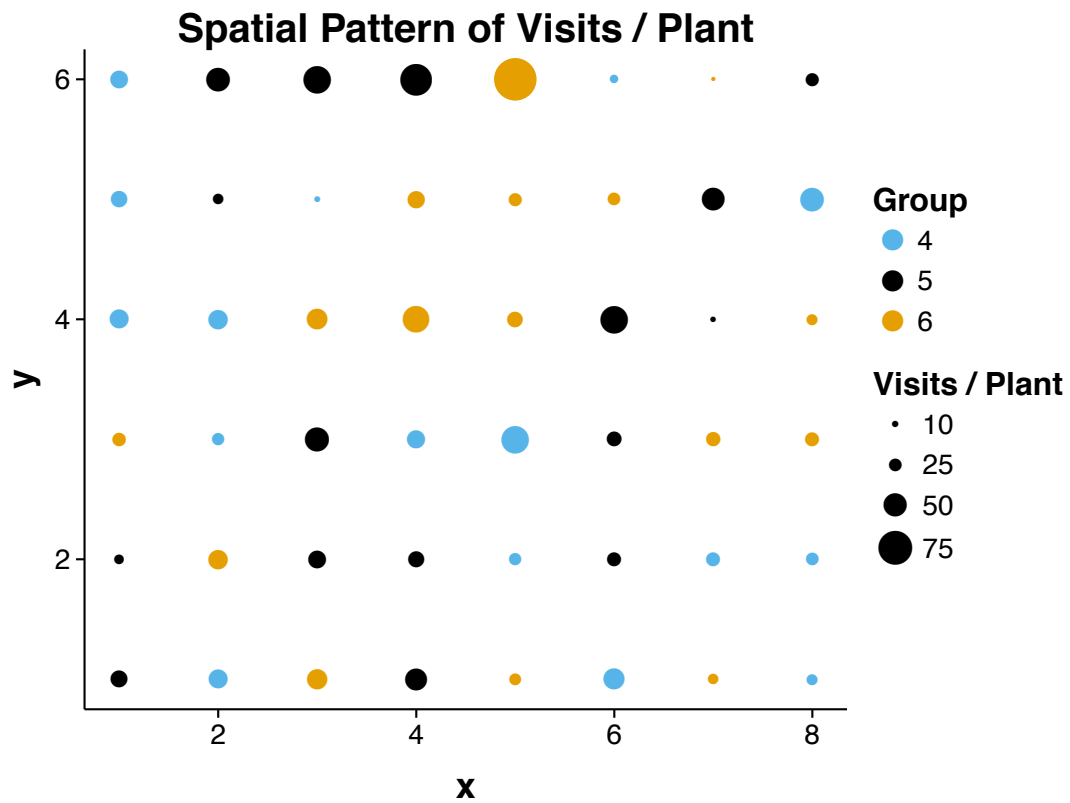


Figure 3.S2: Spatial pattern of visits per plant within the experimental plot. Here, the size of the dots is dictated by the number of visits and colored by the petal number group. While some plants received many more visits, there were no clear spatial patterns.

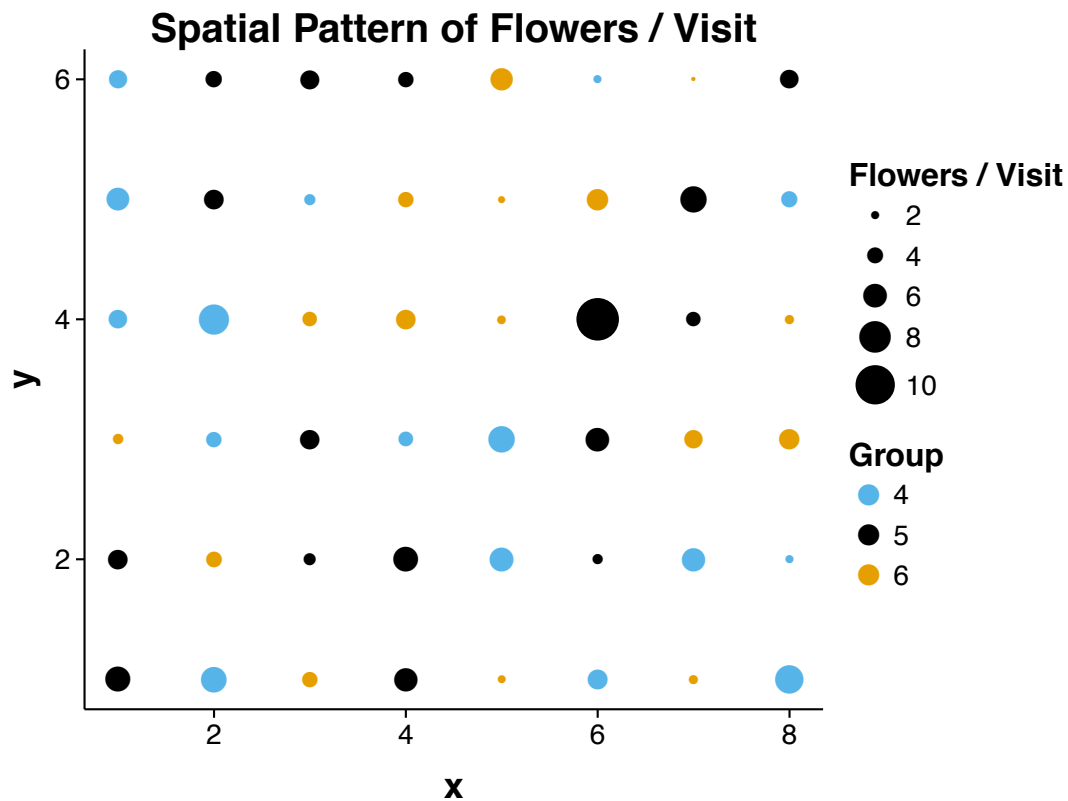


Figure 3.S3: Spatial pattern of flowers visited per visit within the experimental plot. Here, the size of the dots is dictated by the number of flowers visited per visit and colored by the petal number group. While more flowers were visited per visit on some plants, there were no clear spatial patterns.

APPENDIX:
**Differences in Petal Number Variation Among Populations of *Phlox drummondii* in a
Common Garden**

INTRODUCTION

While natural variation in petal number within a species has been documented in several groups (Stark 1918; Lowndes 1931; Roy 1963; Huether 1969; Ellstrand 1983; Lehmann 1987; Ellstrand & Mitchell 1988; Byerley 2006; Mickley 2017, Chapter 2), few studies have compared the amount and direction of this variation among related species or among populations within a species. Such differences in the amount and direction of variation are interesting, as they provide clues as to whether petal number is canalized to the same extent across species, or subject to local adaptation, genetic drift, or environmentally determined. If any of the latter are at play, exploring them might lead to advances in understanding of the underlying genetic architecture or selection on petal number, particularly if differences in variation were biased in one direction toward fewer or more petals.

Species within the Polemoniaceae that are known to express natural variation in petal number vary from 1% abnormally petaled flowers within *Phlox drummondii* (Lehmann 1987) to 16% within *P. longifolia* (Byerley 2006), with other studies reporting values in between those extremes (Huether 1969; Ellstrand 1983; Ellstrand & Mitchell 1988). Differences in intraspecific variation in floral organ number among related species have not been characterized outside the Polemoniaceae.

Surveying populations of *Ipomopsis aggregata* for variation in all four floral parts, Ellstrand and Mitchell (1988) found considerable differences among populations: from 2% to 33% of the flowers sampled deviated from the usual number for at least one organ. Furthermore,

the direction of variation in petal number varied by site (Ellstrand & Mitchell 1988). Huether (1969) found statistically significant differences in petal number variation among populations of *Leptosiphon androsaceus*, though the magnitude of differences was less extreme than those found by Ellstrand & Mitchell (1988), and differences among plants were not controlled for. In contrast, Byerley (2006) found no differences in the amount of petal number variation among five populations of *Phlox longifolia*. Comparing levels of petal number variation in various *P. drummondii* cultivars to a natural population, Lehmann (1987) found between 1.3% and 27% of flowers with an abnormal petal number, with a natural population and an escaped cultivar having the lowest levels of petal number variation. Most of the variation among populations was in the proportion of flowers with six petals as opposed to four petals (Lehmann 1987). Lehmann posited that cultivation led to decanalization of floral organ number (1987), which would imply selection for canalization in natural populations, especially given the low levels of variation in the escaped cultivar. The results from Chapter 2 of this dissertation on petal number variation in *Saltugilia*, *Gilia*, and *Phlox* further show that differences in the amount and direction of variation exist among species (Figures 2.1, 2.2, Table 2.S1). Furthermore, the extent and direction of this variation differed across populations for some species (Figure A1). Within *S. latimeri* there were three-fold differences in the percentage of six-petaled flowers among populations (Figure A1). Two populations of *G. cana* had high levels of six-petaled flowers, while another had almost none (Sm. Quarry, Figure A1).

Huether (1968) found that under conditions of environmental stress (temperature and photoperiod), there was an increase in petal number variation in *L. androsaceus* and suggested that the environment played a significant role in petal number variation, along with genetic differences (Huether 1968). However, C. Schlichting (unpublished) found no such trend in *P.*

drummondii under stressful conditions. Despite Schlichting's finding, *P. drummondii* grown in the greenhouse prior to selection in Chapter 1 of this dissertation had more than six times the percentage of six-petaled flowers found in natural populations of *P. drummondii* in the field (Figure A2).

Therefore, though there is not yet substantial evidence, the bulk of it points to differences in the amount and direction of petal number variation among species and possibly among populations, and at least some of the variation is likely to be environmentally determined. The question then is to what extent these differences among species and populations are due to phenotypic plasticity in response to environment versus variation in genetic factors such as selection and drift. One way to test this question is to compare levels of petal number variation among wild populations of a species to levels in those same populations when grown in a common environment, something that no study on petal number has yet done.

The natural populations studied in Chapter 2 provide the wild populations for this test. The experiments in Chapter 1 provided experience with growing *Phlox* in the greenhouse; therefore, this common garden study focused on the two species of *Phlox* sampled in natural populations: *P. drummondii* and *P. cuspidata*. Growing these *Phlox* species in a common greenhouse environment and comparing to their original field populations, I ask the following questions: 1) Are population-level differences in petal number variation maintained in a common greenhouse environment, implying a genetic basis? 2) Do patterns of petal number variation differ between the greenhouse and the field, implying an environmental basis?

METHODS

Phlox drummondii and *P. cuspidata*, which are normally five-petaled, have already been shown to display natural variation in petal number (Lehmann 1987; Byerley 2006; Mickley

2017, Chapter 2). Seeds from *P. cuspidata* (three populations) and *P. drummondii* (two populations) were collected in May 2014 from a subset of the natural populations of *Phlox* detailed in Chapter 2. Following the methods in Chapter 1, seeds were planted in seedling flats in the greenhouse at the University of Connecticut. Seed germination was poor, especially for *P. cuspidata*, though it improved when seedling flats were moved into a cooler room in the greenhouse ($< 25^{\circ}\text{C}$). However, only 19 plants from 13 maternal lines represented the three populations of *P. cuspidata* compared to 51 plants from 21 maternal lines for the two populations of *P. drummondii* (Table A1). The *P. cuspidata* population C448 was particularly poorly represented with only three plants from two maternal lines (Table A1). The number of petals was counted on 7204 flowers in the greenhouse and compared to 3713 flowers counted in the field (Table A1). The proportions of abnormal petal numbers were compared to the field data from the same five populations collected in Chapter 2.

Analyses were done using R (R Core Team 2015), with the dplyr, tidyr, cowplot, and ggplot2 packages (Wickham & Francois 2015; Wickham 2015; Wilke 2015; Wickham 2009). Statistical modeling followed the methods from Chapter 1 and Chapter 3, using binomial logistic mixed models with the lme4, bbmle, and piecewiseSEM packages (Bates et al., 2015; Bolker & R Development Core Team 2014; Lefcheck 2016), with the proportion of abnormal flowers (abnormal model), four-petaled flowers (four-petaled model), and six-petaled flowers (six-petaled model) as response variables for different sets of models. Model sets were built with plant as a random factor, and the full model included environment, population, and their $G \times E$ interaction as fixed factors. Model sets were run separately for each of the two species. For each set of models, all combinations of fixed factors were compared to a model with only the random factor plant, and a null model. As in previous chapters, marginal and conditional R^2 were

calculated for all models. The marginal R^2 ($R^2_{\text{glmm(m)}}$) shows the proportion of variance explained by fixed factors (environment, population, and the G×E interaction), while the conditional R^2 ($R^2_{\text{glmm(c)}}$) shows the proportion of variance explained by both fixed and random factors (environment, population, G×E interaction, and plant) (Nakagawa & Schielzeth 2013). The difference between the two measures of R^2 is the contribution of the random factor plant to the proportion of variance explained by the model.

RESULTS

The two populations of *P. drummondii* that were grown in the greenhouse had very different profiles of four- and six-petaled flowers in the field. The D290 population had the highest percentage of six-petaled flowers and the lowest percentage of four-petaled flowers in the field of any *P. drummondii* population (Figure A1). The D95 population had no six-petaled flowers at all in the field (Figure A1). The results in the field were not mirrored in the greenhouse. Instead of no six-petaled flowers, the D95 population had nearly twice as many as the D290 population (Figure A3). Furthermore, the D290 population had more four-petaled flowers than the D95 population (Figure A3). Thus, there appeared to be differences between environments and between populations.

For *P. cuspidata*, the three populations grown in the greenhouse all had similar percentages of four- and six-petaled flowers in the field (Figure A3). The C448 population had a low sample size in the greenhouse, but in that small sample there were no six-petaled flowers. The other two populations (C214 and C3011) had more four-petaled flowers and substantially more six-petaled flowers in the greenhouse than in the field (Figure A3). Differences between environments appeared to be larger than differences among populations.

The abnormal and four-petaled models comparing *P. drummondii* populations between the field and the greenhouse common garden showed that variation in petal number was mostly attributed to differences among plants. These differences accounted for 31% of the variation in the abnormal model and 41% of the variation in the four-petaled model ($R^2_{\text{glmm(c)}}$, Tables A2, A3). Environment, population, and the G×E interaction accounted for very little of the variation: 3–6% in the models including all three terms ($R^2_{\text{glmm(m)}}$, Tables A2, A3).

In contrast, the best six-petaled model for *P. drummondii* was the full model, including population, environment, and the G×E interaction, as well as plant (Table A4). This model explained 96% of the variation ($R^2_{\text{glmm(c)}}$, Table A4), nearly all of which was attributable to population, environment, and the G×E interaction ($R^2_{\text{glmm(m)}}$, Table A4). Despite strong support for the full model, none of the factors were statistically significant (Table A5). Parameter estimates were large for both population and the G×E interaction (Table A5), explaining the D95 population having no six-petaled flowers in the field and the highest level of six-petaled flowers in the greenhouse. However, the standard errors on these estimates were nearly as large (Table A5); sample size was not sufficient to show any effect.

As might be expected from qualitative results (Figure A3), none of the *P. cuspidata* models showed strong evidence of population differentiation in either environment (Tables A6–A9). The abnormal model showed a modest effect of environment (Table A6; Table A7), driven by increases in both four- and six-petaled flowers in the greenhouse in the C214 and C3011 populations (Figure A3). For this model, environment explained 11% of the variation ($R^2_{\text{glmm(m)}}$, Table A6), though this was still small relative to what the combination of environment and plant explained ($R^2_{\text{glmm(c)}}$, Table A6). The four-petaled model for *P. cuspidata* was best explained by the null model, and none of the factors, including plant, explained any appreciable amount of

variation (Table A8). The six-petaled model for *P. cuspidata* was best explained by differences among plants (Table A9).

DISCUSSION

Only the abnormal model for *P. cuspidata* was able to show conclusive evidence for effects of environment, population, or a G×E interaction on petal number (Tables A6, A7). Despite a lack of statistical support for environmental or population effects in the four-petaled and six-petaled models for *P. drummondii*, there were some apparent differences between the field and greenhouse environment and among populations. The lack of statistical support for fixed factors in the six-petaled *P. drummondii* model (Tables A4, A5), and the lack of resolution in AICc scores between many of the models, show that sample size was too low to make any strong conclusions. Therefore, effects of environment and population are difficult to rule out.

However, some tentative conclusions can still be made. First, this experiment showed no evidence for differences in the percentage of four-petaled flowers due to either population or environment. Variation in four-petaled flowers seemed to be more random, and even differences among plants were not apparent in *P. cuspidata* (Table A8).

Second, the percentage of six-petaled flowers increased in the greenhouse environment for three of the five populations (exceptions D290 and C448: Figure A3). Furthermore, given the small sample size in the C448 population (Table A1), the lack of six-petaled flowers may be spurious. These results suggest that environment influences at least the proportion of six-petaled flowers in both species, though a larger sample size and perhaps more populations would be needed to show this. In addition to environmental effects on six-petaled flowers, the results are also suggestive of population differentiation in six-petaled flowers in *P. drummondii*, but not in *P. cuspidata*.

Finally, the differences among individual plants were the primary contributors to variance in the four-petaled *P. drummondii* models, six-petaled *P. cuspidata* models, and both sets of abnormal models. More work is needed to determine which environmental parameters influence petal number and whether petal number is influenced only at extremes of environmental stress where canalization breaks down, or at levels that are routinely encountered by plants in their natural environment. It is also not known whether this environmentally determined variation represents adaptive plasticity, maladaptive breakdowns of developmental buffering, or is simply of no consequence.

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TABLES

Table A1: The three *Phlox cuspidata* and two *P. drummondii* populations grown in the common greenhouse environment. Sample sizes for the number of plants and flowers sampled are presented for both the field and the greenhouse, as well as the number of maternal lines represented in the greenhouse plants.

Population	Field		Greenhouse		
	Plants	Flowers	Plants	Flowers	Lines
<i>P. cuspidata</i>					
C214	392	776	9	1129	7
C3011	350	714	7	459	4
C448	274	710	3	231	2
<i>P. drummondii</i>					
D290	136	759	28	2848	10
D95	243	754	23	2537	11

Table A2: Table of models for *P. drummondii* with the proportion of abnormally-petaled flowers as the response variable. Despite the full model having the lowest AICc, the best model is the one with only plant, and no environment or population terms. The ΔAICc differs by only 0.86 between the two models and the plant model has fewer degrees of freedom.

Model terms and reported values: environ = greenhouse or field environment; pop = original field population; (1|plant) = identity of the plant included as a random factor; Model = the fixed and random terms included in the model; AICc = AICc score; ΔAICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(m)}}$ = marginal R^2 (fixed factors); $R^2_{\text{glmm(c)}}$ = conditional R^2 (fixed + random factors).

Model	AICc	ΔAICc	df	weight	$R^2_{\text{glmm(m)}}$	$R^2_{\text{glmm(c)}}$
environ + pop + environ×pop + (1 plant)	1181.65	0.00	5	0.28	0.032	0.332
environ + (1 plant)	1181.73	0.08	3	0.27	0.012	0.343
(1 plant)	1182.51	0.86	2	0.18	0.000	0.313
environ + pop + (1 plant)	1182.75	1.10	4	0.16	0.019	0.359
pop + (1 plant)	1183.54	1.89	3	0.11	0.006	0.328
null model	1260.37	78.72	1	0.00	0.000	0.000

Table A3: Table of models for *P. drummondii* with the proportion of four-petaled flowers as the response variable. The best model is the one with only plant, and no environment or population terms.

Model terms and reported values: environ = greenhouse or field environment; pop = original field population; (1|plant) = identity of the plant included as a random factor; Model = the fixed and random terms included in the model; AICc = AICc score; ΔAICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(m)}}$ = marginal R^2 (fixed factors); $R^2_{\text{glmm(c)}}$ = conditional R^2 (fixed + random factors).

Model	AICc	ΔAICc	df	weight	$R^2_{\text{glmm(m)}}$	$R^2_{\text{glmm(c)}}$
(1 plant)	394.88	0.00	2	0.40	0.000	0.411
environ + (1 plant)	396.54	1.66	3	0.17	0.003	0.416
pop + (1 plant)	396.68	1.80	3	0.16	0.003	0.382
environ + pop + environ×pop + (1 plant)	396.81	1.93	5	0.15	0.059	0.333
environ + pop + (1 plant)	398.38	3.50	4	0.07	0.005	0.382
null model	398.97	4.09	1	0.05	0.000	0.000

Table A4: Table of models for *P. drummondii* with the proportion of six-petaled flowers as the response variable. The best model is the full model with plant, population, environment and the G×E interaction.

Model terms and reported values: environ = greenhouse or field environment; pop = original field population; (1|plant) = identity of the plant included as a random factor; Model = the fixed and random terms included in the model; AICc = AICc score; Δ AICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(m)}}$ = marginal R^2 (fixed factors); $R^2_{\text{glmm(c)}}$ = conditional R^2 (fixed + random factors).

Model	AICc	Δ AICc	df	weight	$R^2_{\text{glmm(m)}}$	$R^2_{\text{glmm(c)}}$
environ + pop + environ×pop + (1 plant)	901.86	0.00	5	0.96	0.936	0.963
environ + (1 plant)	910.81	8.95	3	0.01	0.013	0.543
(1 plant)	911.00	9.14	2	0.01	0.000	0.516
environ + pop + (1 plant)	911.28	9.42	4	0.01	0.029	0.563
pop + (1 plant)	911.64	9.78	3	0.01	0.011	0.533
null model	1004.86	103.00	1	0.00	0.000	0.000

Table A5: The fixed factor coefficients of the full *P. drummondii* six-petaled model in Table A4. Despite strong support for the full model, none of the fixed factors were statistically significant predictors. The reference factor levels for the intercept were population D290 in the field environment.

Model terms: environ = greenhouse or field environment; pop = original field population. Significance code: *** 0.001.

Factor	Estimate	Std. Error	z-value	Pr(> z)	
(Intercept)	-5.10	0.50	-10.29	< 0.001	***
environ	-0.49	0.57	-0.87	0.383	
pop	-29.55	25.93	-1.14	0.254	
environ×pop	30.21	25.93	1.17	0.244	

Table A6: Table of models for *P. cuspidata* with the proportion of abnormally-petaled flowers as the response variable. The best model is one containing environment and plant.

Model terms and reported values: environ = greenhouse or field environment; pop = original field population; (1|plant) = identity of the plant included as a random factor; Model = the fixed and random terms included in the model; AICc = AICc score; Δ AICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(m)}}$ = marginal R^2 (fixed factors); $R^2_{\text{glmm(c)}}$ = conditional R^2 (fixed + random factors).

Model	AICc	Δ AICc	df	weight	$R^2_{\text{glmm(m)}}$	$R^2_{\text{glmm(c)}}$
environ + (1 plant)	762.95	0.00	3	0.83	0.105	0.891
environ + pop + (1 plant)	766.77	3.82	5	0.12	0.110	0.889
(1 plant)	768.87	5.92	2	0.04	0.000	0.896
pop + (1 plant)	772.45	9.50	4	0.01	0.001	0.903
environ + pop + environ \times pop + (1 plant)	778.73	15.78	7	0.00	0.085	0.244
null model	825.73	62.78	1	0.00	0.000	0.000

Table A7: The fixed factor coefficients of the best *P. cuspidata* abnormal model in Table A6. Environment is a statistically significant model predictor, with the greenhouse increasing the proportion of abnormally-petaled flowers. The reference factor level for the intercept was the field environment.

Model term: environ = greenhouse or field environment.

Significance codes: *** 0.001, ** 0.01.

Factor	Estimate	Std. Error	z-value	Pr(> z)	
(Intercept)	-8.25	0.00	-6493	< 0.001	***
environ	3.58	1.19	3	0.003	**

Table A8: Table of models for *P. cuspidata* with the proportion of four-petaled flowers as the response variable. The best model is the null model, with no random or fixed factors explaining much of the variation.

Model terms and reported values: environ = greenhouse or field environment; pop = original field population; (1|plant) = identity of the plant included as a random factor; Model = the fixed and random terms included in the model; AICc = AICc score; Δ AICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(m)}}$ = marginal R^2 (fixed factors); $R^2_{\text{glmm(c)}}$ = conditional R^2 (fixed + random factors).

Model	AICc	Δ AICc	df	weight	$R^2_{\text{glmm(m)}}$	$R^2_{\text{glmm(c)}}$
environ + (1 plant)	384.09	0.00	3	0.41	0.040	0.065
null model	384.68	0.59	1	0.30	0.000	0.000
(1 plant)	386.15	2.06	2	0.15	0.000	0.080
environ + pop + (1 plant)	387.42	3.33	5	0.08	0.050	0.050
pop + (1 plant)	388.97	4.88	4	0.03	0.014	0.074
environ + pop + environ×pop + (1 plant)	389.35	5.26	7	0.03	0.063	0.063

Table A9: Table of models for *P. cuspidata* with the proportion of six-petaled flowers as the response variable. The best model is the one with only plant, and no environment or population terms.

Model terms and reported values: environ = greenhouse or field environment; pop = original field population; (1|plant) = identity of the plant included as a random factor; Model = the fixed and random terms included in the model; AICc = AICc score; Δ AICc = change in AICc relative to the best model; df = degrees of freedom; weight = model weight; $R^2_{\text{glmm(m)}}$ = marginal R^2 (fixed factors); $R^2_{\text{glmm(c)}}$ = conditional R^2 (fixed + random factors).

Model	AICc	Δ AICc	df	weight	$R^2_{\text{glmm(m)}}$	$R^2_{\text{glmm(c)}}$
(1 plant)	458.01	0.00	2	0.57	0.000	0.936
environ + (1 plant)	459.34	1.33	3	0.29	0.008	0.932
pop + (1 plant)	461.75	3.74	4	0.09	0.002	0.935
environ + pop + (1 plant)	463.09	5.08	5	0.04	0.011	0.930
environ + pop + environ×pop + (1 plant)	465.78	7.77	7	0.01	0.993	0.999
null model	557.47	99.46	1	0.00	0.000	0.000

FIGURES

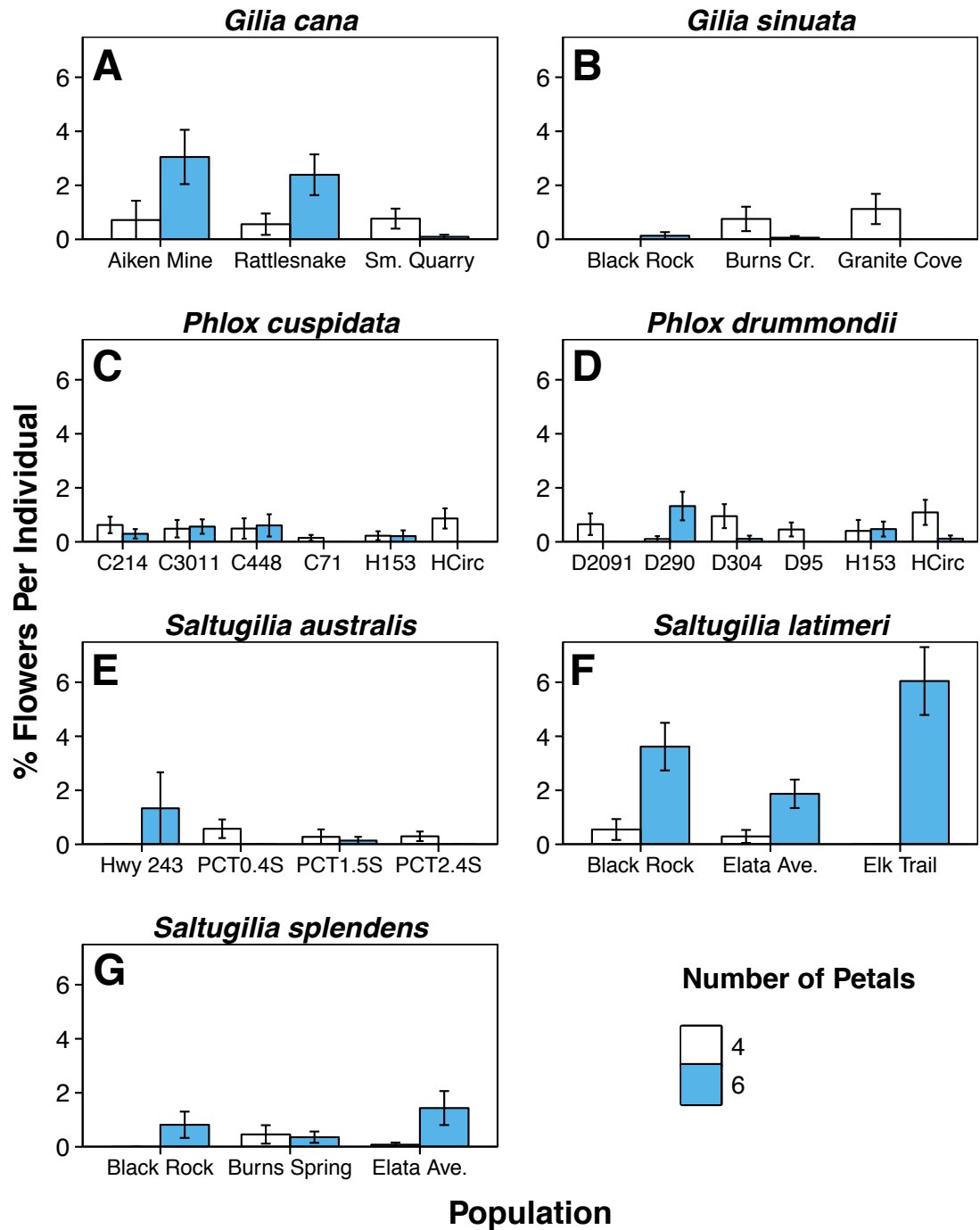


Figure A1: Differences in the percentage of four- and six-petaled flowers among populations within species and among species. Data from Chapter 2.

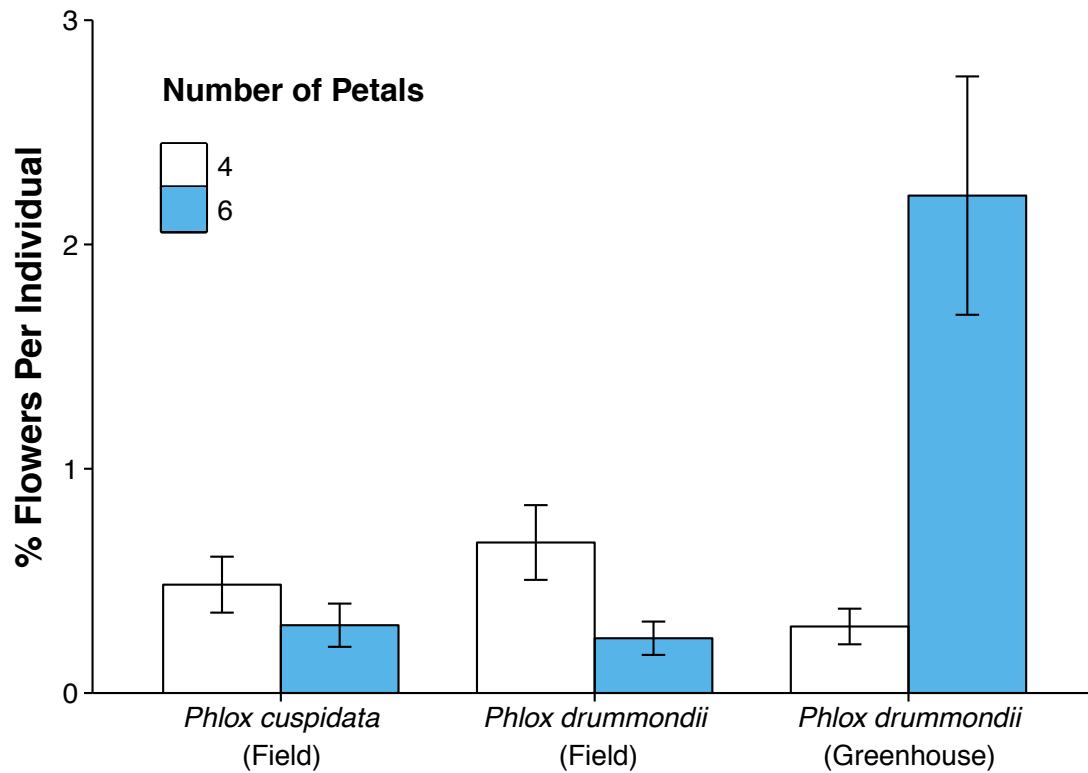


Figure A2: A comparison of petal number variation in wild populations of *Phlox* from Chapter 2, to greenhouse-grown *P. drummondii* from Chapter 1 prior to selection. Greenhouse-grown *P. drummondii* exhibit more than six times the percentage of six-petaled flowers found in wild populations.

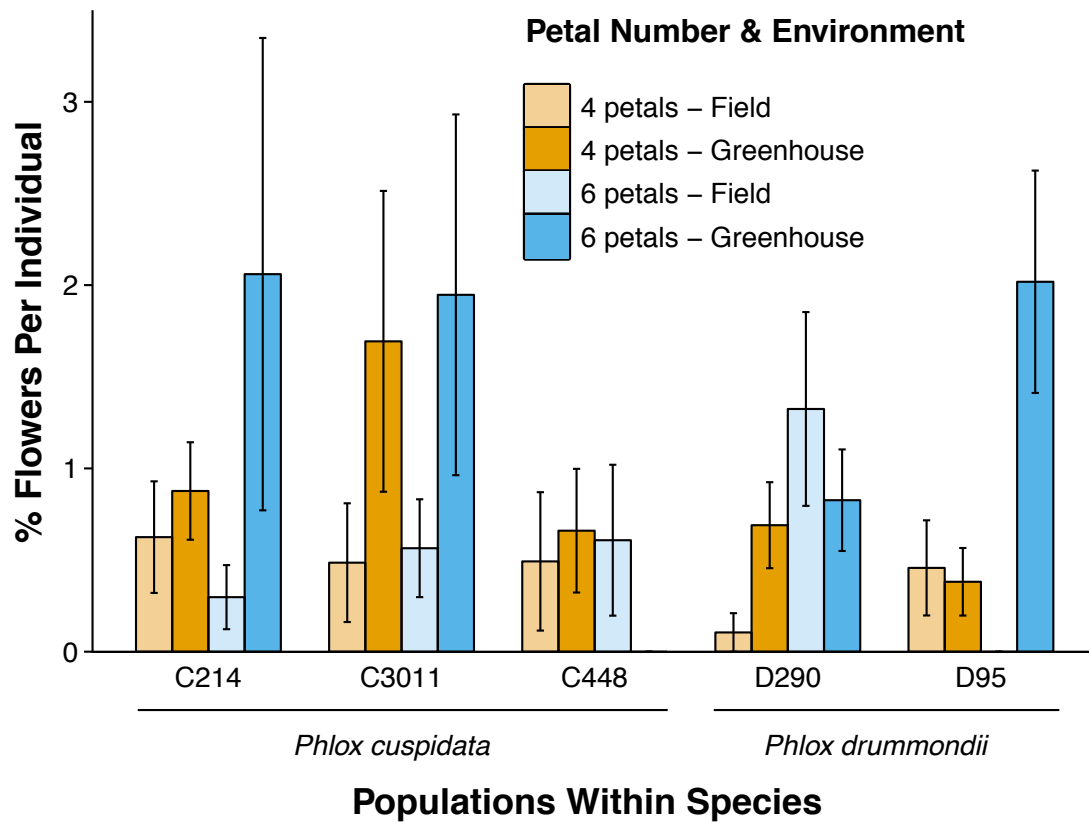


Figure A3: The per-individual percentages of four- and six-petaled flowers among populations of *P. drummondii* and *P. cuspidata* comparing data from the field to the same populations grown in the greenhouse.

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