UNDERSTANDING NATURAL SELECTION ON FLORAL TRAITS: VARIATION, AGENTS AND CONSEQUENCES

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UNDERSTANDING NATURAL SELECTION ON FLORAL TRAITS: VARIATION, AGENTS AND CONSEQUENCES

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For animal pollinated plants, flowers are highly interactive organs expected to be under strong and potentially diverse selection pressures. Plants not only need to attract their pollinators but also deter their enemies, as well as keep the costs of these structures low. Thus natural selection on floral characters is an important area of inquiry. The following series of studies examines multiple aspects of natural selection on plants with a focus on floral characters. The first paper asks whether natural selection is more variable in space or time for both a focal species (*Penstemon digitalis*) and for in plants in general. Indeed, selection is variable, but not more so in either of these dimensions, suggesting that constraints to local adaptation and speciation are equally as likely as their occurrence. Knowing the agents of selection on traits can inform the direction and expectations for evolutionary change. The second paper discusses the finding that pollinators were driving selection for larger flowers and displays in *P. digitalis.* Furthermore, selection on floral traits was generally stronger when pollinators are present than when they were excluded for multiple species for which there is data. These results suggest that pollinators may indeed be major agents of natural selection on flowers. However, with so few studies directly testing agents of selection, this assumption should be applied with caution in contemporary populations. The third paper looks at an understudied trait in an

evolutionary context. Although scents have been characterized for many plant species, very few studies examine the variation in this trait or how natural selection acts on it. Scents vary among populations of *P. digitalis* on a small geographic scale and there was significant natural selection to produce more scent in a common garden. Finally, the last paper examines the expected outcomes of selection for later flowering in *Lobelia siphilitica* by pre-dispersal seed predators. As predicted by optimal defence theory, flowering time and latex are correlated in this system suggesting that selection on flowering time could also effect selection on defence.

BIOGRAPHICAL SKETCH

Amy Parachnowitsch completed a Bachelor's of Science degree in 2003 at Simon Fraser University (British Columbia, Canada), with a double -major in both Biology and Psychology. She was also participated in the Science Co-operative Education program where she was first introduced to biological research in two Simon Fraser labs. Her work on greenhouse tomato pollination in Mark Winston's bee lab led to an interest in pollination systems. She went on to work with Elizabeth Elle in her plant evolutionary ecology lab on pollination in the endangered Garry Oak ecosystem and sex allocation in *Collinsia parviflora*. She then pursued a Masters degree with Christina Caruso at the University of Guelph (Ontario, Canada). There, she first became fascinated with the potential of antagonists (such as herbivores) to impose selection on floral traits, and oppose selection by pollinator mutualists. In *Lobelia siphilitica*, she found that pre-dispersal seed predators were mediating selection on flowering time rather than pollinators. Amy came to Cornell University to study with André Kessler and continue her interests in pollination and evolutionary ecology. She followed up some of her results in *Lobelia siphilitica* and started studying natural selection in *Penstemon digitalis*, including examining selective agents, floral scents, and variation. She was also involved in collaborations examing community diversity effects in *Oennis biennis*, as well as looking at floral scents and seed predation in Colorado *Penstemon spp*.

I dedicate this dissertation to my daughter, Maiken Aina Gyllström. I hope some day she can do and accomplish whatever she wants.

ACKNOWLEDGMENTS

Scientific research is rarely a solo endeavor, and mine is no exception. Cornell has been a wonderful atmosphere for collaboration and feedback. I would like to thank my committee: André Kessler, Anurag Agrawal, Monica Geber and Kelly Zamudio for their support and intellectual input throughout. I would also like to acknowledge all of the wonderful collaborators I have been able to work with through the course of my doctorate: Anurag Agrawal, Susan Cook, Stuart Campbell, Elizabeth Elle, Monica Geber, Scott McArt, Robert Raguso, Jennifer Thaler. I would especially like to thank my advisor, André for his kindness, understanding and collaboration. I have benefited greatly from his insight and viewing my work from a different perspective. We may not have always agreed but our discussions have broadened my scope and strengthened my abilities as a scientist. Most of all, André's ability to see beyond the science and view life from a holistic perspective allowed me to both finish my dissertation and care for my daughter, without feeling the need to compromise either. I feel lucky to have had the pleasure of working with him.

I once wrote in an autobiography for a grade 6 project that I wanted to grow up to work with animals and plants. I have been very fortunate to be able to do just that. However, without the love and support of my parents, Myrna King and Peter Parachnowitsch, I don't think I would have continued to pursue my interests in the natural world all the way to a PhD. Thank you for always being there. It's allowed me to run off and do crazy things like study floral evolution (and so much more), because I knew you'd be there to catch me if I needed it.

A PhD is a lengthy journey in anyone's life and I have so many friends that have

helped along the way. The network of people in Ithaca has been so good to me. People have shown me kindness in so many large and small ways, without which I may have given up when things got tough. I don't dare mention names, lest I leave someone out, but I hope that you know I was thinking of you. Thank you.

Finally, the writing of this dissertation would truly not be possible without Mikael. Not only did he take time out of his work to care for our daughter while I was writing but he was also all a girl could ask for in a housewife. Thank you, my love. It just goes to show that good things can come out of dressing up and being silly for Halloween.

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CHAPTER 1

Comparing spatial and temporal variation in natural selection: a field example with *Penstemon digitalis* and a meta-analysis of plants

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Abstract

Variation in natural selection can have important evolutionary implications. Spatial variation in selection can lead to local adaptation and ultimately speciation. However, temporal variation may constrain such adaptation, leading to phenotypic variation and/or plastic phenotypes. The majority of natural selection studies measure selection in a single location and time, making comparisons of spatial and temporal variation difficult. Here we measured natural selection in *Penstemon digitalis* across space and time to explicitly ask whether selection is more variable in one of these components. We then reviewed the literature for studies that measured selection in multiple locations and/or times to examine the same question across many plant species. Selection was variable in both space and time for *P. digitalis* and plants in general. However selection was no more variable in space than in time, suggesting that local adaptation is no more likely than constraints to it.

Introduction

That environments vary spatially and temporally is a truism and this variation can have profound effects on evolutionary processes. For example, one mechanism of speciation can be the differential adaption of populations to local environments (Schluter, 2000) and divergent selection can lead to genomic divergence although this is often heterogeneous (reviewed in Nosil *et al.*, 2009). Within species, spatial heterogeneity can lead to variation in biotic interactions and therefore coevolutionary processes (Thompson, 1999; Thompson & Cunningham, 2002). For example, abiotic variation can impact biotic interactions and natural selection by pre-dispersal seed predators on a perennial herb (Kolb & Ehrlen, 2010). An important caveat to these outcomes of spatial variation in selection is that degree of divergence can depend on gene flow and phenotypic plasticity (Crispo, 2008). Phenotypic variation within

species is common and populations are frequently but not always locally adapted to their environment (Hereford, 2009). If the phenotypes represented in a particular location are the result of adaptive natural selection, rather than other processes such as genetic drift or microhabitat variations, then we would expect a link between phenotypes and divergent natural selection (e.g. Alcantara *et al.*, 2010).

A recent review of temporal variation in selection suggests that the strength of selection varies considerably between years and that shifts in the direction of selection may also be common (Siepielski *et al.*, 2009). Furthermore, natural selection in a few years might not be informative over longer time scales because of stochastic environmental variation (e.g. Grant & Grant, 2002). Temporal variation, depending on its scale relative to the life span of individuals, can result in the maintenance of genetic variation in populations or phenotypic plasticity (e.g. Agrawal, 2001) and may limit or slow local adaptation. Temporal change in the environment that exceeds the lifespan of individuals can lead to genetic polymorphism, especially when there is storage of dormant stages across generations (e.g., seed banks), while environmental change during the lifespan of individuals can favor phenotypic plasticity.

Both spatial and temporal variation in natural selection could therefore be important determinants of micro-evolutionary processes. Furthermore, spatial and temporal variation in selection can result in evolutionary divergence, a generalist phenotype or phenotypic plasticity, depending on their scale relative to the life history of an organism. However, little is know about the relative magnitude and strength of these two sources of variation. Because variation across space and time are expected to have different outcomes for the phenotypes present within and among populations, an important question for predicting phenotypic variation is whether one is more variable than the other.

In this paper, we use a wild flower (*Penstemon digitalis*) to ask whether

phenotypes and patterns of natural selection varied across populations and three consecutive years, and whether selection was more variable in space or in time. The populations were chosen deliberately to be near each other and in similar habitats to test whether phenotypes and selection varied despite no obvious differences in environment. We then compared our findings to an analysis of spatial and temporal variation in selection coefficients drawn from a survey of the literature. We compiled a database of selection coefficients in plants from studies that either measured selection over multiple time periods (life history stages or years) and/or across multiple sites, including across a "randomly" chosen set of populations, and a set populations from distinct habitats. We used the database to quantify and compare the magnitude of variation in the pattern (direction) and strength of selection in space and time. We also asked, with respect to time, whether selection is as variable among life history stages as inter-annually, and, with respect to space, whether selection is equally variable across "random" locations vs. across locations chosen specifically for habitat differences.

Methods

Field Study

Study System—*Penstemon digitalis* (Plantaginaceae) Nutt. ex Sims is a native wild flower found in the meadows and prairies of North America (Wolfe *et al.*, 2006). The flowers are protanderous and visited generally by small and large bodied bee pollinators (Clinebell & Bernhardt, 1998; Mitchell & Ankeny, 2001; Dieringer & Cabrera, 2002). An unidentified micro-lepidopteran is a known pre-dispersal seed predator in both Ohio (Mitchell & Ankeny, 2001) and New York (Parachnowitsch, *personal observation*).

Data Collection—We measured phenotypic traits and fitness in four different

populations of *P. digitalis* for three years. Populations were chosen based on the presence of *P. digitalis* and the accessibility of the site rather than any particular hypothesis for differences among them. All populations were in old fields and within 10 km of each other (Table 1.1). In each population, 99 plants were flagged after the plants had bolted and formed flower buds but prior to flowering with the exception of TH in 2008. In 2008, 150 plants were measured in TH and used for an experiment examining pollinators as selective agents (Parachnowitsch & Kessler, 2010), however in all other respects these plants were treated the same as our other collections. Plant loss, often due to missing flags and late season mammal herbivory, led to variation in final sample sizes (see Appendix 2). Mammalian herbivory led to a complete loss of fitness estimates for TH in 2006 and therefore we added BB to the data collection in 2007.

Table 1.1 List and locations of *Penstemon digitalis* populations used in our survey of variation in natural selection.

Population Name	Abbreviation	Location
BB Hunting Club	BB	N 42° 30.965', W 76° 22.522'
Neimi Road	NR	N 42° 30.092', W 76° 26. 204'
Turkey Hill	TH	N 42° 26.428', W 76° 25.743'
Whipple Farm	WF	N 42° 26.436', W 76° 25.892"

We measured six phenotypic traits that we hypothesized could be under natural selection in this species: flower size, flower colour, number of flowers, inflorescence

length, aborted flowers, and plant height. We measured flower size by taking six dimensions of the flower as in Parachnowitsch and Kessler (2010). To avoid additional variation in measurements, one researcher took all floral measurements. We attempted to measure at least three flowers per plant and calculated the mean of the measured dimensions that were then reduced into a single flower size trait by taking the geometric mean of the six mean flower dimensions (as in Williams & Conner, 2001; Parachnowitsch & Caruso, 2008).

Penstemon digitalis flowers can be white or have purple striping that appears black under UV light and may act as nectar guides for pollinators (Silberglied, 1979). To estimate colour, we counted the number of lines on the corolla for flowers measured for size and scored the intensity of the colour on a four-point scale (no colour, 0; light, 1; medium, 2; dark purple, 3). Flower colour was then estimated by multiplying the number of lines by the intensity and averaged across flowers on the same plant.

We also measured display size by counting the number of flowers per plant. Display size is commonly assumed to be attractive to pollinators and the number of flowers a *P. digitalis* plant produces was correlated with daily display size for WF in 2006 when daily display size was also measured ($r = 0.637$, $P < 0.0001$, $N = 61$). We measured flower/fruit abortion because plants may respond to pre-dispersal seed herbivory by aborting infested fruits rather than investing in them (e.g. Thompson & Cunningham, 2002). Plant height can be a target of selection (e.g. Cariveau *et al.*, 2004; Parachnowitsch & Caruso, 2008) therefore we measured the final plant height and inflorescence length at the end of the growing season.

We estimated pre-dispersal seed predator damage by opening mature fruits and scoring them as undamaged or damaged. In 2006 we examined all fruits produced by plants but in subsequent years we randomly selected five fruits per plant to assess

damage and used this proportion to estimate the number of fruits damaged on the plant.

Female fitness was estimated by measuring fruit diameter (mm) of all the fruits. In 2006, we measured fruit diameter and length, as well as seed number for all WF fruits. Fruit diameter was an accurate predictor of seed number when plant identity is included as a random factor in a general linear model for both undamaged ($R^2 = 0.75$, $F_{1,526} = 710$, $P < 0.0001$), and damaged fruits ($R^2 = 0.72$, $F_{1,116} = 26$, $P < 0.0001$). Additionally, seed predators consumed on average half the seeds (undamaged fruits: 111 ± 2 seeds, $N = 527$; damaged fruits: 56 ± 5 seeds, $N = 116$). Thus we used the fruit width and damage frequencies to estimate female fitness in all other populations and years and the estimates we present are total fruit volume. Although we have the actual seed number for WF fruits in 2006, we use the volume estimate of female fitness to allow for comparisons to the other populations and years.

Statistical Analyses—To test whether phenotypic traits, seed predator damage and fitness measures varied across populations and/or years, we used ANOVA. Models included traits as the dependent variable and population, year and the population by year interaction term as explanatory variables. To summarize variation in our six traits, one damage estimate, and two fitness estimates, we calculated the coefficients of variation (CV). To compare variation among populations with variation among years, we first averaged across the one factor (e.g. populations) and then calculated the CV for the other and repeated in the reciprocal.

We estimated natural selection on the six phenotypic traits following the methods outlined in Lande and Arnold (1983) where selection gradients (β) measure selection by accounting for correlations among traits through multivariate regressions of standardized traits on relative fitness. Models calculating selection gradients included the six phenotypic traits and were done for each population and year

separately. Because our sample sizes were small in many populations and years and therefore we lack power to detect non-linear selection, we do not include it. To test whether selection differed across years and/or populations we used an ANCOVA model which included the six phenotypic traits, a term for population, year and their interaction, the interactions between traits and population, the interactions between traits and year, and the three-way interaction terms. To visualize the variation in selection across years and populations, we calculated the SD averaged across the one factor (e.g. years) and repeated in the reciprocal for the other factor.

Literature Survey

Survey—We compiled studies of natural selection from the literature that included measurements of standardized selection differentials and/or gradients on the same traits at multiple times, or different spatial locations. We refer hereafter to time, space or experimental treatments as "factors" and to the multiple times/spatial locations or experimental treatments within a study as "levels". Our purpose was to estimate variation in the pattern (i.e., direction) and strength of selection among levels of a factor and compare whether variation in pattern or strength differs between factors.

We used only studies conducted in natural environments or in common gardens that, according to the authors, approximated natural environments for the species. Studies in the database were a subset of those used in Geber and Griffen (2003) that included papers published between 1985 and 2002 and that reported selection on vegetative characters. This database was supplemented with studies of selection on reproductive characters and extended to include studies published since 2002 until 2008 found using the search engine 'Web of Science'. Our database was also supplemented by studies sent to us by Dr. John Stinchcombe. We do not claim to

have been exhaustive in our identification of suitable papers.

Database compilation—There were two sources of temporal variation in selection. The first was variation across episodes in a life history, including such stages as survival to reproduction, survival from flowering to fruiting, and components of male or female mating success (e.g., pollen removed from a flower and pollen deposited on a stigma), fruit set (fruits per flower or total fruit number), seed set (seeds per fruit or total seed number). The second temporal source was inter-annual variation (i.e., years). There were also two sources of spatial variation in selection. (1) variation across "sites" chosen by the authors without regard to habitat differences or (2) across "habitats" selected specifically for differences in abiotic or biotic conditions.

For each study, we recorded the species (a few studies included two species which were analyzed separately), the plant family, life form (annual, herbaceous perennial, woody), the specific characters under selection, the type of character (see below), type of factor (time: episode/year and/or space: site/habitat), and, within each source factor, the level of the factor (e.g., year 1, year 2), the standardized selection differentials (s_{ik}) and/or gradients (β_{ik}) measured on each character, i, and level k of a factor, and whether the selection coefficients were estimated from phenotypic or genotypic analysis. Thus, for a given study and species, the database contained i x k lines, for each species in a study, one line for each specific character and level of the factor(s). For studies that included multiple crossed factors (e.g., episode and site, or episode and year) with levels k and m, respectively, there were i x k x m lines in the database; if the multiple factors were not crossed, the database contained i x (k + m) lines. Specific characters were also classified into broader types: chemistry, physiology, morphology, phenology, size, and herbivory (resistance or tolerance).

This database was then used to calculate measures of variation in the pattern

and strength of selection, for each study, species within a study, and factor(s). The pattern or direction of selection refers to both positive and negative values of selection coefficients while the strength is equal to the absolute value of the selection coefficients. Although mean standardized, rather than variance standardized selection coefficients have been suggested as a better way to compare differences in selection our database contains publications before this recommendation (Hereford *et al.*, 2004). However, as in Siepielski *et al.* (2009), rather than focusing on whether selection is strong or not, we are interested in the comparison between groups and do not expect this to limit our interpretation. Variation in selection for each character (*i*) was measured as the standard deviation (σ_i) of selection coefficients (actual or absolute values). For studies that included only one factor (e.g., episodes) the standard deviation (σ_i) was calculated across the *k* levels of the factor. In studies that included two factors, such as episodes and years, with levels *k* and *m*, respectively, we estimated variation in selection across factor 1 as follows: selection coefficients for each character were averaged across the *m* levels of factor 2 and then calculated the standard deviation of these averages across the *k* levels of factor 1. The reverse order was used to calculate variation in selection across factor 2.

As a final step, we averaged the standard deviations of the characters of the same type (e.g., vegetative morphology, *vm*). We did so in order to reduce the influence of studies in which many individual characters were measured. In sum, the data for our analyses included only one estimate of variation in the pattern and strength of selection per character type and per factor for each species in a study.

Statistical analyses—We used multivariate mixed model ANOVAs to determine whether variation (SD) differed in our series of comparisons. The factors recorded for each study were considered independent fixed effects, with study included as a random effect. We log transformed values (+ 1) to meet the assumptions

of normality. For each question, we asked whether variation differed for the pattern of selection and strength of selection. All analyses were used SAS (v. 9.1) and had an α–level of 0.05.

Standard deviations did not depend on whether the selection estimates were calculated using phenotypic or genotypic selection analyses, therefore we pooled across these analyses to increase our sample size. To avoid double counting any studies, we only included selection estimates from the genotypic analyses when both estimates were reported. To determine whether we could pool estimates from both selection differentials and gradients, we compared the 30 studies that reported both types of coefficients. Selection type was the independent variable and study was a random effect. Variation between differentials and gradients differed significantly for the pattern of selection ($F_{1,149} = 6.30$, $P = 0.01$) and marginally for the strength of selection ($F_{1,149} = 3.89$, $P = 0.0503$), therefore we analyzed these selection estimates separately for all subsequent questions.

We first tested whether plant family, life form, environment (natural or artificial), or character type were able to explain any of the variation in selection. With the exception of environment in one comparison (strength of differentials), none of these variables significantly explained the variation in selection and including them did not alter the main effects of our models, therefore we present the models without these factors for simplicity (but retain study as a random effect). We tested whether selection was more variable across episodes of the life history or across multiple years, and separately whether selection was more variable among "random" sites or habitats explicitly chosen for some difference (abiotic or biotic). When we found no differences in SD for either of these comparisons, we pooled episodes and years to create a ‘time’ variable and sites and habitats to create a ‘space’ variable. These were then used to ask whether there was greater variation in selection across space or time.

Results

Field Study

We found significant phenotypic variation across years and populations for every measured trait of *P. digitalis* (Figure 1.1). Although there were significant population by year interactions in our models, the rank patterns of populations were consistent among years for most traits (Appendix 1). We could not test explicitly whether the CV's of the traits across years differed from the coefficients of variation across space, however the CV's among populations were generally greater than the CV's across years for most traits suggesting that inter-population differences were more greater than inter-annual variation.

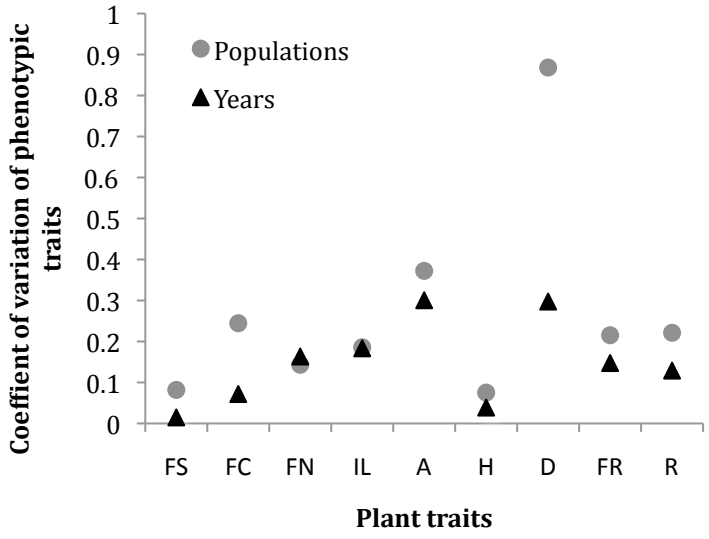

Figure 1.1 Coefficient of variation for six phenotypic traits, one fruit damage estimate and two fitness estimates of *Penstemon digitalis* from four populations in three consecutive years. FS= flower size, FC = flower colour, FN = number of flowers, IL = inflorescence length, A = aborted flowers, H = plant height, D = number of fruits with pre-dispersal seed predator damage, FR = fruit set, R = female fitness.

We found significant natural selection on four of the six phenotypic traits in at least one population and/or year (Appendix 1). There was selection for larger flowers, longer inflorescences, more flowers and fewer aborted flowers (Figure 1.2). However, there was significant population by year variation in natural selection for inflorescence length ($P = 0.006$), the number of flowers produced ($P < 0.0001$) and aborted ($P < 0.0001$), suggesting that selection was variable across both time and space. Additionally, temporal and spatial variation in selection was higher for these compared to other traits, with selection more variable across populations for the number of flowers and across years for inflorescence length and aborted flowers (Figure 1.2).

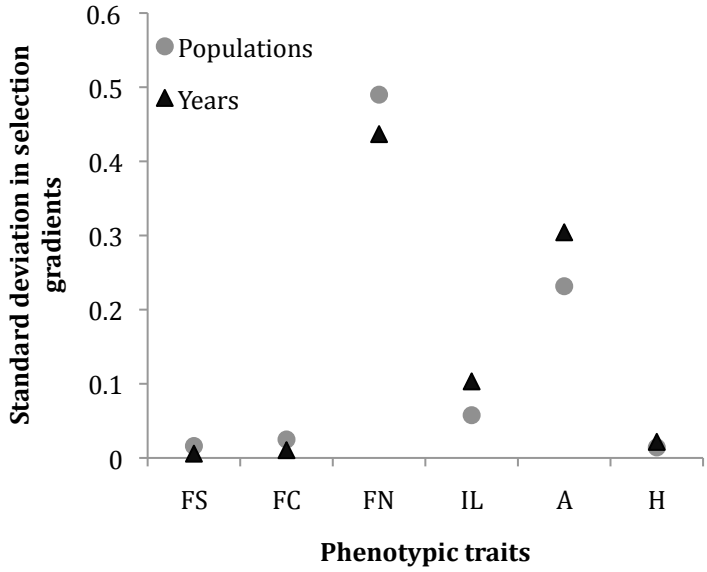

Figure 1.2 Variation (standard deviations) in selection gradients on six traits of *Penstemon digitalis* across four populations and three years. FS= flower size, FC = flower colour, FN = number of flowers, IL = inflorescence length, A = aborted flowers, H = plant height.

Literature Survey

The literature survey yielded 69 studies that reported selection in two or more levels of at least one of our factors of interest. These studies represented a wide range of plant taxa although the dataset was biased towards herbaceous plants (Table 1.2). We calculated 57 standard deviations of selection differentials across time and 69 across space. Selection gradients yielded 73 standard deviations of time and 92 of space (Appendix 2).

Table 1.2 Summary of literature included in the database of selection coefficients.

Number in survey	Differentials	Gradients
Studies	41	56
Species	29	36
Families	20	25

Overall, the pattern and strength of selection varied for both differentials and gradients (Figure 1.3). Because the pattern of selection includes both positive and negative values, it was more variable than the strength selection (Figure 1.3). However the general outcome of all comparisons of temporal and spatial variation in selection was the same whether we used the pattern or strength of selection. In particular, selection was equally variable between life history episodes vs. inter-annually, and between "random" sites vs. across habitats chosen to be distinct (Appendix 2). Moreover, selection was no more variable in time vs. space.

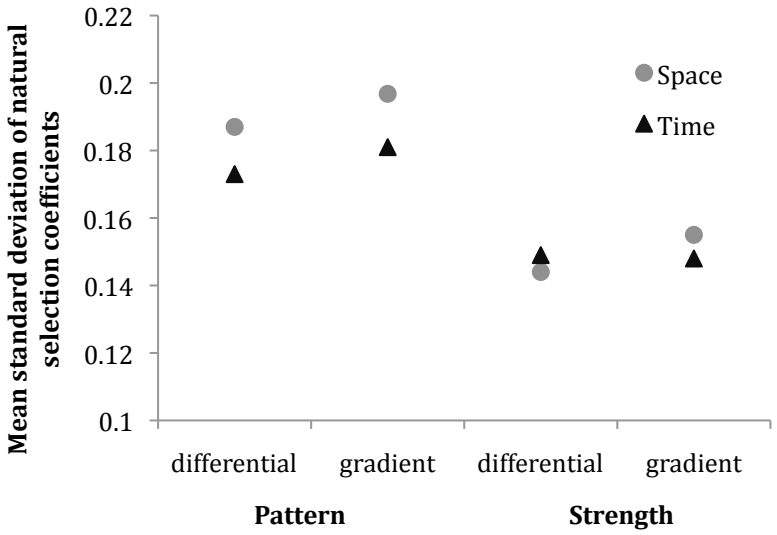

Figure 1.3 Mean variation (standard deviations) in selection coefficients for both the pattern and strength of selection. Errors are not included but were completely overlapping for all comparisons and standard deviations do not differ between space and time for any of the comparisons ($P > 0.75$).

DISCUSSION

That natural selection is variable is not overly surprising, for both our study system and those in the literature. Many authors have noted that natural selection varies across space (e.g. Campbell & Dooley, 1992; Caruso *et al.*, 2003; Gómez *et al.*, 2008) and a recent review has examined temporal variation (Siepielski *et al.*, 2009). However, the general pattern that selection was no more variable in space than in time for plants warrants some consideration. As we outlined in our introduction, variation across space and time can have very different evolutionary outcomes. That we find equal variation in these two factors, suggests they may balance one another in nature.

On a broad scale, this could mean that local adaptation and population differentiation via spatially differential selection are no more likely than within population maintenance of genetic variation via temporally variable selection.

Clearly, plant ecotypes and species have arisen from spatially different natural selection (Linhart & Grant, 1996). For example, pollinator adaptation and differential selection pressures by pollinators is thought to have led to much of the plant diversity we see (e.g. Fenster *et al.*, 2004; Harder & Johnson, 2009). However, it is also clear that species are both phenotypically and genetically variable (e.g. Jansen *et al.*, 2009; Keurentjes, 2009). Our survey of the available selection coefficients for plants suggests that for any given system, the ecology will need to be explored before any predictions of whether selection would be more variable across space or time can be made.

We examined natural selection in *P. digitalis* and found that there was variation in selection across both space and time for three traits (flower number, inflorescence length and aborted flowers). However, it is important to note that selection was always in the same direction for each of these traits and only the strength, not direction, was altered (Appendix 1). This suggests that selection would continue to push the populations in a particular direction although it would vary in the speed in different populations and years. Like Siepielski et al. (2009), we also found that variation in selection was greater for those traits with stronger selection. We did not have any *a priori* predictions for variation in selection among these populations. The populations were all in old fields with some disturbance, pollinators were generally observed to be the same and although many differences likely exist among the sites, no obvious factors stand out to predict differential selection. Therefore we would not expect strong local adaption to be operating in *P. digitalis* at this scale. Thus, it may be surprising that we found phenotypic variation as well as variation in

natural selection across these populations.

The three years we measured selection varied as they would be expected to. For example, June (when *P. digitalis* flowers) in Tompkins County varied across all three years; 2006 was cooler but wet (monthly mean temperature = 18.2°C, total precipitation = 191mm), 2007 was hot and dry (19°C, 73mm), and 2008 was hot and moderately dry (19.9°C, 113mm) (based on weather data from the Northeast Regional Climate Center). Generally selection was stronger in 2006, although it is not known whether temperature or precipitation drive these patterns. For example in contrast to June, July 2006, when fruits are maturing, was the hottest and wettest of the three years and April 2006 (growth period) had moderate temperatures but low precipitation.

Patterns in natural selection were not sufficient to explain the patterns of phenotypic differences we found. For example, there was a strong pattern of population variation in flower colour, however natural selection was never detected on this trait (Appendix 1, 2). Furthermore, for traits under selection such as flower number, which was consistently under positive natural selection in all populations and years, the populations that produced the most flowers were not necessarily those with the strongest selection coefficients (Appendix 1, 2). However, the closer these populations are to an optimum will reduce our ability to detect selection. Therefore these differentiated phenotypes may reflect past selection pressures rather than the current ones we observed.

Our survey suggests that variation in selection is no more variable in space or time for plants. However, our data could be limited and/or biased. First, like many reviews of selection in plants, our survey is strongly biased towards herbaceous and annual species (e.g. Geber & Griffen, 2003). Unfortunately, estimating lifetime fitness in long-lived organisms is beyond the scope of most research projects, so the

focus of many studies remains on short-lived species but this does limit our ability to generalize these patterns for all plants with confidence. Second, it is important to consider that it is possible to have largely differentiated phenotypes across populations, which are the result of past selective sweeps and that do not experience current natural selection because they are adapted to their current location. If this were the case, than we would expect that selection would not vary across these populations (zero in both cases), although it would have, if we had caught the populations during the adaptive selection pressure. Therefore the variation in selection we surveyed may underestimate the importance of divergent selection across space.

If we are to predict the distribution of phenotypes across landscapes, it is essential to know something about how natural selection has acted on those phenotypes. Variation in biotic and abiotic environment across space and time may give us a hint as to what trajectory populations will have. Our survey suggests, at least for herbaceous plants, that temporal variation in natural selection is just as likely to constrain population differentiation, as spatial variation is to drive it.

Acknowledgements

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CHAPTER 2

Pollinators exert natural selection on flower size and floral display in *Penstemon digitalis*

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Abstract

A major gap in our understanding of floral evolution, especially micro-evolutionary processes, is the role of pollinators in generating patterns of natural selection on floral traits. Here we explicitly test the role of pollinators in selecting floral traits in an herbaceous perennial, *Penstemon digitalis*. We manipulated the effect of pollinators on fitness through hand pollinations and compared phenotypic selection in open- and hand-pollinated plants. Despite the lack of pollen limitation in our population, pollinators mediated selection on floral size and floral display. Hand pollinations removed directional selection for larger flowers and stabilizing selection on flower number, suggesting that pollinators were the agents of selection on both of these traits. We review studies that measure natural selection on floral traits by biotic agents and generally find stronger signatures of selection imposed by pollinators as compared to herbivores and co-flowering plant species.

Introduction

Pollinators are often thought to be driving floral evolution (Fenster *et al.*, 2004). Indeed, pollinator specialization seems to have driven rapid evolution in some systems (e.g. Kay *et al*., 2005) and is the main hypothesis put forth to explain the diversity of flowering plants (e.g. Fenster *et al*., 2004). Moreover, key innovations that allow for greater pollinator specialization seem to lead to diversification, as is the case for nectar spurs in *Aquilegia* (Hodges, 1997). Pollination syndromes (a collection of floral traits associated with attracting a particular group of pollinators) can also explain floral trait variation (Wilson *et al.*, 2004), suggesting that pollinator specialization has been an important driver in floral evolution. Although pollinators seem to be important drivers of floral evolution on a macro-evolutionary scale and natural selection on floral traits is common (although not consistent) on a micro-evolutionary scale (Harder &

Johnson, 2009), a major gap in our knowledge is whether pollinators are the agents of natural selection within populations (Ashman & Morgan, 2004).

Because flower size and display size are likely to be attractive to pollinators, these floral phenotypes are often assumed to be the result of selection by pollinators (Barrett & Harder, 1996; Ashman & Morgan, 2004). Furthermore, a recent review of phenotypic selection on floral traits shows that selection for larger flowers is common and flower production even more so (Harder & Johnson, 2009). However, it is also clear that floral signals used for pollinator attraction can be perceived and used by other organisms (Raguso, 2009), making them particularly vulnerable to conflicting selection (Strauss & Irwin, 2004). For example, antagonists such as herbivores and pre-dispersal seed predators, as well as abiotic factors can drive natural selection on floral traits (Strauss & Whittall, 2006). To conclusively determine the agents of selection, the selective environment must be manipulated (Wade & Kalisz, 1990; Conner & Hartl, 2004). However, there are only six studies that employ this approach to testing the role of pollinators in natural selection.

We explicitly set out to test whether pollinators were acting as agents of selection on floral traits of *Penstemon digitalis*. Pollinators are thought to have played an important role in the diversification of the genus *Penstemon* (Wilson *et al.*, 2004) and following the traditional pollination framework this could suggest that pollinators are acting as selective agents in particular *Penstemon* species. Therefore, we compared natural selection in open- and hand-pollinated *P. digitalis* to assess whether pollinators were exerting selection on floral traits. To gauge the generality of our findings, we evaluated the role of pollinators as selective agents for multiple species by reviewing studies that specifically manipulated pollination and measured natural selection. We then compared pollinators to two other potential agents of selection (herbivores and co-flowering species).

Materials and Methods

Study system

Penstemon digitalis Nutt. ex Sims (Plantaginaceae) is a native wild flower found in the meadows and prairies of North America. The flowers are protandrous and, while bagged to prevent pollinators, do not set fruit although geitonogamy within a plant is possible (Parachnowitsch, unpublished). This plant can also be pollen-limited (Zorn-Arnold & Howe, 2007). Flowers are visited by small- to large-bodied bees throughout the geographic range (Clinebell & Bernhardt, 1998; Mitchell & Ankeny, 2001; Dieringer & Cabrera, 2002). The flowers are mainly white with variable purple striping and sticky trichomes covering the flowers and flowering stems (but not leaves). An unidentified micro-lepidopteran is a pre-dispersal seed predator in both Ohio (Mitchell & Ankeny, 2001) and New York (Parachnowitsch, personal observation).

Field Experiment

In June 2008, we selected a total of 300 *P. digitalis* plants in an old-field population in Tompkins County, New York (N 42° 26.428' W 76° 25.743'). To limit biases, we used transects to choose individuals for inclusion in the study. Plants were paired (5m apart) along four parallel transects in two spatial blocks of 150 plants (~ 25m apart) and assigned to either an open-pollinated or hand-pollinated treatment. The spatial blocks were chosen to encompass variation within the population. Our population is situated on a gentle slope ending in a valley, and the two blocks were qualitatively different from each other. The lower block floods more frequently, is generally more open but *P. digitalis* density is higher (Parachnowitsch, personal observation).

Open-pollinated plants were left unmanipulated. Hand-pollinated plants were

supplemented with pollen every 2-3 days throughout flowering (eight times for approximately three weeks). Field-collected pollen was applied to the stigmas with wooden toothpicks. We collected pollen from plants at least 5m away to ensure outcrossing on the day of the pollinations. Generally 2-4 donors were used per plant per day, depending on the number of open flowers. Pollinated flowers were also marked with correction fluid at the base to ensure each flower was supplemented at least once. Hand-pollination allowed us to 1) assess whether plants were pollen limited (Ashman *et al.*, 2004) and 2) determine whether pollinators were the agents of selection on floral traits (Conner & Hartl, 2004). We expected that selection on floral traits would be stronger in open- rather than hand-pollinated plants if pollinators were the agents of selection because hand-pollinations remove the benefit of being attractive to pollinators through two mechanisms. Hand-pollinated plants are no longer pollen limited but in addition to receiving excess pollen, hand-pollinations provide "haphazard" contributions of pollen that may differ from that pollinator-deposited pollen.

Phenotypic Measurements

We estimated seven phenotypic traits that we hypothesized could be under natural selection in this species: flower size, flower colour, total number of flowers, flower density, aborted flowers, plant height and biomass. These traits were not chosen to represent an exhaustive list of traits potentially under selection by pollinators in this species but rather were the hypothesized pollinator-selected traits that we were able to measure in addition to manipulating pollination. For example, floral scents could also be cues under selection by pollinators (Raguso, 2009) and we are studying selection on scents as a part of a separate study. We did not measure flowering phenology which could also experience pollinator-mediated selection (Sandring & Ågren, 2009)

because we wanted to limit our impacts on pollinator behaviour by reducing our visitation to the plants (e.g. Cahill *et al.*, 2001; Hik *et al.*, 2003).

We measured flower size by recording six dimensions of the flower for three haphazardly chosen flowers per plant (Figure 2.1). Whenever possible the three flowers were measured over multiple days to capture variation within a plant (generally two days per plant). Moreover, one researcher did all floral measurements to reduce error. To estimate the visual display of the petals, we measured the width and length of the centre lower lip where the petals are not fused. Pollinating bees enter the flower tube, brushing the sexual organs with their backs (Dieringer and Cabrera, 2002), therefore we measured the width and length of the bell of the tube where the pollinator body fits into the flower, as well as the full length of the tube. *Penstemon* flowers have a constricted floral tube around the ovary, which could limit access to the nectaries, as well as the ovaries (potentially important for pre-dispersal seed predators), therefore we measured the width of the constriction. We then took a plant average for the measured flowers and reduced the six measurements into a single size variable by calculating the geometric mean (as in Williams & Conner, 2001; Parachnowitsch & Caruso, 2008). The geometric mean was strongly correlated with the first principle component of the six flower measurements ($r = 0.85$, $P < 0.0001$) and the patterns of selection were similar whether we used the geometric mean or principle component (data not shown). However, we present the geometric mean for its ease of interpretation (i.e. selection on the mean is selection on overall size).

Figure 2.1 Floral morphology and colour variation in *Penstemon digitalis*. The two flowers represent extremes of the floral colour phenotypes.

Penstemon digitalis flowers vary in the presence, quantity and intensity of purple striping on the corolla (Figure 2.1). The purple appears black under UV light and may act as a nectar guide for pollinators (Silberglied, 1979) and therefore could be under selection (e.g. Irwin & Strauss, 2005). We counted the number of purple lines on flowers measured for size and scored the intensity of the colour on a four-point scale (0 = no lines, 1 = light, 2 = medium, 3 = dark purple). To give a single numeric value to colour, we multiplied the number of lines by the intensity. Again, these values were averaged per plant to give a plant estimate of colour.

We also measured display size by counting the total number of flowers per plant based on end of the season estimates (easily assessed from the senesced flowering stalk). Display size is commonly assumed to be attractive to pollinators and the total number of flowers was positively correlated with daily display size for a close

population of *P. digitalis* in 2006 ($r = 0.637$, $P < 0.0001$, $N = 61$). *Penstemon digitalis* floral architecture varies from dense inflorescences to a more open plan (Parachnowitsch, personal observation) and we estimated this trait as the number of flowers/length of the inflorescence. Pre-dispersal seed herbivores may select for higher abortion rates (Thompson & Cunningham, 2002) and because *P. digitalis* has both high abortion rates and pre-dispersal seed herbivores, we measured the total number of aborted flowers. Plant height can be a target of selection (Cariveau *et al.*, 2004; Parachnowitsch & Caruso, 2008) and we measured the final plant height at the end of the growing season. Some pollinator attractive traits such as flower size and display size could be correlated with general plant vigour and therefore selection on these traits maybe due to correlations with vigour rather than selection by pollinators (Andersson, 1996). We estimated selection on vigour by measuring above ground biomass (dry mass in g).

After senescence when all fruits had matured, we collected plants for estimates of fitness and pre-dispersal seed damage. To estimate the number of damaged fruits, we assessed five randomly chosen fruits per plant and then multiplied the proportion of damaged fruits by the total number of fruits to give the number of damaged fruits per plant. Pre-dispersal seed predator damage was scored as either present or absent based on evidence found within the fruit (larvae, pupae and/or frass). Female fitness was estimated by first measuring fruit diameter (mm) of all the fruits. Fruit diameter was an accurate predictor of seed number when plant identity was treated as a random factor for both undamaged ($R^2 = 0.75$, $F_{1,526} = 710$, $P < 0.0001$), and damaged fruits ($R^2 = 0.72$, $F_{1,116} = 26$, $P < 0.0001$) in a nearby population counted in 2006. Pre-dispersal seed herbivores consumed on average half the seeds (undamaged fruits: 111 ± 2 seeds, $N = 527$; damaged fruits: 56 ± 5 seeds, $N = 117$). Therefore, we calculated female fitness as the total diameter of undamaged fruits plus one half the diameter of

damaged fruits. Although *P. digitalis* is perennial, local populations in Tompkins Co., NY generally are semelparous (Parachnowitsch, personal observation), suggesting that our measures were lifetime fitness for all but the most robust plants. We divided fitness by the treatment mean to give relative fitness values for each plant.

Statistical Analyses

Plant loss and missing data led to a total sample size of 281 plants ($N = 141$ open-pollinated; $N = 140$ hand-pollinated). We used Pearson's correlations to test for relationships among our traits. All analyses were conducted using SAS version 9.02.

Pollinators could affect absolute fitness in our plants in two ways that we measured. First, they could alter the number of fruits to successfully mature (fruit set = successful fruits/total number of flowers). Second, they could alter the seeds per fruit (fruit size). We used these two measures to determine whether there was pollinator limitation in *P. digitalis*. We compared fruit set and mean fruit size between open- and hand-pollinated plants using an ANOVA model with spatial block and plant pair (within block) as random effects. Both fruit set and mean fruit size met the assumptions of ANOVA and therefore were not transformed. Additional ANOVA models of the same form tested for block differences in the seven phenotypic traits.

We measured directional (β) and non-linear or quadratic (γ) selection gradients using multivariate regression models of standardized traits (mean of 0, variance of 1) on relative fitness as in Lande and Arnold (1983). The multivariate models control for correlations among the traits included in the analysis and therefore measure direct rather than total selection on each trait. Thus, selection gradients allow identification of the targets of selection among the measured traits (Conner & Hartl, 2004). Standardized traits, relative fitness and selection coefficients were calculated within each pollination treatment. Directional selection models included the seven

phenotypic traits. Non-linear selection measures potentially stabilizing or disruptive selection and was estimated with regression models that included the linear and quadratic terms for the seven traits. The non-linear selection gradients reported are a doubling of the regression coefficients (Stinchcombe *et al.*, 2008). The patterns of selection were qualitatively the same whether we included block as a random factor in the model or not, so we present selection estimates from the models without block for simplicity.

To determine whether pollinators were selecting on our seven phenotypic traits, we used ANCOVA to compare the selection between treatments (Sokal & Rohlf, 1995). If pollinators were the agents of selection then we would expect selection to be weaker in the hand-pollinated treatment (Figure 2.2a). The multivariate model included the linear and quadratic terms for all of the traits as well as a categorical term for the treatment and all the interaction terms with pollination treatment. Again, block did not affect the pattern of selection so the models without block are presented.

Literature Survey

To assess the general effect of pollinator-driven phenotypic selection across many taxa and studies, we reviewed selection studies that specifically tested for pollinators as selective agents by manipulating pollination through open- and hand-pollinated plants using Lande and Arnold's methods (1983). This method allows for comparisons among studies and has been used in a number of broad surveys of natural selection (Kingsolver *et al.*, 2001; Geber & Griffen, 2003; Harder & Johnson, 2009; Siepielski *et al.*, 2009). We first searched papers included in a recent review of phenotypic selection on flowers (Harder & Johnson, 2009), as well as an expanded dataset of Geber and Griffen (2003) collected to examine variation in selection (Geber &

Parachnowitsch, unpublished). An additional literature search with Web of Science used the key words 'selection' and 'pollen limitation' or 'hand pollination'.

To compare selection by pollinators to other agents of selection on floral traits, we searched for papers which manipulated an agent of selection in an analogous way to hand pollinations (agent present/agent absent) and measured some aspect of selection on floral traits. Here we first searched through the expanded database for appropriate studies (Geber & Griffen, 2003). Additional papers were found searching papers citing Lande and Arnold (1983) because we assumed that papers which measured selection using their methodology would cite the paper. We then examined papers with titles that suggested they would meet our criteria. We do not assert that this is an exhaustive search of all published examples, however, we attempted to be as complete as possible. Although there are examples that manipulate abiotic factors (such as nutrients or shade) and measure selection on floral traits we excluded these from our comparisons for two reasons. Manipulations of abiotic factors examine a gradient of a selective agent (not the complete absence versus presence) and predictions about the strength of selection in each treatment are difficult to generalize.

We then categorized studies by the three agents with reported selection estimates on floral traits: pollinators, herbivores or co-flowering species. Whenever possible, we used selection gradients (β) which controlled for correlations among traits and estimated direct selection. We recorded selection estimates in the presence and absence of the agent of selection and categorized traits as: display (generally number of flowers), flower morphology (estimates of flower size and distances within a flower), phenology, flower type (male or female or protandry), colour, and nectar (nectar production). For three studies, selection was estimated in multiple populations, years and/or treatments, and we averaged across the replicates to avoid biasing our dataset to any particular study. To increase our power to detect trends in

the pollinator manipulations, we included our results from *P. digitalis*.

We estimated the general trend of the relationship of selection between treatments with and without agents by regression. To test whether the regression slope was significantly different than one (the equivalency line where selection was equal in the presence and absence of the agent), we used the 'test' option in PROC REG (Littell *et al.*, 2002). If the slope was significantly greater than one, selection was stronger in the presence rather than absence of the agent. The opposite was true if the slope was significantly less than one.

Results

General Biology

The seven phenotypic traits were variable within the population. Our flower size estimate ranged from 6.48 – 9.44 mm (mean: 7.78). Flower colour varied from 0 – 48 on our numeric scale, with an average of 21. Interestingly, flower colour was the only trait that did not correlate with any of the others ($P > 0.48$). The mean flower production was 26 flowers/plant, however this trait was quite variable (range: 7 – 73). Plants generally aborted 9 flowers, although some aborted zero while others aborted as many as 23. Those plants with higher flower numbers generally had higher abortion ($r = 0.661$, $P < 0.0001$). Plants were on average 76 cm tall (range: 38 – 119), with 13 cm of that the inflorescence (range: 3.5 – 29). Biomass ranged from 0.40 – 5.29 g (mean: 1.89) and generally all the size measurements were positively correlated with each other (flower size, flower number, two lengths and biomass).

We also found phenotypic variation between the two blocks. Plants produced smaller (7.71 versus 7.86 mm) and fewer flowers (24 versus 28) in the lower part of the field. The lower block was also generally significantly smaller (for inflorescence length, height and biomass). Conversely, aborted flowers and flower colour did not

vary across the field.

Pre-dispersal seed predator damage was extremely low in this population; only five plants had damage, each with only one of five fruits attacked. Although all of these plants were in the hand-pollinated treatment and most were in the upper block (4/5), there are too few damaged plants to interpret whether our hand-pollinations increased attack or whether damage was spatially variable.

Pollen limitation

We found no pollen limitation in our population of *P. digitalis*. Fruit set did not differ between open- and hand-pollinated plants ($F_{1, 280} = 1.22$, $P = 0.27$), however there was spatial variation in fruit set between our two blocks ($F_{1, 280} = 26.11$, $P < 0.0001$). Likewise, the mean fruit size per plant did not differ between the pollination treatments ($F_{1, 280} = 0.46$, $P = 0.50$), but also differed at the block level ($F_{1, 280} = 15.62$, $P < 0.0001$). Fruit set (0.60 versus 0.70) and mean fruit size (4.77 versus 4.57 mm) was higher in lower block.

Natural Section

Natural selection differed between the open- and hand-pollinated plants (Table 2.1). In open-pollinated plants, we found significant directional selection on four of seven phenotypic traits. Plants with larger, more, and fewer aborted flowers, as well as larger size had higher relative fitness. We found no detectable directional selection on flower colour, inflorescence length or plant height. There was also significant stabilizing selection on flower number and disruptive selection on floral density in the open-pollinated plants but no significant quadratic selection was detected on the other five traits. However, sample size in our treatments may have limited our power to measure non-linear selection. Conversely, we found significant phenotypic selection

on only three of seven traits in the hand-pollinated plants. There was directional selection for more flowers, fewer aborted flowers and larger plants but no significant quadratic selection in the hand-pollinated population. Furthermore, natural selection was significantly stronger in the open-pollinated plants for flower size and flower number as well as stabilizing selection on flower number, suggesting that pollinators were agents of selection on these traits. Selection on flower size was marginally different between our two treatments ($P < 0.06$), but when we used inflorescence length rather than our composite flower density trait, selection on flower size was the same within treatments and was significantly different between treatments ($P < 0.05$). We found that selection for fewer aborted flowers and greater biomass did not differ between pollination treatments, suggesting that pollinators were not the agents of selection for these two traits.

Table 2.1 Comparisons of natural selection gradients (directional, β and quadratic, γ) between open- (N = 141) and hand-pollinated (N = 140) *Penstemon digitalis*. Phenotypic selection (± 1SE) is followed by statistics from an ANCOVA testing whether the selection estimates differed between treatments. Bold indicates significant selection within a pollination treatment.

Phenotypic Trait	Open-pollinated	Hand-pollinated	*F*	*P*
		β		
Flower size	**0.030 ± 0.010**	0.0007 ± 0.008	3.58	0.06
Flower colour	-0.003 ± 0.009	-0.005 ± 0.007	0.08	0.78
Number of flowers	**0.745 ± 0.018**	**0.601 ± 0.014**	43.85	<0.0001
Floral density	-0.004 ± 0.010	0.009 ± 0.009	0.72	0.40
Aborted flowers	**-0.319 ± 0.012**	**-0.306 ± 0.010**	0.93	0.34
Plant height	-0.014 ± 0.012	-0.0002 ± 0.011	1.54	0.22
Biomass	**0.077 ± 0.017**	**0.060 ± 0.012**	0.00	0.99
		γ		
Flower size	0.008 ± 0.005	-0.012 ± 0.007	1.17	0.28
Flower colour	0.004 ± 0.007	-0.006 ± 0.007	0.00	0.98
Number of flowers	**-0.068 ± 0.009**	-0.006 ± 0.006	9.05	0.03
Floral density	**0.026 ± 0.009**	0.012 ± 0.006	0.68	0.41
Aborted flowers	-0.008 ± 0.007	-0.006 ± 0.006	0.04	0.84
Plant height	-0.0006 ± 0.006	0.014 ± 0.007	0.58	0.45
Biomass	0.024 ± 0.009	-0.012 ± 0.007	2.52	0.11

Survey of Experimental Evidence for Biotic Agent-mediated Natural Selection

In addition to the current study, we found 15 studies that manipulated an agent of selection and measured selection on floral traits for 12 species spanning 11 plant families (Table 2.2). Six additional studies explicitly tested whether pollinators were

agents of selection by manipulating the pollination environment (Andersson, 1996; Galen, 1996; Totland *et al.*, 1998; Fishman & Willis, 2008; Parachnowitsch & Caruso, 2008; Sandring & Ågren, 2009), five manipulated herbivory (Juenger & Bergelson, 1998; Juenger & Bergelson, 2000; Gómez, 2003; Juenger *et al.*, 2005; Wise & Cummins, 2007) and a further four studies manipulated the presence of co-flowering species (Caruso, 2000; 2001; Moeller & Geber, 2005; Smith & Rausher, 2008), although one study examined facilitation by congeners rather than competition for pollinators (Moeller & Geber, 2005). Measures of flower morphology were by far the most common, followed by display and phenology. Very few studies examined flower type, colour or nectar traits.

Natural selection was stronger when pollinators were present rather than absent (Figure 2.2b) and the slope of the regression line was significantly different from one ($F_{1,22} = 5.78$, $P = 0.026$). Conversely, selection in the absence of co-flowering species was stronger than in their presence, and this trend was not affected by the nature of the study (competition versus facilitation) (Figure 2.2d; $F_{1,20} = 5.85$, $P = 0.026$). Finally, natural selection on flower traits was equivalent in the presence or absence of herbivory (Figure 2.2c; $F_{1,9} = 0.33$, $P = 0.58$). Among the differences in selection observed, most biotic alterations of selection resulted in changes in the strength (not direction) of selection; reversals in the direction of selection were most prevalent when comparing the presence and absence of co-flowering species (7 comparisons; Figure 2.2d).

Table 2.2 Summary of a literature survey of experimental manipulations of agents of natural selection on floral traits. Pollinator manipulation refers to experiments with open- vs. hand-pollinated treatments (and include this study), herbivores were manipulated either by simulating herbivory or excluding herbivores, co-flowering species were experimentally present or absent. Selection gradients (which control for correlations among traits) are presented when available.

Number in survey	Complete dataset	Manipulation		
		Pollinators	Herbivores	Co-flowering species
Studies	16	7	5	4
Plant Families	11	7	3	3
Species	12	7	3	3
Selection estimates:	53	23	9	21
Display	10	6	1	3
Floral morphology	29	12	3	14
Phenology	9	4	5	0
Flower type	3	0	1	2
Colour	1	1	0	0
Nectar	2	0	0	2

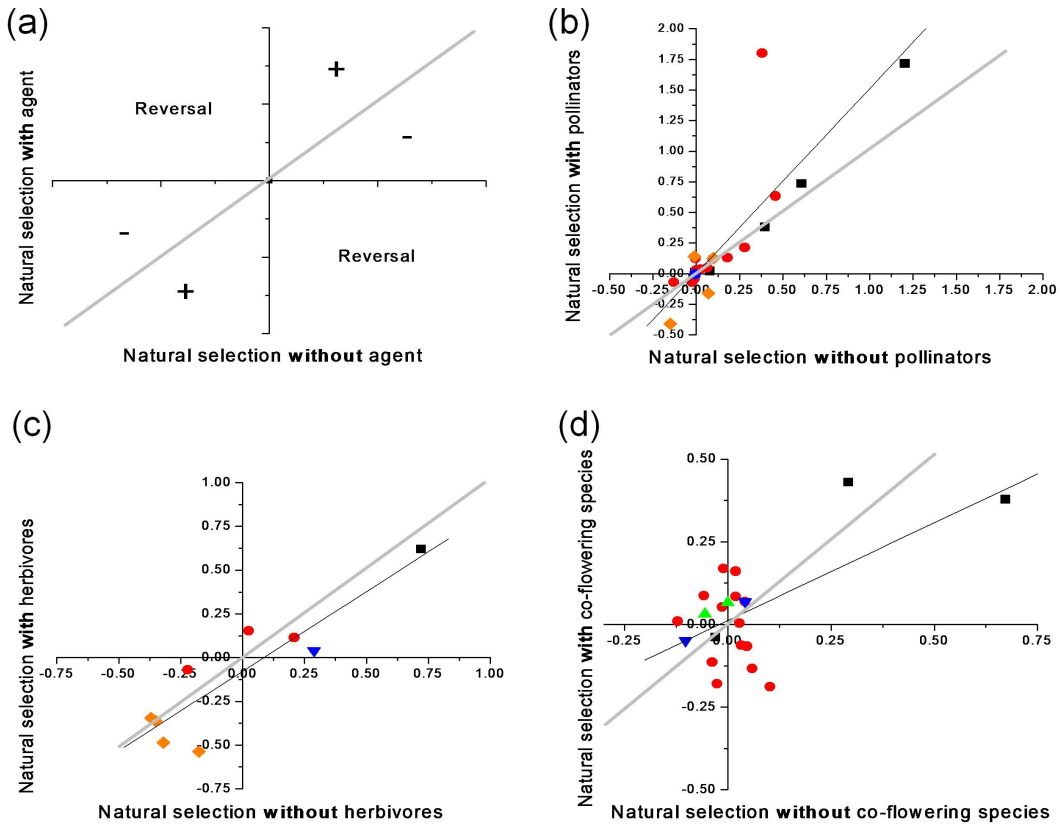

Figure 2.2 Comparisons of directional selection (s or β) on floral traits in experiments that manipulated an agent of selection; either pollinators (b), herbivores (c) or co-flowering species (d). The general pattern of the plots follows (a): + selection is stronger when the agent is present, – selection is stronger when the agent is absent, reversals = change in the direction of selection. Trait classifications: flower display ■, flower morphology (size) ●, nectar ▲, flower type ▼, phenology ◆, petal colour ⬢.

Discussion

We found pollinator-mediated selection in *P. digitalis* on two traits thought to be important for pollinator attraction: flower size and display size (Table 2.1). Selection for larger flowers was present in the open- but not hand-pollinated plants. Natural

selection on flower morphology selected for larger values (Figure 2.2; Harder & Johnson, 2009) and fits with the general hypothesis that pollinators select for larger flowers because they are more conspicuous and/or maybe associated with larger rewards (e.g. Blarer *et al.*, 2002). Natural selection for larger displays is also common (Figure 2.2; Harder & Johnson, 2009) and we found stronger directional selection on flower number in open-pollinated *P. digitalis* (Table 2.1). Because flower number sets the upper limit for fruit number, in many systems such as *P. digitalis*, there is a direct positive relationship between flower and fruit number. Thus, positive selection would be expected on flower number independent of pollinator-mediated selection. However, we found stronger directional selection in the open-pollinated plants, suggesting that the difference in strength of selection was due to an added benefit of larger displays attracting pollinators. We also found stabilizing selection on flower number in the open- but not the hand-pollinated plants, suggesting a cost to having too many flowers. Large displays can have increased geitonogamy, which could be costly through reduced fitness of selfed seeds (Harder & Barrett, 1995). Because hand-pollinations likely minimized the number of selfed seeds, we would expect the cost of large displays to be likewise reduced, suggesting that the difference in stabilizing selection between our treatments was due to stabilizing selection by pollinators.

We found natural selection by pollinators despite the lack of pollen limitation. Although stronger selection via female fitness can be correlated with a greater degree of pollen limitation (Ashman & Morgan, 2004), selection by pollinators is not always associated with pollen limitation. Galen (1996) found selection by pollinators without pollen limitation and two studies found pollen limitation but no selection by pollinators (Andersson, 1996; Totland *et al.*, 1998). Thus it may be more likely to find selection by pollinators in pollen-limited populations because selection is likely to be stronger. However it cannot be assumed to be true for all populations.

Field estimates of phenotypic selection can be biased due to environmental covariance between traits and fitness (Rausher, 1992). We attempted to control for this bias by both physically pairing our treatments and using a blocked design. There were phenotypic differences between our two blocks that suggest pollination, competition, and/or resources may have differed in these parts of the population. However, when we included block as a random factor in our selection models, the significant patterns remain, suggesting that an environmental gradient was not responsible for the overall selection we found.

If biotic agents frequently exert natural selection on plants, than we would expect stronger selection when the agents are interacting with the plant, compared to when they are experimentally removed. For pollinators specifically, we expect selection on floral traits to be stronger when pollinators are selecting plants than when experimenters hand-pollinate them. Indeed, we found that natural selection on floral traits was stronger in the presence rather than absence of pollinators (Figure 2.2b). Furthermore, we do not necessarily expect herbivores to be strong selective agents on flowers although they could influence floral evolution in a number of ways (Strauss & Irwin, 2004). We found that selection on floral traits was equivalent whether herbivores were present or absent (Figure 2.2c). However, there were few selection coefficients to test this hypothesis so these findings should be interpreted with caution. Co-flowering species could either have competitive or facilitative effects on the focus species, which could lead to divergent or convergent evolution of floral traits (Caruso, 2001). Therefore, it is difficult to predict across systems whether selection should be stronger with or without a co-flowering species. When co-flowering species have been manipulated, selection was stronger in the absence of the co-flowering plant (Figure 2.2d). However, there were also many more reversals when co-flowering species were removed (i.e. positive selection became negative or vice versa),

suggesting that community context could alter selection on floral traits (Caruso, 2000). Surprisingly, we found significant trends for both pollinator and co-flowering species manipulations despite our small sample size and the fact that selection is frequently weak in natural populations (Kingsolver *et al.*, 2001; Knapczyk & Conner, 2007). Indeed, the majority of the selection coefficients included were close to zero and non-significant. However, our ability to detect trends suggests that these patterns could be strong in nature.

Our survey data could be biased by three major limitations. First, although our complete dataset included plants spanning multiple functional pollinator groups, pollinator manipulations have been limited to hymenopteran and dipteran pollinators. Pollinator shifts have been proposed as a major driver of speciation (e.g. Fenster *et al.*, 2004) and some shifts seem more common than others. For example, bee to bird pollination is more common than the reverse (Thomson & Wilson, 2008). However, it is uncertain whether one functional group would exert stronger selection on floral traits than another. Thus, the addition of plants pollinated by other functional groups such as birds, beetles, moths, etc. may alter the pattern we detected. Second, although our data set spans almost as many families and species as studies, it only includes herbaceous plants. Studying selection in herbaceous plants is a bias common to the plant literature, however, it is important to note that it may affect our ability to generalize to all flowering plants (e.g. Geber & Griffen, 2003). Because natural selection by pollinators is of general interest in floral evolution, this suggests that measuring selection by pollinators in non-herbaceous plants and/or non-bee/fly pollinated plants will provide further insights into their role as agents of natural selection. Lastly, our survey was necessarily limited to studies that measured Lande and Arnold (1983) selection coefficients and directly manipulated an agent of selection. This allowed for direct comparisons across studies but could also introduce

biases.

We were unable to directly compare the impacts of pollinators with selection by other floral visitors such as florivores (McCall & Irwin, 2006) or pre-dispersal seed herbivores (these data have simply not been collected, but see Wise & Cummins, 2007). In particular, pre-dispersal seed predators are expected to conflict with pollinator selection on floral traits because they should visit fertilized flowers (or those that will be fertilized). Moreover, they can exert selection on floral traits in multiple systems (Pilson, 2000; Cariveau *et al.*, 2004; Rey *et al.*, 2006; Parachnowitsch & Caruso, 2008), making a direct comparison between seed predator- and pollinator-mediated selection a relevant question. For our population of *P. digitalis*, we found no evidence that pre-dispersal seed predators influenced selection on flower size or number, nor were they affecting selection on aborted fruits. Only five of the 281 plants in this experiment received any fruit damage and our results were robust to excluding these plants (data not shown). Pre-dispersal seed herbivores can be much more frequent in *P. digitalis* (Mitchell & Ankeny, 2001; Parachnowitsch, unpublished) and it is possible that they do exert natural selection in other populations and/or years.

An important limitation of our and the studies reviewed here is that they generally only measure female fitness in hermaphroditic plants in one season. Natural selection can vary in time (Siepielski *et al.*, 2009). Therefore, to fully understand the evolutionary trajectory for a population, selection should be measured in multiple years. Moreover, if most of the natural selection on flowers by pollinators or other agents is via male function, then these types of experiments may fail to detect their impact (see Conner (1997) for a theoretical discussion and counter example). Although few natural selection studies examine both male and female fitness estimates, the general pattern from the literature does not support the male function hypothesis. That is, selection on attractive floral traits was not always stronger via

male fitness but rather selection through male and female fitness was context dependent (Ashman & Morgan, 2004). Thus, hand-pollinations can provide valuable information about selection by pollinators but are still only half of the story. Manipulations of the agents of selection via both male and female fitness are necessary to give a more complete picture and reveal functional differences.

Conclusion

Flowers offer a conundrum to evolutionary ecologists. On one hand it seems fairly obvious that flowers are adapted to their pollinators (reviewed in Harder & Johnson, 2009) and that shifts in pollination systems have lead to diversification in plants (e.g. Kay *et al.*, 2005). Moreover, there are a limited number of examples of pollinator-mediated natural selection such as we found in *P. digitalis*. However, the majority of selection estimates on floral traits from manipulations of pollination do not support selection by pollinators (Figure 2b) and studies which have used path analyses often find that pollinators weakly effect fitness (Conner *et al.*, 1996; Gómez, 2000; Rey *et al.*, 2006; Ashman & Penet, 2007). Harder and Johnson (2009) argue that natural selection studies may be measuring the effects of pollinators during periods of relative stasis and that we should expect selection by pollinators on novel traits or in new environments. In other words, the lion's share of pollinator-mediated natural selection shaping flowers may have occurred during and shortly after speciation. While this view needs further testing, we are left to explain what the agents of selection are in cases when there is significant selection on floral traits, but not by pollinators (e.g. Andersson, 1996; Totland *et al.*, 1998; Fishman & Willis, 2008; Parachnowitsch & Caruso 2008; Sandring & Agren, 2009). If pollinators truly exert most of their selection on flowers during cladogenesis, then an interesting question follows. During a period of stasis, how far from these adaptive peaks can non-pollinator agents push

floral traits within a particular population or species?

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CHAPTER 3

Variation and natural selection of plant volatile emission in *Penstemon digitalis*

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Abstract

Floral scent is an important and complex character of flowering plants. However, little is known about its evolutionary ecology. We characterized floral scents in a wild flower, *Penstemon digitalis*, in a common garden planted with individuals from three near-by source populations. We found that even on a small geographic scale, floral scents vary among populations, suggesting that scents can be highly variable and maybe under differential selection pressures. Moreover we found natural selection to increase five different volatiles of the blend, suggesting that smelling stronger can be beneficial in this plant. Our data suggest that different components of a scent can be under natural selection. However, further research is needed to integrate scents into our broader understanding of floral evolution.

Introduction

Floral scent is an important but frequently ignored component of the floral phenotype (Raguso, 2008a). Although scents have been cataloged for many species (e.g. Knudsen *et al*., 2006) and their direct and indirect roles in plant-animal interactions have been elucidated (e.g. Dobson, 2006; Raguso, 2008b; Junker *et al*., 2010), far less is known about the variation of floral volatile organic compound (VOC) emission within a species and the ecological and evolutionary consequences of that variation (Whitehead & Peakall, 2009). Floral traits that have been extensively studied, such as morphology and colour, tend to vary within a species, even to the extent that variation and natural selection can be seen on family diagnostic characters (e.g. Conner *et al*., 2003). Thus, one could predict that scents would be no different. However, like much of the phenotypic variation in morphological traits, environment can play a large part in phenotypic differences. Volatile compounds can be particularly sensitive to microhabitat and climate variables, making it challenging to assess the magnitude of

observed differences (Majetic *et al*., 2009).

To date, natural selection on plant secondary chemicals has focused mainly on compounds with suggested direct defensive function. There are few studies that have empirically examined natural selection on chemical defences, and thus far, the results have been varied. Selection can act in favor of increasing secondary compounds (Berenbaum *et al*., 1986; Latta & Linhart, 1997; Johnson *et al*., 2009) or to decreasing them (Zangerl & Berenbaum, 1997; Shonle & Bergelson, 2000; Johnson *et al*., 2009). Furthermore, natural selection on chemical defences can depend on the community context (Lankau & Strauss, 2008), whether herbivores were present (Zangerl & Berenbaum, 1997) and whether the herbivores were specialists or generalists (Lankau & Strauss, 2007). Chemical defences are predicted to be costly although this is not always empirically evident (e.g. Strauss *et al*., 2002), therefore researchers have predicted that these chemicals should only be selected for in the presence of herbivores. It has been suggested that some of the costs associated with defensive secondary metabolite production are ecological and may, for example, compromise interactions with mutualistic organisms, such as predators and parasitoids of herbivores (Kessler & Halitschke, 2007 and citations therein) or pollinators (Kessler & Halitschke, 2009). The organisms mediating ecological cost of secondary metabolite production are therefore potential agents of selection on those traits. Thus, the production of floral VOCs, as part of the overall secondary metabolite production may be under similar diffuse natural selection.

Similar to non-volatile secondary metabolites, VOCs can be costly if they attract antagonists (e.g. Raguso, 2009; Baldwin, 2010). Volatile compounds can certainly be used as cues or signals by many and diverse organisms interacting with the plant. Pollinators can use volatile cues to locate plants (Wright & Schiestl, 2009), however, antagonists such as herbivores can use floral volatiles to cue into a suitable

host (e.g. Baldwin *et al*., 1997). Therefore we might expect diverse selection pressures on scents depending on the community context. However, to date, no study has measured natural selection on volatile compounds, which is key to understand their function as signals or cues (Allison & Hare, 2009).

We used a native North American wild flower, *Penstemon digitalis*, to explore the evolutionary ecology of its scent. This species has no strong obvious scent chemistry therefore we had no *a priori* expectations of the role of scent in the system. The genus *Penstemon* contains a diverse array of species with multiple transitions from bee to humming-bird pollinated plants (Wolfe *et al*., 2006). The role of flower colour and morphology both within species and across pollination syndromes has been studied extensively (e.g., Castellanos *et al*., 2004; Wilson *et al*., 2004; Thomson & Wilson, 2008). However, to date, no study has examined floral scent in this genus and it is unknown whether scent plays any role in the transitions from bee to bird pollination. Bee-pollinated flowers are generally expected to have stronger scents and insects likely use floral scents as foraging cues and may play a role in their evolution (Wright & Schiestl, 2009). Therefore, we measured scents in bee-pollinated *P. digitalis*. Specifically in a common garden experiment, we asked:

1. How much do plant volatiles vary within this species?
2. Do floral scents correlate with pigment variation?
3. Is there natural selection on plant volatiles?

Materials and Methods

Study system

Penstemon digitalis Nutt. ex Sims (Plantaginaceae) is a native wild flower found in the meadows and prairies of North America. *Penstemon digitalis* is visited by a number of bee pollinators throughout its range varying in size from small to large

bodied bees (Clinebell & Bernhardt, 1998; Mitchell & Ankeny, 2001; Dieringer & Cabrera, 2002). Flowers are protandrous and self-compatible (Zorn-Arnold & Howe, 2007), however, bagged flowers fail to set seed in our NY populations, suggesting that pollinators are necessary for seed set. An unidentified micro-lepidopteran is a pre-dispersal seed predator in both Ohio (Mitchell & Ankeny, 2001) and New York (Parachnowitsch, personal observation). The flowers are mainly white with variable purple striping inside the throat of the corolla tube. Sticky trichomes cover the flowers and flowering stems but not leaves (Thomas, 2003).

Field Experiment

We planted a common garden population of *P. digitalis* in 2007, made up of plants from 3 populations (NR, TH, WF) known to differ in morphological floral traits Parachnowitsch *et al*. (Chapter 1). The common garden was used to eliminate environmental variation in scent among our populations. Population descriptions, locations, phenotypic variation in morphological traits and natural selection on those traits can be found in Chapter 1. Plants were dug from each population after bolting but prior to flowering to prevent biased collecting. There was at least a meter between each plant to ensure each plant was from a separate genet and plants were collected from across each population. In the transplant population, plants were arranged in a complete block design with 35 plants from each population. Spacing was 0.5 meters between plants in rows of 15 plants with a meter between each row. Plants flowered for approximately seven weeks and were visited by naturally recruited bumblebees (*Bombus spp*. generally *Bombus impatiens*) and other small bodied bees, as well as the occasional hummingbird (*Archilochus colubris*) (*personal observation*). Plant mortality, failure to flower and missing data led to a sample size of 88 plants (NR = 30, TH = 26, WF = 32).

Flower colour variation

Penstemon digitalis flowers can be white or have purple striping that appears black under UV light and may act as nectar guides for pollinators (Silberglied, 1979). To estimate colour, we counted the number of lines on the corolla for flowers measured for size and scored the intensity of the colour on a four-point scale (no colour, 0; light, 1; medium, 2; dark purple, 3). Flower colour was then estimated by multiplying the number of lines by the intensity and averaged across flowers on the same plant.

Plant volatile collections

We collected volatile organic compounds (VOC) using an open-flow dynamic headspace trapping design described in Kessler and Baldwin (2001) where inflorescences and leaves are separately enclosed in 500ml polyethylene cups that functioned as trapping chambers. Air is pulled through the chambers and activated charcoal absorbent vials (ORBO-32, SIGMA-Aldrich) at about 450-500ml min^{-1}. Collections were made over four days at the peak of flowering and the number of flowers open was recorded for each plant. For each day two air controls and three leaf controls (one from each population) were collected in addition to the 27 floral bouquets. On the fourth day, in addition to sampling plants that had yet to be measured, we re-sampled six plants that had been measured previously to assess temporal and environmental variation in volatile emission.

VOC quantification

ORBO-32 absorbent vials were each eluted with 350μL and samples were stored in 1.5ml GC-vials with glass inserts. Samples were analyzed using a Varian 2200

GC/MS equipped with an Altech WAX-column (30 m, 0.25 mm internal diameter, 0.25 μm film thickness; Alltech, USA). Helium was used as carrier gas at a constant flow of 1 ml/min and the following column temperature gradient: 45 °C for 6 min, increased to 130 °C at 10 °C/min, increased to 180 °C at 5 °C/min, increased to 230 °C at 20 °C/min with a 5 min hold at 230 °C, increased to 250 °C followed by a final hold at 250 °C for 5 min. Peak area was used for VOC quantification and standardized with tetraline as an internal standard. We then subtracted the average of the two air controls from the samples for a given day and assigned the peaks as either a floral or leaf volatile. Floral volatiles were defined as those peaks emitted from the flower samples on average three times that of the emission in the leaf controls. Because scent emissions from a plant could simply be a function of differences in the number of flowers open at a given time, in addition to examining total scent, for floral specific compounds we also looked at emission per flower by dividing the total scent by the number of flowers open.

VOC identification

We identified compounds by comparing the mass spectra with those in the NIST compound library (National Institute of Standards and Technology, Gaithersburg, MD) and by comparing retention times and mass spectra with known standards.

Fitness and damage estimates

After the fruits had matured (mid-August), we collected plants to assess fitness and pre-dispersal seed predation. We measured fruit diameter (mm) of all the fruits and then assessed fruits for damage by opening them and scoring damage as present or absent. As in Parachnowitsch and Kessler (2010), we estimated female fitness as the diameter of undamaged fruits plus one half the diameter of damaged fruits. Fruit

diameter is correlated with seed set and on average, pre-dispersal seed predators consume half of the seeds of undamaged fruits (Parachnowitsch & Kessler, 2010).

Statistical Analyses

To determine whether population origin influenced volatiles (total emission and emission per flower), we used two approaches. First we applied traditional methods of a MANOVA followed by ANOVAs on individual compounds. Both block and day of measurement were included in the models as controls and the Ryan, Elinot, Gabriel, Welsh's *post hoc* test was used to determine the differences among populations. However, plants produce many volatiles and this method gives population differences for the single compounds but does not give a picture of the complete odour plume. Furthermore, it can be difficult to ascertain with of the volatiles are driving differences among the populations. Classification methods such as principle component analyses (example) and non-metric multidimensional scaling analyses (e.g. Majetic *et al*., 2008) have been used to address these issues. However, they too have their drawbacks and new methodology borrowed from bioinformatics has been suggested as a way to deal with the vast quantity of data generated by volatile collections (van Dam & Poppy, 2008).

Thus to determine global differences in volatile profiles among our three populations we used a Random Forests classification algorithm as described in Ranganathan and Borges (2009). This technique determines a minimum set of predictor volatiles that distinguish each population from the others and gives objective criteria for determining the relative importance of each volatile in population differentiation. To find the predictor volatiles that separated each population from the others we ran three Random Forest analyses with two categories 1) the population of

interest and 2) the other two populations classified as others. This allowed us to determine the unique set of volatiles that distinguished a population from the rest. We used 200 bootstrap iterations in each analyses as recommended in Ranganathan and Borges (2009). The package *varselRF* in R was used to compute these values. The probability of a sample belonging to a group (population or others) was calculated using the average out of bag probability of membership (Ranganathan & Borges, 2009). This procedure assigns a mean probability of group membership to each plant. The mean decrease in accuracy for removal from the models was calculated for each VOC.

To examine female fitness, we first tested whether seed set and seed herbivore damage differed among populations using ANOVA with block included in the model. We used Pearson correlations followed by the Dunn-Šidák correction for multiple comparisons to determine whether seed herbivore damage was correlated with plant volatiles.

We used selection differentials (S) to measure the strength and direction of the total natural selection on plant volatiles. For these analyses, we simply regressed relative fitness on each standardized volatile using univariate generalized linear models. Due to the large number of volatiles, we did not have the power to detect direct selection (selection gradients, β) using multivariate regression including all volatiles (Lande & Arnold, 1983). However, we did measure direct selection on the individual volatiles by controlling for correlations with total flower number.

Results

Volatile emissions

We found 21 plant VOCs emitted by *P. digitalis*; 12 leaf volatiles and 9 floral specific volatiles. Many compounds we detected are common floral scents. Linalool, methyl

salicylate and *trans*-β-ocimene are some of the most common floral volatiles across all flowering plants (Knudsen *et al*., 2006). In addition, we found the *cis*- isomer of β-ocimene, β-citronellol, cis-jasmone, α-cedrene, α-copaene, an unknown alkene and an unknown compound that were all higher in the flower bouquets relative to the leafs. The bouquets also emitted the following compounds that were equivalent in the leaf-only samples: germacrene-D, bergamotene, α-farnesene, trans-2-octenal, cis-6-nonenal, thujol, nerolidol, methyl benzoate, as well as one unidentified sesquiterpene and two unknown compounds (Table 3.1). All of these compounds or compound classes have been found in floral scents in other species (Knudsen *et al*., 2006) and therefore may be informative to general pollinators such as bumblebees. Corolla and/or other reproductive tissue likely produce the floral specific volatiles and the octenals appear to be emitted from the sticky trichomes exudates. Volatile emission was variable among individuals (Table 3.1), however the mean variation in all volatiles was higher across sampling days (CV = 1.271) than within plants (CV = 0.949), for those plants with two sampling days (N = 6 plants).

Table 3.1 (following page) Volatile compounds from *Penstemon digitalis* quantified with GC-MS. Results from ANOVAs testing population differences in VOCs among plants from three populations planted in a common garden are shown for the total emission and emission per flower. For simplicity, only those compounds with significant differences are included (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$), differences between populations were determined using Ryan, Elinot, Gabriel, Welsh's *post hoc* test. Peak area was standardized to an internal standard ($N = 88$).

Compound	Retention time	Mean peak area ± SE	Total emission
Leaf VOCs			
bergamotene	15.83	0.396 ± 0.063	
germacrene-D*	17.76	0.254 ± 0.046	NR>WF≥TH*
alpha-farnesene	18.03	0.097 ± 0.019	NR>TH=WF****
thujol*	19.62	0.098 ± 0.013	NR≥WF≥TH**
nerolidol	23.39	0.045 ± 0.010	NR>WF=TH****
unknown	24.26	0.082 ± 0.015	WF=NR=TH*
trans-β-ocimene	10.58	0.352 ± 0.065	
unknown-2	22.65	0.111 ± 0.021	
methyl benzoate	16.50	0.136 ± 0.045	NR>TH=WF**
unknown sesquiterpene	16.15	0.174 ± 0.043	NR>TH=WF****
Floral VOCs			
Corolla			
unknown	11.61	0.849 ± 0.141	NR=TH=WF*
cis-β-ocimene	10.25	0.086 ± 0.012	NR=WF>TH**
linalool	15.26	0.467 ± 0.068	
β-citronellol	18.41	0.140 ± 0.023	WF=NR=TH*
methyl salicylate	19.04	0.233 ± 0.068	NR>TH=WF***
cis-jasmone	21.8	0.012 ± 0.002	NR>TH=WF*
alpha-cedrene	15.58	0.096 ± 0.017	NR>WF=TH**
alpha-copaene	14.53	0.085 ± 0.010	NR>WF=TH**
Trichomes			
unknown alkene	14.45	1.552 ± 0.779	
trans-2-octenal	13.58	0.610 ± 0.086	TH>WF=NR****
cis-6-nonenal	13.87	0.527 ± 0.102	

Population variation in scent

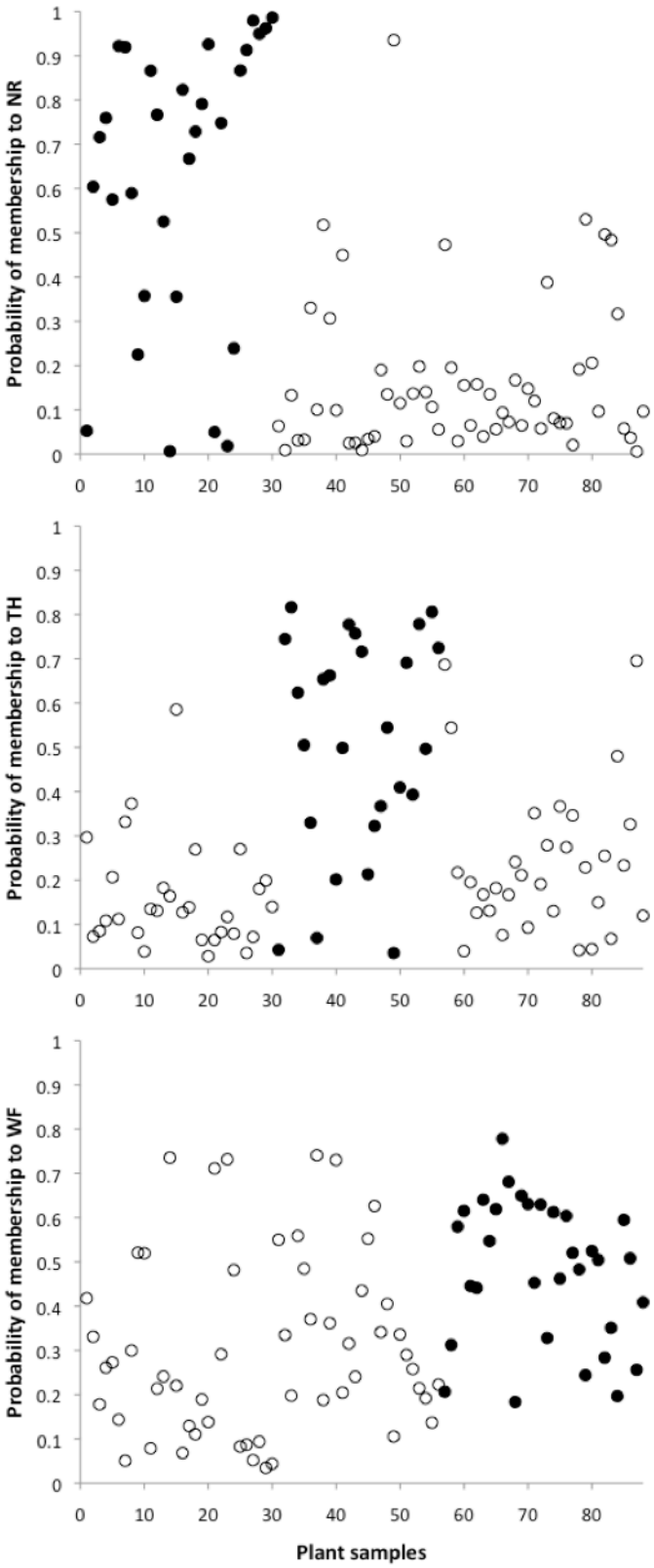
Our three *P. digitalis* populations differed in the amounts of the 21 VOCs emitted in a common garden ($P = 0.0093$) and the population differences remained when we examined the per flower emissions ($P = 0.032$). Moreover, populations did not differ in the number of open flowers on the sampling days ($P = 0.096$) nor in the total number of flowers produced over the season ($P = 0.85$), suggesting that the differences in scent were not simply driven by flower production. Eight of the leaf volatiles and seven of the floral specific volatiles varied among populations in total emission (Table 3.1). However, many fewer volatiles were found to be predictors for their respective populations in the Random Forest analyses (Table 3.2). Three volatiles were necessary to distinguish NR (α-farnesene, methyl benzoate and the unknown floral VOC) and WF (*trans*-2-octenal, α-farnesene and the unknown sesquiterpene) from the other populations, and only two were necessary for TH (*trans*-2-octenal and nerolidol).

Not surprisingly, due to their close proximity, there was considerable overlap in the scents from the three populations. In general, each population has a number of plants that were likely to be classified as 'other' and not belonging to their own population in the average out of the bag probability (Figure 3.1). However, for both NR and TH, more than half of the individuals were on average considered different than the others and few of the others were classified as belonging to that population.

Table 3.2 Predictor volatiles that distinguish each population of *Penstemon digitalis* from the other two, their frequency in the Random Forest models, and the mean percentage in the headspace.

Population	Predictor volatile	Model frequency	% in the headspace
NR	α-farnesene	0.970	1.52
	unknown floral VOC	0.845	13.26
	methyl benzoate	0.650	2.12
TH	*trans*-2-octenal	1.000	9.53
	nerolidol	0.245	0.70
WF	*trans*-2-octenal	0.810	9.53
	α-farnesene	0.710	1.52
	unknown sesquiterpene	0.245	2.72

Figure 3.1 (following page) Comparisons of volatile emissions from three populations of *Penstemon digitalis* grown in a common garden using the out of the bag probability of membership. Each population was compared to the other two in a separate analysis. Each point represents a single plant sample (the same position in each of the graphs) and the focal population is in filled circles in their respective graph. Complete separation of populations would have all samples from each treatment falling on opposite sides of the 0.5 line.


Probability of membership to NR
Probability of membership to TH
Probability of membership to WF
Plant samples

Flower colour and scent

The amount of flower colour (as estimated from the number and intensity of purple lines) was positively correlated with four *P. digitalis* volatiles. Plants with more pigmented flowers also had higher emission of cis-jasmone ($r = 0.28$), bergamotene ($r = 0.24$), α-farnesene ($r = 0.26$), the unknown leaf sesquiterpene ($r = 0.22$).

Fitness and predispersal seed predator damage

Female fitness did not differ among populations ($P = 0.52$) grown in the common garden, however seed predator damage did and followed the pattern found in the natural populations (Chapter 1). WF plants had the most damage, followed by NR, with TH receiving the least amount of damage ($P < 0.0001$). However, after controlling for multiple comparisons seed predator damage was not correlated with any of the plant volatiles, suggesting that volatile emission did not drive the patterns of damage.

Natural selection

We found significant total natural selection on one leaf and four floral volatiles (Figure 3.2). Plants that produced more linalool, *cis*-β-ocimene, β-citronellol, the unknown alkene and cis-6-nonenal had higher relative fitness than those that produced low amounts of these compounds. When we controlled for correlations between these volatiles and the total number of flowers a plant produced, selection remained for linalool, *cis*-β-ocimene, and the unknown alkene. However, after controlling for correlations with flower number, there was no significant selection on β-citronellol and cis-6-nonenal.

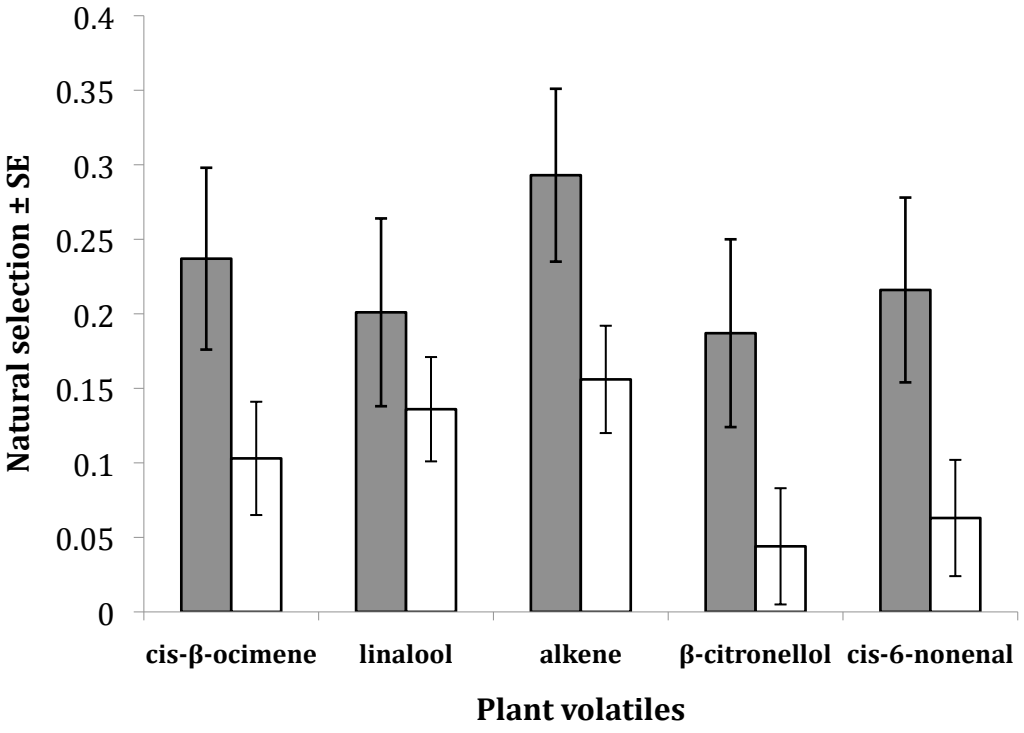


Figure 3.2 Natural selection (± 1 SE) on plant volatiles in *Penstemon digitalis*. Grey bars represent univariate selection differentials, whereas white bars represent selection gradients (β) controlling for correlations between scents and the total number of flowers produced. All selection coefficients are significant with the exception of for –citronellol and cis-6-nonenal.

Discussion

Population variation in scent

Three geographically close populations of *P. digitalis* differed in the amounts of scent produced when grown in a common garden (Table 3.1). Furthermore, because different scent compounds showed different patterns in amounts among the three populations (Table 3.1), not only would overall scent differ but the ratios among the volatiles would as well. Taken together, these plants would likely be perceived as smelling differently (Raguso, 2008b). We also found within population variation for all compounds and if this variation were heritable then we would expect populations to be able to respond to natural selection on scents.

Geographic variation in floral scents has been characterized for only a few species thus far, however variation within and among populations may be common. Floral scents are variable among populations of *Geonoma macrostachys* (Arecaceae) (Knudsen, 2002), *Herperis matronalis* (Brassicaceae) (Majetic *et al*., 2008), *Linanthus dichotomus* (Polemoniaceae) (Chess *et al*., 2008), *Magnolia kobus* (Magnoliaceae)(Azuma *et al*., 2001), *Orchis mascula* and *O. pauciflora* (Orchidaceae) (Salzmann *et al*., 2007) and *Silene latifolia* (Caryophyllacceae) (Dötterl *et al*., 2005). However, this population variation was generally found over much larger geographic ranges than the *P. digitalis* populations we sampled (but see Salzmann *et al*., 2007). Conversely, *Yucca filamentosa* (Agavaceae) scents do not vary over a large geographic range despite pollinator and presumed abiotic differences (Svensson *et al*., 2005). Much like morphological variation in floral phenotypes, we would expect volatiles to vary both within and among populations depending on the ecological interactions and evolutionary history of the species. However, unlike morphological and visual traits, very little is known about the general patterns and/or any causal links

for floral volatiles.

Scent and corolla colour

Four scents were positively correlated with corolla colour in *P. digitalis*. Phenolic compounds could be related to the amount of anthocyanins in the corolla tissue through shared pathways, however none of the volatiles correlated with colour where phenolic. The relationship between cis-jasmone, bergamotene and terpenoids with colour is generally unknown. *Penstemon* species generally contain much more pigment than *P. digitalis*, with hymenopteran flowers generally having yellow or blue-violet petals (Wilson *et al*., 2004), suggesting that the mostly white petals of this species may represent a loss of pigment. However, we did not observe selection to reduce colour (not shown) or any of these volatiles, suggesting that in this current mixed population, colour is a stable trait.

Natural selection on *Penstemon digitalis* scents

There was natural selection to linalool, *cis*-β-ocimene, β-citronellol, cis-6-nonenal and the unknown floral alkene in the common garden (Figure 3.2). These are common floral scents, and may experience direct pollinator-mediated selection in some systems. For three of these volatiles, selection was independent of correlations with a strong predictor of fitness, flower number, suggesting direct selection on these volatiles. However, when we included total flower number in our selection models, selection on two volatiles was close to zero, suggesting that selection on volatile production of these compounds was driven by correlations with flower number. Because selection for more flowers is common in wild populations of *P. digitalis* (Chapter 1), correlations with flower number may drive natural selection for increased production of these volatiles as well. Additionally, none of these volatiles were

predictor compounds that distinguished the populations from each other (Table 3.2) suggesting that these compounds may play consistent role in all the populations. Electrophysiologically active compounds can show more variation and higher population differentiation than non-active ones in deceptive orchids (Mant *et al*., 2005). The activity of the different compounds is likely to effect any pollinator- or seed predator-mediated selection in *P. digitalis*. However, unlike the orchid system, the pollinators present in the three wild populations are similar (*personal observation*). Thus, key compounds that are tied to basic functions may not vary but compounds not so strongly correlated to these other traits may be free to vary due to differential selection in the populations or to random processes such as genetic drift.

Implications of natural selection on floral scents

The finding of natural selection on floral scents leads to questions on how we interpret these results. Like morphology, correlations among traits may constrain responses to selection (e.g., Caruso, 2004). However, floral scents may act differently than other traits. Changes in emission to just one compound can alter the entire scent of the plant (Raguso, 2008b), suggesting that selection on a single compound could lead to dramatically different scent bouquets, as perceived by pollinators and possibly antagonists. Depending on whether pollinators discriminate against new phenotypes or not, this could constrain the evolution of scents by reducing pollinator visits to novel forms. Pollinator discrimination could also accelerate population change if pollinator constancy functions as a mechanism of assortative mating.

In addition to increasing our ability to detect population variation by growing plants in a common garden, we were able to increase our power to detect natural selection on scents by expanding the phenotypes available for selection to act on by including variation from three populations. However, it is important to note that

natural selection is context dependent and scents under natural selection in the common garden may not be those that experience selection in the wild populations.

Conclusions

Natural selection is thought to have shaped the expression of scents in the angiosperms (and other contexts). However, we are a long way from understanding how selection functions in current populations. This study shows that there can be variation in floral scent over very short distances, even without obvious divergent selection pressures in the different populations. Additionally, we could detect natural selection on scents. However, how these pressures play out in wild populations and who the agents of selection are, is largely unknown. Furthermore, our data suggest that selection can act on single compounds. But we know from empirical work that changes to single compounds in a blend can alter the perception of scent (Raguso, 2008b), making the evolution of scents within populations an interesting and open question.

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CHAPTER 4

Two ways to lose your seed herbivore? Consequences of flowering phenology for the evolution of latex in *Lobelia siphilitica*

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Abstract

Optimal defence theory predicts that plants which escape herbivory should reduce costly and unnecessary defences. This prediction has been tested in plants that escape herbivory in space by growing in novel environments. However, plants may also escape in time by exposing vulnerable tissues when herbivores are absent. We examined whether delaying flowering, which allows escape from pre-dispersal seed herbivory in *Lobelia siphilitica,* affected the production of a latex defence. We estimated phenotypic and genetic correlations among flowering phenology, latex production and four fitness correlates for *L. siphilitica* growing in the absence of seed herbivores. Consistent with optimal defence theory, later flowering was phenotypically and genetically correlated with reduced latex. In addition, delayed flowering, but not latex production, was negatively correlated with fitness, suggesting that escaping herbivory in time is costly. Our results suggest that when herbivores attack reproductive tissues, changes in flowering phenology can also influence the evolution of herbivore defences.

Introduction

Although chemical defences allow plants to resist herbivores, these defences can vary between individuals, both within and among populations (e.g., Stamp, 2003). Optimal defence theory suggests that this variation can be explained by the relative costs and benefits of defence: plants that can escape herbivory should invest less in costly defences and more in growth and reproduction, relative to plants that cannot escape attack (Feeny, 1976; McKey, 1979; Rhoades, 1979). Spatial escape from herbivory has been extensively studied (e.g., Orians & Ward, 2010). In contrast, variation in defence in plants that escape herbivory in time has received less attention, even though temporal escape may also be common (Elzinga *et al.*, 2007). For example, plants can

escape herbivory by outgrowing your herbivores (Allcock & Hik, 2004) or presenting vulnerable tissues such as young, expanding leaves (Kursar & Coley, 2003) and reproductive structures (Juenger *et al.*, 2005; Atlan *et al.*, 2010) when herbivores are absent.

A plant's reproductive structures may be particularly likely to escape herbivory in time for two reasons. First, flowers, unlike many vegetative structures, are often present for only a portion of the plant's adult life cycle. Consequently, altering flowering phenology can allow plants to escape herbivory on reproductive structures, even if it means switching pollinators (e.g., Kessler *et al.*, 2010). In contrast, escape in time from herbivores that attack structures such as stems and roots may be more difficult due to the continuous exposure of those tissues. Second, because damage to reproductive structures directly affects fitness, plants should be under particularly strong selection to either escape or resist this damage. There can be strong selection against flowering when florivores or pre-dispersal seed herbivores are abundant (Schemske, 1984; Pilson, 2000; Parachnowitsch & Caruso, 2008), supporting the escape hypothesis. However, floral tissues can also be highly defended (e.g., Strauss et al., 2004), supporting the resistance hypothesis. If these alternatives are costly, than we would predict a correlation between flowering phenology and physical and/or chemical defences. Specifically, plants that flower when herbivores are abundant should invest more heavily in defences than plants that flower when herbivores are rare. However, the relationship between flowering phenology and defence has rarely been studied (but see Berenbaum *et al.*, 1986; Juenger *et al.*, 2005).

We used the wildflower *Lobelia siphilitica* L. (Lobeliaceae) to test whether flowering phenology is correlated with herbivore defences. *Lobelia siphilitica* is attacked by the specialist pre-dispersal seed herbivore *Cleopmiarus hispidulus* LeConte (Coleoptera: Curculionidae; Anderson, 1973) throughout much of its range in

eastern North America. This herbivore attacks significantly more flowers on *L. siphilitica* plants that flower earlier (Figure 4.1), resulting in direct selection for later flowering (Parachnowitsch & Caruso, 2008). Consequently, delayed flowering is a mechanism by which *L. siphilitica* can escape *C. hispidulus* herbivory. *Lobelia siphilitica* also produces an alkaloid-rich latex exudate upon damage (Kesting *et al.*, 2009), which likely functions as a plant defence (Agrawal & Konno, 2009). Latex can act as a physical defence by gumming insect mouth parts, in addition to containing defensive compounds (Agrawal & Konno, 2009). Vegetative damage by other herbivores is generally low (Parachnowitsch, *personal observation*), suggesting that seed herbivores may be a strong selective force for *L. siphilitica* defence in addition to escape.

We measured flowering phenology and latex production in greenhouse-grown *L. siphilitica* to test the predictions of optimal defence theory when plants escape herbivory in time. Using a known family structure we estimated phenotypic and genetic correlations between the number of days to flower and latex production, as well as the heritability for these traits. We hypothesized that the number of days to flower and latex production would be negatively correlated because in the field late-flowering *L. siphilitica* escape most *C. hispidulus* damage (Parachnowitsch & Caruso, 2008) and therefore should produce less latex than early-flowering plants. In addition, we measured four fitness correlates to test whether delayed flowering and high latex production are costly in the absence of *C. hispidulus*, a key assumption of defence theories.

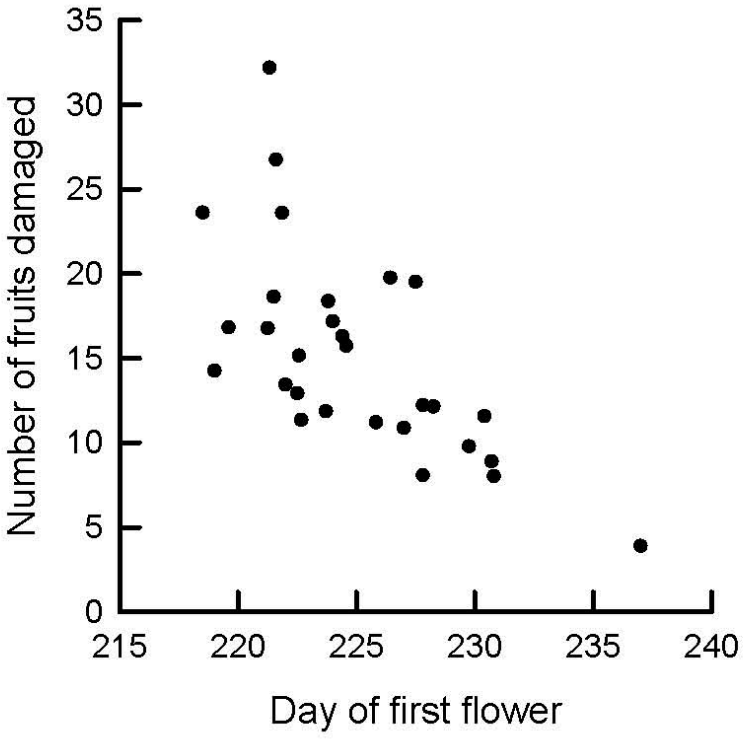

Figure 4.1. Relationship between flowering phenology and pre-dispersal seed herbivore damage in *Lobelia siphilitica* from a 2004 field experiment. Each point represents a maternal mean of two to eight plants ($N = 29$) from which 23 families were drawn for this study. Figure redrawn from Parachnowitsch & Caruso (2008). Day refers to Julian date where January 1 = 1.

Materials and methods

Study system

Lobelia siphilitica is a short-lived, herbaceous perennial native to eastern North America. It reproduces by a single racemose inflorescence, although some individuals produce additional lateral inflorescences. *Lobelia siphilitica* is self-compatible but cannot autonomously self-fertilize, making pollinators essential for seed set (Johnston, 1992). In Ontario, Canada, plants flower from late July into September and fruits ripen from September to early October (Parachnowitsch & Caruso, 2008). Although *L. siphilitica* is gynodioecious (Dudle *et al.*, 2001), female plants are rare in the northern part of its range (Caruso & Case, 2007).

Study design

The seeds used for our study were offspring of *L. siphilitica* included in a field experiment designed to estimate the strength of phenotypic selection on flowering phenology and identify the agents of this selection (Parachnowitsch & Caruso, 2008). To generate plants for this field experiment, we collected open-pollinated seeds (hereafter grand-maternal families) from an *L. siphilitica* population near Guelph, Ontario, Canada. The seeds were grown to flowering in the greenhouse and then returned to their source population, where they were also open-pollinated. We selected 46 of these open-pollinated maternal families, two from each of 23 grand-maternal families, for the experiment described in this paper. Given this design, offspring of each maternal family were half- or full-siblings. The offspring within each grand-maternal family were cousins.

We rinsed seeds in a distilled water, bleach and ethanol solution (16:1:1) to break dormancy (Dudle et al., 2001). All seeds from each fruit were germinated on wet filter paper in Petri dishes and the day of first germination for each dish was

recorded. To ensure that we had enough seedlings, we germinated seeds from two fruits per maternal family. If both dishes had successful germination, we chose the fruit that began germinating earlier. However, both fruits were used from two of the maternal families to ensure that we had enough seedlings. We transplanted 24 seedlings per maternal family to 72-well plug trays and replaced any dead seedlings after four days. Families were randomly assigned to a position within trays and trays were rotated within the greenhouse weekly. Seedlings were grown for six weeks before transplanting 12 plants per maternal family to 10 cm diameter pots filled with greenhouse potting soil (Promix®). We randomly assigned plants to trays and bottom-watered to maintain flooded soil conditions. Plants were treated for common greenhouse pests (thrips, whiteflies and fungi) and fertilised as necessary.

Phenotypic measurements

To determine whether herbivore escape and defence were correlated in *L. siphilitica*, we measured flowering time and latex production. We censused plants daily to determine the day of first flower. To estimate latex production, we clipped two of the first 5-10 flowers per plant with scissors and collected the latex exudate on pre-dried and pre-weighed filter paper (Whatman's No. 1; as in Agrawal *et al.*, 2008). We weighed the latex-soaked filter paper both prior to and after drying at 60°C for at least 24 h to estimate wet and dry latex mass, respectively. Wet and dry latex mass were strongly positively correlated in *L. siphilitica* ($r = 0.735$, 95% CI = 0.691 – 0.773, $N = 478$, $P < 0.0001$), and therefore we only present dry mass. Because flowers within a plant are not independent of each other, we used the mean latex exuded by the two flowers collected from each plant for all of our analyses.

In addition, we non-destructively estimated three traits that are correlates of female fitness in *L. siphilitica*: flower size, inflorescence height, and rosette number.

Lobelia siphilitica plants with larger flowers produce more seeds (Caruso & Yakobowski, 2008; Parachnowitsch & Caruso, 2008). We measured petal width, petal length and corolla tube width (as in Parachnowitsch & Caruso, 2008) for at least five flowers per plant. We then took the geometric mean of these three measurements (e.g. Williams & Conner, 2001) to get an overall estimate of flower size. Height is also positively correlated with total seed set in *L. siphilitica* (Parachnowitsch & Caruso, 2008). When all plants had finished flowering, we measured inflorescence height and the number of rosettes produced. Because rosettes can overwinter and produce a flowering stalk in the following year (Beaudoin Yetter, 1989), they estimate the potential for asexual reproduction in *L. siphilitica*.

When all plants had finished flowering, we destructively estimated final biomass as an additional fitness correlate. Unlike the field, in the greenhouse, the flowering season was not ended by frost, therefore we likely overestimate the fitness of late-flowering plants by allowing them to fully flower. We clipped the inflorescence and any rosettes and dried them at 45 °C for 24 h to measure aboveground biomass. To estimate belowground biomass, we washed, dried, and weighed the roots of a subset of the plants ($N = 90$) in the study. Initially, mass was estimated separately for the roots that were contained in the pot and those that emerged out of the pot into the water-filled tray. Because the mass of contained and emerged roots was positively correlated ($r = 0.411$, df = 89, $P < 0.0001$), we estimated belowground biomass for the remaining plants based on the mass of their emerged roots (belowground root biomass = 2.79 + 1.88 × emerged root mass + emerged root mass). We estimated final biomass as the sum of the aboveground and estimated belowground biomass for each plant. This estimate of final biomass was strongly positively correlated with the sum of aboveground biomass and emerged root biomass ($r = 0.976$, df = 89, $P < 0.0001$).

Statistical analysis

Our final data set ($N = 483$ plants) was unbalanced, with 7-12 offspring per dam. We eliminated 16 female plants plus one unsexed plant from our data set because female *L. siphilitica* can differ phenotypically from hermaphrodites (Caruso *et al.*, 2003). In addition, 48 plants died prior to the end of the study and four plants were excluded because of missing flowering time data. Finally, some traits were not measured on all plants and thus had an $N < 483$ (see Table 1). All analyses were done using either SAS (version 9.1) or SPSS (version 18).

Prior to analyzing our data, we used ANOVA to confirm that differences in flowering time between grand-maternal families or nested maternal families were not due to differences in germination time. Germination time did not differ significantly among grand-maternal families ($F_{22,86} = 2.10$, $P = 0.09$) or nested maternal families ($F_{21,86} = 1.94$, $P = 0.11$). Consequently, we did not include germination time as a covariate in any of our analyses.

We also used ANOVA to test whether there was a genetic basis to variation in flowering phenology, latex, and our four fitness correlates (flower size, inflorescence height, rosette number, and total biomass). Our model included terms for grand-maternal family and maternal family nested within grand-maternal family. In addition, we included a term for planting tray to control for any effect of location in the greenhouse on phenotype. If the term for grand-maternal family and/or maternal family was significant, then we concluded that there was a genetic basis to variation in that trait.

We estimated phenotypic correlations among flowering phenology, latex and four fitness correlates (flower size, inflorescence height, rosette number, and biomass) as the Pearson correlation coefficient. To test whether there was a genetic basis to

these phenotypic correlations, we estimated the genetic correlation as the Pearson correlation coefficient among maternal family means. Family mean correlations can be biased estimates of the true genetic correlation (Lynch & Walsh 1998). However, for our data set family mean correlations were quite similar to genetic correlations estimated using restricted maximum likelihood approaches (data not shown), suggesting that our conclusions are robust to the estimation technique used. We maintained an experiment-wide error rate of $\alpha = 0.05$ for each matrix of correlations using the sequential Bonferroni correction by the Dunn-Šidák method (Sokal & Rohlf, 1995). We expect day of first flower and latex mass to be negatively phenotypically and/or genetically correlated. If herbivore escape or defence is costly, then also we expect these traits to be negatively phenotypically and/or genetically correlated with one or more fitness correlates in the absence of seed herbivory.

Four features of our design could have inflated our estimates of genetic variation and genetic correlations. First, because we have maternal rather than paternal families, our estimates of these genetic parameters include not only additive genetic variance, but also common maternal effects. If common maternal effects are substantial, then our estimates will be inflated relative to estimates of genetic parameters calculated from paternal family designs (reviewed in Lynch & Walsh, 1998). Second, because we germinated seeds from open-pollinated plants, our families consist of an unknown mixture of full- and half-siblings. Consequently, our estimates of genetic variation and genetic correlations may include dominance genetic variance in addition to additive genetic variance (reviewed in Lynch & Walsh, 1998). Third, our open-pollinated families could have included offspring produced through geitonogamous self-pollination. Such inbreeding is expected to decrease the standing genetic variation within populations (e.g., Kristensen et al., 2005). Fourth, we measured genetic variation and genetic correlations for plants grown in a greenhouse

environment. Greenhouse estimates of genetic variation for plant functional (Geber & Griffen, 2003) and floral (Conner *et al.*, 2003).traits are generally higher than greenhouse estimates. However, greenhouse and field estimates of genetic correlations are quite concordant, at least relative to estimates of genetic variation (Conner *et al.*, 2003).

Results

There was considerable phenotypic (Table 4.1; Figure 4.2a) and genetic (Table 4.2; Figure 4.2b) variation in flowering phenology, latex production, and fitness correlates of *L. siphilitica*. Day of first flower and dry latex mass varied significantly among both grand-maternal families and maternal families. In addition, we detected effects of grand-maternal family and maternal family on three of the four fitness correlates (flower size, inflorescence height, and biomass). In contrast, rosette number varied significantly among maternal families, but not among grand-maternal families.

Table 4.1 Summary statistics for flowering phenology, latex defence and four fitness correlates of greenhouse-grown *Lobelia siphilitica*.

Phenotypic traits	Mean (SD)	Range	*N*
Days to first flower	123 (11)	101-153	483
Dry latex mass (mg)	0.32 (0.33)	0-2.25	478
Flower size (mm)	12.00 (0.87)	9.89-14.91	483
Inflorescence height (cm)	61 (12)	28-106	477
Rosette number	10 (5)	0-25	477
Total biomass (g)	16.76 (5.49)	5.40-36.17	397

Table 4.2 Effects of grand-maternal family, maternal family and planting tray on variation in flowering phenology, latex defence and four fitness correlates of greenhouse-grown *Lobelia siphilitica*.

Phenotypic traits	Grand-maternal family	Maternal family	Planting tray
Days to first flower	$F_{22,454} = 4.15$****	$F_{23,454} = 4.47$****	$F_{93,454} = 1.38$*
Dry latex mass	$F_{22,451} = 1.88$*	$F_{23,451} = 2.61$****	$F_{93,451} = 1.36$*
Flower size	$F_{22,454} = 2.74$****	$F_{23,454} =2.00$**	$F_{93,454} = 1.13$
Inflorescence height	$F_{22,450} = 2.83$****	$F_{23,450} = 2.34$***	$F_{93,450} = 2.21$****
Rosette number	$F_{22,450} = 1.04$	$F_{23,450} = 21.73$*	$F_{93,450} = 1.24$
Total biomass	$F_{22,372} = 2.49$***	$F_{23,372} = 3.45$****	$F_{93,372} = 1.63$**

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Both genetic and phenotypic correlations suggest that flowering time and latex production are correlated in *L. siphilitica*, as predicted by optimal defence theory (Table 3; Figs. 2a, b). *Lobelia siphilitica* plants and families that flowered later had significantly lower latex mass.

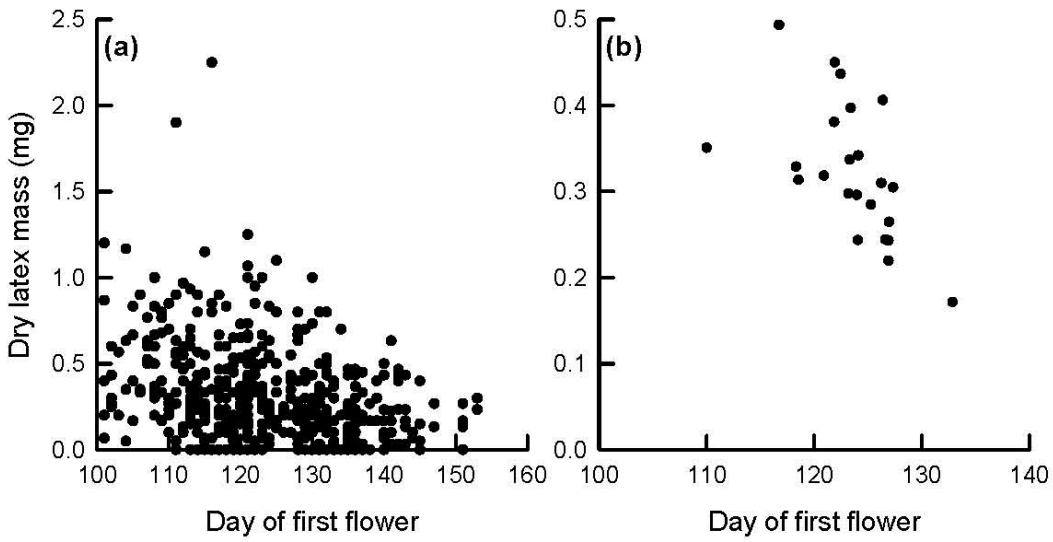

Figure 4.2 Relationship between flowering phenology and latex defence in greenhouse-grown *Lobelia siphilitica*. **(a)** Phenotypic correlation between days to first flower and dry latex mass ($N = 478$). **(b)** Grandmaternal family mean correlation between days to first flower and dry latex mass ($N = 23$).

Plants that flowered earlier produced larger flowers, taller inflorescences and accumulated a greater final biomass than *L. siphilitica* that flowered later (Table 4.3). In contrast, day of first flower was not phenotypically correlated with rosette number. Of the three fitness correlates that were phenotypically correlated with day of first flower, final biomass was significantly genetically correlated after Bonferroni correction.

In contrast, latex mass was not significantly negatively correlated with any of the four fitness correlates that we measured (Table 4.3). Instead, plants with greater latex mass produced significantly taller inflorescences and greater biomass. The correlation between inflorescence length and latex mass was independent of positive

correlations between these traits and day of first flower (partial correlation: 0.003, P = 0.009, N = 472), however the relationship between biomass and latex was not (0.0007, P = 0.8, N = 392) . Neither correlation between latex mass and the two fitness correlates (inflorescence height and biomass) had a significant genetic basis.

Table 4.3 Phenotypic and genetic correlations for flowering phenology, latex defence, and four fitness correlates of greenhouse-grown *Lobelia siphilitica*. Phenotypic correlations are above and genetic correlations are below the diagonal. N = 392-483 for phenotypic correlations. N = 46 for genetic correlations. Phenotypic and genetic correlations in **bold** are significantly ($P < 0.05$) different from zero after Bonferroni correction by the Dunn-Sidak method. Genetic correlations in *italics* were significant prior to but not after Bonferroni correction.

	Days to first flower	Dry latex mass	Flower size	Inflorescence height	Rosette number	Total biomass
DF	-	**-0.31**	**-0.17**	**-0.29**	-0.07	**-0.50**
LM	**-0.56**	-	0.11	**0.20**	-0.03	**0.16**
FS	*-0.31*	0.19	-	**0.17**	-0.04	0.10
IH	*-0.36*	0.22	*0.31*	-	-0.06	**0.35**
RN	-0.09	0.01	0.15	-0.13	-	**0.26**
TB	**-0.54**	*0.40*	0.25	*0.41*	*0.31*	-

Discussion

As we predicted based on optimal defence theory (Rhoades, 1979), *L. siphilitica* plants that flowered later and therefore would escape pre-dispersal seed herbivory in the field (Parachnowitsch & Caruso, 2008) also produced less latex defence. To our knowledge, this is the first study to detect a significant, genetically-based negative correlation between herbivore escape in time and defence. Our results contrast with those of Juenger *et al.* (2005) and Berenbaum *et al.* (1986), who found that plants that escaped herbivory in time produced more, rather than less, chemical defence against these herbivores. One potential explanation for why a negative correlation between herbivore escape and defence is evident in *L. siphilitica*, but not in *Ipomopsis aggregata* (Juenger *et al*. 2005) or *Pastinaca sativa* (Berenbaum *et al*. 1986) is that *L. siphilitica* flowers later in the season. In fall-flowering species such as *L. siphilitica*, any selection to delay flowering to escape herbivores might leave inadequate time to mature seeds prior to the onset of winter. Thus the biotic and seasonal selection pressures may oppose one another in *L. siphilitica*. This could result in strong selection for resistance to herbivory among early-flowering *L. siphilitica* plants, a hypothesis that could be tested by measuring phenotypic selection on latex production in the field.

Escaping herbivory through delayed flowering could be costly for *L. siphilitica* plants. Plants that flowered later produced smaller flowers, shorter inflorescences and accumulated a lower final biomass than *L. siphilitica* that flowered earlier (Table 3). *Lobelia siphilica* plants may need to acquire a certain size before they will flower, therefore biomass or resource acquisition may drive flowering time. The reduced biomass of the later flowering plants suggest that this is possible. However, our plants were harvested after flowering was completed, suggesting that whether it is biomass or some other mechanism determining flowering time, those plants which flower later are

unable to obtain the same fitness as early flowering plants in a herbivore free environment. Escape in time, particularly from herbivores that attack reproductive tissue, has been documented in other species (e.g., English-Loeb & Karban, 1992; Bishop & Schemske, 1998), however, this is the first study to demonstrate that such escape can be costly. Moreover, our estimate of the cost of herbivore escape in time for *L. siphilitica* is likely an underestimate because it only reflects resource-based trade-offs ("direct costs"; Strauss *et al*., 2002). Costs can also arise from interactions with the biotic or abiotic environment ("ecological costs"; Strauss *et al*., 2002). In *L. siphilitica*, we hypothesize that delayed flowering would also incur such ecological costs because in this fall-flowering species, plants that flower later might not have adequate time to mature seeds before the onset of winter. More generally, our results suggest that in addition to the direct and ecological costs of defence traits such as phytochemicals (Strauss, 1997), traits that allow plants to escape herbivory in time may also carry costs. Thus, plant phenology maybe an important factor to consider when examining the correlated evolution of alternative defence strategies ("defence syndromes"; Agrawal & Fishbein, 2006).

In contrast to herbivore escape, we found no evidence that latex production was costly in *L. siphilitica*. Instead, *L. siphilitica* with high latex production had taller inflorescences and a larger final biomass than plants with low latex production (Table 3). However, like most studies of costs of herbivore resistance (reviewed in Strauss *et al.*, 2002), we tested whether these costs were expressed through female fitness correlates. It is possible that costs of latex production in *L. siphilitica* are expressed through pollen production, a male fitness correlate. Both *L. siphilitica* pollen (Dudle, 1999) and the alkaloids in *Lobelia* spp. latex (Kesting *et al.*, 2009) are rich in nitrogen. In addition female *L. siphilitica* plants, which by definition do not incur male fitness costs, had 64% higher latex production than hermaphrodites (unpaired *t*-test assuming

unequal variances; $t = 2.413$, df = 15, $P = 0.029$), as expected if they allocate nitrogen not used for pollen production to herbivore resistance.

Although latex production has evolved multiple times in the angiosperms and is a key innovation in some clades, little is known about its evolutionary ecology (Agrawal & Konno, 2009). We found that there was significant genetic variation for latex production of *L. siphilitica* (Table 2). The only other study (Agrawal, 2005) that estimated quantitative genetic parameters for latex production also detected significant genetic variation for this trait, suggesting that it could evolve in response to selection by herbivores. However, Agrawal (2005) found that herbivore-mediated selection for increased latex production was weak, even though latex has no known functions other than as a defence against herbivory (Agrawal & Konno, 2009). Measuring selection on latex production in species such as *L. siphilitica* could indicate whether this weak relationship between latex and fitness is common in plant populations.

Our studies on *L. siphilitica* suggest that changes in flowering phenology can not only affect interactions with herbivores, but may also alter natural selection on chemical defences. Specifically, any herbivore-mediated selection for later flowering in *L. siphilitica* should result in indirect selection for reduced latex production. Flowering phenology has often been considered only in terms of its ecological and evolutionary effects on interactions for pollinators (Elzinga *et al.*, 2007). However, our data suggest that a broader view is necessary to understand the full implications of changes to flowering time.

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APPENDIX 1

Phenotypic variation and selection gradients in *Penstemon digitalis*

Table A1.1 Phenotypic variation for six traits, pre-dispersal seed predator damage, and two measures of female fitness across four populations of *Penstemon digitalis* in three years. Population abbreviations and locations found in Table 1.1. There was a significant population by year interaction for all traits, except for inflorescence length that varied across populations and years but had no interaction.

	Populations			
Phenotypic traits	BB	NR	TH	WF
		2006		
		$N = 57$		$N = 55$
Flower size	-	7.36 ± 0.08	-	7.06 ± 0.08
Flower colour	-	22 ± 1.2	-	17 ± 1.5
Number of flowers	-	48 ± 4	-	29 ± 2
Inflorescence length	-	19.5 ± 1	-	15 ± 1
Aborted flowers	-	23 ± 2	-	16 ± 1
Plant height	-	78.5 ± 2	-	72.5 ± 1.5
Damage	-	2.14 ± 0.31	-	2.13 ± 0.27
Fruit set	-	0.53 ± 0.02	-	0.42 ± 0.03
Female fitness	-	113.51 ± 11.58	-	53.43 ± 5.36
		2007		
	$N = 65$	$N = 83$	$N = 85$	$N = 48$
Flower size	6.69 ± 0.05	6.98 ± 0.05	8.02 ± 0.08	6.35 ± 0.08
Flower colour	12 ± 1	20 ± 1	18 ± 1	19 ± 2
Number of flowers	31 ± 2	32 ± 2	32 ± 2	28 ± 3
Inflorescence length	10.5 ± 0.5	13 ± 1	14.5 ± 0.5	10.5 ± 1
Aborted flowers	9 ± 1	13 ± 1	8 ± 1	14 ± 1

Plant height	64 ± 1.5	68.5 ± 1.5	76.5 ± 1.5	72 ± 2.5
Damage	0.46 ± 0.12	2.12 ± 0.30	0.40 ± 0.08	1.63 ± 0.29
Fruit set	0.72 ± 0.02	0.61 ± 0.21	0.73 ± 0.16	0.47 ± 0.03
Female fitness	92.16 ± 7.15	82.33 ± 4.85	100.60 ± 7.06	61.35 ± 8.12
		2008		
		N = 88	*N* = 145	*N* = 73
Flower size	-	6.96 ± 0.05	7.84 ± 0.04	6.70 ± 0.05
Flower colour	-	24 ± 1	21 ± 1	14 ± 1
Number of flowers	-	33 ± 2	28 ± 1	24 ± 1
Inflorescence length	-	16 ± 0.5	14 ± 0.5	11 ± 0.5
Aborted flowers	-	19 ± 1	9 ± 0.5	12 ± 1
Plant height	-	68 ± 1	77.5 ± 1	68 ± 1.5
Damage	-	2.68 ± 0.34	0	3.07 ± 0.46
Fruit set	-	0.41 ± 0.02	0.67 ± 0.01	0.50 ± 0.02
Female fitness	-	61.76 ± 5.39	86.90 ± 3.75	50.48 ± 3.93

Notes: Flower size is the geometric mean of six floral dimensions, colour was obtained by multiplying the number of lines by the intensity, inflorescence length and height are in cm, damage is the number of fruits with pre-dispersal seed predator damage, and female fitness is the number of fruits multiplied by fruit size (accounting for damaged fruits) see text for further details.

Table A1.2 Selection gradients (± 1 SE) in four populations of *Penstemon digitalis* for three consecutive years. There was significant population by year variation in natural selection on inflorescence length ($P = 0.006$), the number of flowers produced ($P < 0.0001$) and aborted ($P < 0.0001$). Bold values represent significant selection gradients within a particular population and year (* $P < 0.05$; ‡ $P < 0.10$), sample sizes follow Table A1.1.

	Populations			
Phenotypic traits	BB	NR	TH	WF
Flower size	-	0.02 ± 0.09	-	0.006 ± 0.01
	0.01 ± 0.03	0.03 ± 0.02	-0.001 ± 0.02	-0.02 ± 0.02
	-	-0.02 ± 0.02	**0.05 ± 0.01***	-0.03 ± 0.02
Flower colour	-	0.03 ± 0.08	-	-0.02 ± 0.01
	-0.04 ± 0.02‡	0.02 ± 0.02	-0.005 ± 0.01	-0.02 ± 0.02
	-	0.01 ± 0.02	-0.008 ± 0.013	0.03 ± 0.02
Number of flowers	-	**2.5 ± 0.21***	**-**	**1.3 ± 0.03***
	1.4 ± 0.04*	**1.3 ± 0.03***	**0.08 ± 0.02***	**1.5 ± 0.03***
	-	**1.5 ± 0.03***	**1.1 ± 0.03***	**1.0 ± 0.03***
Inflorescence length	-	**0.33 ± 0.15***	-	0.03 ± 0.02‡
	-0.007 ± 0.04	0.01 ± 0.02	0.01 ± 0.002	-0.04 ± 0.02‡
	-	0.008 ± 0.03	0.02 ± 0.02	-0.000 ± 0.03
Aborted flowers	-	**-1.4 ± 0.2***	**-**	**-0.83 ± 0.02***
	-0.62 ± 0.03*	**-0.67 ± 0.03***	**-0.28 ± 0.02***	**-0.74 ± 0.02***
	-	**-0.75 ± 0.03***	**-0.45 ± 0.02***	**-0.49 ± 0.03***
Plant height	-	0.030 ± 0.13	**-**	-0.005 ± 0.02
	0.02 ± 0.04	-0.007 ± 0.03	0.02 ± 0.17	0.07 ± 0.03
	-	-0.05 ± 0.03‡	0.01 ± 0.02	-0.01 ± 0.02

APPENDIX 2

Summary of selection survey data and comparisons

Table A2.1. Number of estimates of variation in selection across multiple levels time or space. Episodes refer to selection across life history stages vs. years. "Sites" refers to multiple populations of a given species without regard to habitat differences whereas "habitat" refers to populations from locations that were specifically chosen by the authors based on some abiotic or biotic difference.

	Selection differentials		Selection gradients	
Factor	Number of estimates	Number of studies	Number of estimates	Number of studies
Time	57	23	73	29
	31	15	41	18
Episode				
Year	26	12	32	15
Space	69	28	92	35
Site	31	13	38	16
Habitat	38	15	54	19

Table A2.2 Mean standard deviations and statistics from mixed model ANOVAS comparing temporal and spatial variation in selection.

Comparison	Differential	Gradient
	Pattern of selection	
Episode versus year	0.132 vs 0.233	0.141 vs 0.264
	$F_{1,33} = 2.78, P = 0.10$	$F_{1,43} = 1.57, P = 0.22$
Site versus habitat	0.316 vs 0.174	0.283 vs 0.203
	$F_{1,41} = 0.98, P = 0.33$	$F_{1,57} = 0.46, P = 0.50$
Space versus time	0.237 vs 0.179	0.236 vs 0.239
	$F_{1,84} = 1.10, P = 0.30$	$F_{1,112} = 0.93, P = 0.34$
	Strength of selection	
Episode versus year	0.114 vs 0.205	0.112 vs 0.311
	$F_{1,33} = 1.35, P = 0.25$	$F_{1,43} = 1.23, P = 0.27$
Site versus habitat	0.258 vs 0.136	0.204 vs 0.167
	$F_{1,41} = 0.80, P = 0.38$	$F_{1,57} = 0.21, P = 0.65$
Space versus time	0.191 vs 0.155	0.201 vs 0.199
	$F_{1,84} = 0.89, P = 0.35$	$F_{1,112} = 0.03, P = 0.87$