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GENERALIZATION IN PLANT POLLINATION SYSTEMS AND ITS EFFECT ON FLORAL EVOLUTION WITHIN AND AMONG POPULATIONS OF WILD RADISH (*RAPHANUS RAPHANISTRUM*)

By

Heather Farrah Sahli

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ABSTRACT

GENERALIZATION IN PLANT POLLINATION SYSTEMS AND ITS EFFECT ON FLORAL EVOLUTION WITHIN AND AMONG POPULATIONS OF WILD RADISH (*RAPHANUS RAPHANISTRUM*)

By

Heather Farrah Sahli

While specialists are thought to be well adapted to their resources, generalists are thought to be a jack of-all-trades but master of none. However, few studies have examined how selection by multiple resources influences the evolution of morphological traits in generalists. As most plants are dependent upon pollinators for reproduction, pollinators are important, and often essential, plant resources. I examined the degree of generalization in wild radish (*Raphanus raphanistrum*) and how selection by multiple pollinators influences adaptation to each taxon individually, both within and among populations.

Although previous studies of generalization in plant-pollination systems have focused primarily on estimates of species richness, pollinators exhibit great variation in visitation rates. I found that diversity indices, which take into account both species richness and evenness, provide a more useful estimate of pollinator generalization. I characterized generalization in the pollinator assemblage of the widespread agricultural weed, wild radish, and studied how pollinators differed in their selection on several floral traits. Although wild radish is effectively pollinated by at least 14 pollinator genera, few of these taxa actually selected on floral traits. My findings suggest that wild radish is able to adapt to a subset of its pollinators without trading off the ability to effectively use other taxa.

Finally, I examined how selection and drift have contributed to differentiation of floral and phenological traits of wild radish by comparing estimates of population

differentiation in quantitative traits (Q_{ST}) to population differentiation in allele frequencies (F_{ST}). I found that differences in most floral traits among populations can be explained by drift alone. I also found that life history traits such as ovule number and flowering time were under strong divergent selection across populations, suggesting life history traits may have been important in colonization and adaptation to new habitats.

Overall, my results indicate that wild radish has successfully evolved to use many pollinators to effect reproduction, that it can adapt to a subset of these pollinators without trading off the ability to use others effectively, and that its success as a generalist may be one reason for its successful invasion in many non-native environments.

Dedicated to my family, especially my parents, Brenda and Muhammad Sahli, who first planted the idea of getting a Ph.D. in my head before I even knew what one was. Their love and support are with me always. And to my grandmother, Nancy Payne, who helped make me the person I am today.

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CHAPTER 1

INTRODUCTION

Background

Interactions among organisms and their resources can lead to the evolution of specialization, which occurs when an organism uses only a subset of available resources. Specialists are thought to be well adapted for this subset of the resource pool, and specialization has been widely studied in ecology and evolution. Generalization, on the other hand, occurs when an organism uses many resources, and is thought to come at a cost because generalists are considered to be less well-adapted to each of their resources (Futuyma and Moreno 1988; Via 1991). In other words, generalists are often considered a 'jack-of-all-trades, master of none' (MacArthur 1972; Futuyma and Moreno 1988).

The degree to which plants specialize on pollinators has received much recent attention (e.g. Ollerton 1996; Waser et al. 1996; Johnson and Steiner 2000). Because pollinators are such an important resource for reproduction in many angiosperms (Tepedino 1979), pollinator-mediated selection is a major driving force in floral adaptation. Since the 1700s biologists have studied specialized plants and made connections between floral traits and pollinator usage (e.g. Darwin 1862; Grant and Grant 1965; Stebbins 1970; Faegri and van der Pijl 1979; Sprengle 1996). Faegri and van der Pijl (1979) used pollinator-mediated selection to explain their concept of pollination syndromes, where convergent suites of floral characters evolved in disparate plant taxa in response to similar pollinators. Furthermore, Stebbins (1970) predicted that plants adapt to the most effective and abundant pollinator, thus resulting in pollinator specialization. Orchids, the phlox family, and numerous other taxa exhibit pollinator syndromes (Darwin 1862; Grant and Grant 1965; Faegri and van der Pijl 1979; Wyatt 1983), leading to the widely adopted assumption that plants exhibiting these syndromes only use a subset of the available pollinators. Until the mid 1990s, pollinator specialization was considered to be a common pattern in nature (Waser 1996; Johnson and Steiner 2000).

However, more generalized pollination systems are also common and the discovery of an abundance of generalized pollination systems has lead many to question how and why generalization has evolved and persists in pollination systems (Herrera 1988; Waser et al. 1996; Gomez and Zamora 1999). Furthermore, we do not yet understand how selection by different pollinators in generalist plants influences floral adaptation to different pollinators. If trade-offs are common, generalist plants may not be adapted to any of their pollinators (Herrera 1996). My dissertation research focuses on measuring selection by different pollinators on floral traits of a generalist plant, wild radish (*Raphanus raphanistrum*), to understand the importance of trade-offs in influencing adaptations in generalist pollination systems. I also examine how divergent selection on floral traits of wild radish has contributed to floral differentiation between populations over a large geographic area.

Organization of the dissertation

Chapter 2: In collaboration with Jeffrey Conner, I examined the roles that pollinator visitation and pollinator effectiveness play in influencing a pollinator's total importance to plant reproduction, where importance is measured by a pollinator's visitation rate multiplied by its effectiveness. I reviewed previous studies of 17 different plant species, all of which measured effectiveness and visitation of the majority of visiting pollinator taxa, and tested whether importance was influenced more by effectiveness versus visitation. I also characterized diversity of each plant's pollinator assemblage using Simpson's index, which takes into account not only species richness, but species evenness as well. Using an index of diversity to characterize generalization in plant pollination systems is a unique approach to comparing the degree of generalization among plant species. For most plant species included in our study, pollinator importance was largely determined by visitation rates rather than effectiveness of different pollinators. Often, pollinator effectiveness is emphasized as being important to measure in order to determine a pollinator's importance. However, our research shows that visitation rates are much more useful, and that the labor-intensive methods of estimating a pollinator's effectiveness may be unnecessary for most plant species. We also found that pollinator richness explained only 57-65% of the variation in diversity, and that the rank of specialized versus generalized taxa changed when evenness was incorporated into estimates of generalization. This chapter was published in *Oecologia* (Sahli and Conner 2006).

Chapter 3: I estimated pollinator effectiveness and visitation for 15 visiting genera of wild radish to determine which pollinators are important for plant reproduction in this species. I also compared each pollinator's ability to effectively remove pollen versus its ability to effectively cause seed set during a single visit, and examined how body size influenced a pollinator's effectiveness. The small sweat bee, *Dialictus*, is the most important pollinator of wild radish at Kellogg Biological Station, although all but one of the visiting genera are able to effectively pollinate. Differences among pollinators in their ability to effect seed set was influenced by a pollinator's body size, with larger pollinators setting more seed than smaller pollinators. However, body size did not influence pollen removal. I also found little temporal variation in the pollinator assemblage across the two years of this study, suggesting stability among pollinators from year to year.

Chapter 4: To understand how trade-offs influence adaptation of floral traits in generalist plants, I estimated selection on anther exsertion, stamen dimorphism, flower size, and flower number by the seven most important pollinator genera in wild radish. My findings show that trade-offs in adapting to different pollinators are rare, and that some pollinators select similarly on traits. Thus, although a generalist plant is experiencing different selection pressure by some pollinators, it may also adapt to multiple pollinators at once. I also estimated the total average selection gradient for each floral trait by weighting estimates of selection by each pollinator by the estimates of pollinator importance from Chapter 3. Our findings reveal that floral evolution may be primarily occurring in response to selection by the most important pollinator, *Dialictus*. However, due to the large variance around our estimate of selection, we could not detect significant selection on traits using the weighted average selection gradients

Chapter 5: In collaboration with Jeffrey Conner and Frank Shaw, I examined the degree to which differentiation in floral traits among nine populations of wild radish was due to natural selection versus genetic drift. We estimated Q_{ST} for floral and phenological traits of wild radish, which is an estimate of the amount of the total additive variation in a quantitative trait that is due to differences among populations. I compared estimates of Q_{ST} to F_{ST} at presumptively neutral microsatellite loci, which serves as the null expectation of differentiation among populations due to genetic drift. I found that most floral traits have not diverged more than neutral loci, indicating that we cannot rule out drift as the primary cause for floral differentiation among populations. However, flowering time and ovule number have diverged among populations due to natural selection, suggesting that these two traits may have been important in the spread of wild radish. Wild radish has successfully invaded a large geographic area with little adaptation of its floral traits, indicating this species has been able to be effectively pollinated despite colonization in new locations, and that divergent selection on floral traits among populations is not very strong.

Sahli, Heather F., and Jeffrey K. Conner. 2006. Characterizing ecological generalization in plant-pollination systems. Oecologia 148: 365-372.

CHAPTER 2

CHARACTERIZING ECOLOGICAL GENERALIZATION IN PLANT-POLLINATION SYSTEMS

Abstract

Despite the development of diversity indices in community ecology that incorporate both richness and evenness, pollination biologists commonly use only pollinator richness to estimate generalization. Similarly, while pollination biologists have stressed the utility of pollinator importance, incorporating both pollinator abundance and effectiveness, importance values have not been included in estimates of generalization in pollination systems. In this study we estimated pollinator generalization for 17 plant species using Simpson's diversity index, which includes richness and evenness. We compared these estimates with estimates based on only pollinator richness, and compared diversity estimates calculated using importance data with those using only visitation data. We found that pollinator richness explains only 57-65% of the variation in diversity, and that, for most plant species, pollinator importance was determined primarily by differences in visitation rather than by differences in effectiveness. While simple richness may suffice for broad comparisons of pollinator generalization, measures that incorporate evenness will provide a much more accurate understanding of generalization. Although incorporating labor-intensive measurements of pollinator effectiveness are less necessary for broad surveys, effectiveness estimates will be important for detailed studies of some plant species. Unfortunately, at this point it is impossible to predict *a priori* which species these are.

Introduction

Recent empirical studies have shown that many plant species are pollinator generalists; i.e., visited by many potential pollinator species (Herrera 1989; Waser et al. 1996; Olsen 1997; Memmott 1999; Kandori 2002). This observation sparked a debate over the relative frequency of generalization in plant-pollination systems in nature

(Waser et al. 1996; Vazquez and Aizen 2003) and how to appropriately characterize generalization (Johnson and Steiner 2000; Vazquez and Aizen 2003; Kay and Schemske 2004; Herrera 2005). These questions are relevant for basic questions such as understanding plant-pollinator webs (Memmott 1999; Memmott and Waser 2002) and measuring spatio-temporal variation in plant-pollinator interactions (Feinsinger 1978; Waser et al. 1996; Gomez and Zamora 1999; Fenster and Dudash 2001). The debate has applied importance as well. The heavy reliance of many plants on animal pollinators for reproduction (Tepedino 1979; Burd 1994) has caused growing concern that pollinator declines will cause the extinction of specialized plant species or reduce crop yield of economically important plants (Allen-Wardell et al. 1998; Kearns et al. 1998). In addition, some weedy and invasive plants rely heavily upon pollinators for reproduction and population growth (Parker 1997; Barthell et al. 2001), and their spread may be influenced by how generalized their pollination is.

While virtually all previous studies of pollinator generalization have only measured generalization as the number of visiting taxa (i.e., **pollinator richness**; see Table 2.1 for definitions), visitors may not be equal in either their **visitation** rates or their ability to effect seed set (**pollinator effectiveness**); thus, pollinator species can differ in their contribution to reproduction. Previous authors have suggested that many flower visitors are not actually pollinating, thus are not contributing to plant reproduction (e.g., Spears 1983; Johnson and Steiner 2000; Fenster et al. 2004). Furthermore, to understand how generalized a plant's pollinator assemblage is, pollination biologists have pointed out the need to characterize both quantity (visitation rates) and quality (effectiveness) of different pollinators (Johnson and Steiner 2000). The product of visitation rate and effectiveness for each pollinator species is **pollinator importance**, a measure of a pollinator's contribution to plant reproduction (Primack and Silander 1975; Waser and Price 1983; Lindsey 1984; Schemske and Horvitz 1984; Inouye et al. 1994; Kearns and Inouye 1997).

Table 2.1. Definitions of terms used in this paper

Term	Definition
Pollinator visitation	The proportion of total flower, inflorescence, or plant visits made by a pollinator taxon during a unit of time
Pollinator effectiveness	The average number of seeds produced, percent fruit set, or pollen grains deposited by a pollinator taxon during a single visit to a plant
Pollinator importance	The proportion of the total number of seeds or fruits produced by a pollinator taxon, or the proportion of the total number of pollen grains deposited by a pollinator taxon. This is often estimated as the product of visitation rate and effectiveness
Visitation richness	The number of different genera that visit a plant species
Effective richness	The number of visiting pollinator genera that are effective pollinators of a plant species
Visitation diversity	The diversity of the pollinator community of a plant species calculated using the proportion of total visits made by each pollinator taxon
Importance diversity	The diversity of the pollinator community calculated using the proportion of total number of seeds or fruits produced, or pollen grains deposited, by each pollinator taxon

However, because pollinator importance is calculated for each visiting taxon separately, no one has previously suggested a method of combining estimates of importance for each visitor to produce one estimate of generalization for a plant species.

Although pollination biologists have discussed the need to measure visitation frequency and pollinator effectiveness when characterizing pollinator diversity, pollination studies rarely use diversity indices developed over five decades ago by community ecologists, which incorporate not only species richness, but also the relative abundance of each species (evenness) (Feinsinger et al. 1987; but see Parrish and Bazzaz 1979; Bosch et al. 1997; Balvanera et al. 2005). In a recent paper, Herrera (2005) pointed out that pollinator evenness deserves consideration in future studies of pollinator generalization. When visitation is uneven, diversity indices provide a more accurate depiction of pollinator generalization than species richness alone. If pollinator richness is increased mainly by the addition of infrequently visiting species, increasing richness may have little effect on pollinator diversity and plant reproduction.

Importance and diversity can be combined into a single measure by calculating diversity indices using pollinator importance values rather than pollinator visitation. We call this **importance diversity** as opposed to **visitation diversity**. Because pollinator importance characterizes each pollinator's contribution to plant reproduction, a diversity estimate calculated using the relative importance of each pollinator is the best estimate of the diversity of pollinators contributing to plant reproduction. If pollinator visitation data misrepresent a pollinator's actual importance, then visitation diversity will poorly predict importance diversity.

We used Simpson's diversity index to calculate pollinator diversity for 17 plant species. Visitation diversity, importance diversity, and **visitation richness** (the number of pollinator taxa visiting a plant species) were calculated to assess how well visitation richness, the typical measure in studies of pollinator generalization, predicts diversity. The evenness of pollinator assemblages was calculated for each plant species to

understand differences between richness and diversity. Finally, to test the commonly held view that incorporating pollinator effectiveness provides a better understanding of a pollinator's contribution to plant reproduction than using only visitation rates, we estimated how visitation rates and effectiveness each contribute to variation in pollinator importance.

Methods

Datasets that contained comprehensive estimates of both pollinator effectiveness (measured as pollen removal/deposition or number of seeds set during a single visit) and pollinator visitation frequency were reviewed for 17 plant species (Table 2.2). Pollinator diversity for each plant species was calculated using Simpson's (1949) diversity index:

$$\frac{1}{D} = \frac{1}{\sum_{i=1}^{S} p_i^2},$$

where p_i is the proportional visitation or importance for pollinator species i and S is richness, the number of pollinator taxa visiting or pollinating a plant species . If all pollinators are equally abundant or important (i.e., evenness = 1), diversity reaches a maximum of S. Simpson's index is commonly used to characterize diversity because it takes into account richness of the pollinating assemblage as well as pollinator evenness, but is weighted more heavily towards common species rather than rare ones (Magurran 1988). Other indices measuring niche breadth or generalization incorporate relative abundances of the resources (e.g. pollinators) in a given community (see Krebs 1989 for a review) because a pollinator may visit a plant more than others simply because it is more abundant in that community. We chose Simpson's index for our study because very little pollinator community data is currently available to calculate such niche breadth indices and such indices can misrepresent how reproduction is impacted by pollinator generalization. For example, if very few pollinator taxa are present in a community, but a plant uses all of them, it would be generalized according to a niche breadth index.

Table 2.2. Plant species and studies for which pollinator data was used to calculate diversity and evenness. P=Perennial, A=Annual.

Plant Species	Life habit	# yrs	Family	Location	Habitat	# pops	Reference
Asclepias incarnata[*]	P	2	Asclepiadaceae	USA	Wetland	1	Ivey et al. 2003
Asclepias tuberosa[‡]	P	2	Asclepiadaceae	USA	Wetland	1	Fishbein and Venable 1996
Calathea ovandensis	P	3	Marantaceae	Mexico	Rainforest	4	Schemske and Horvitz 1988;Schemske and Horvitz 1984
Citrullus lanatus[§]	A	1	Curcurbitaceae	USA	Agricultural	3[†]	Kremen et al. 2002
Coffea canephora[§]	P	1	Rubiaceae	Indonesia	Agricultural	15	Klein et al. 2003
Dieffenbachia longispatha[‡]	P	3	Araceae	Costa Rica	Rainforest	1	Young 1988
Geranium thunbergii	P	2	Geraniaceae	Japan	Temperate field	1	Kandori 2002
Heterotheca subaxillaris	A	1	Asteraceae	USA	Temperate field	1	Olsen 1997
Hormathophylla spinosa [*]	P	4	Brassicaceae	Spain	Montane	3[†]	Gomez and Zamora 1999
Ipomopsis aggregata [*]	P	5	Polemoniaceae	USA	Montane	3	Mayfield et al. 2001
Lavandula latifolia[*]	P	6	Lamiaceae	Spain	Mixed woodland	1	Herrera 1987;Herrera 1989
Lithophragma parviflora[*]	P	2	Saxifragaceae	USA	Steppe	2[†]	Thompson and Pellmyr 1992
Macromeria viridiflora[*]	P	1	Boraginaceae	USA	Montane	2[†]	Boyd 2004
Prosopis velutina	P	1	Fabaceae	USA	Desert	1	Keys et al. 1995
Raphanus raphanistrum[§]	A	1	Brassicaceae	USA	Temperate field	1	Sahli and Conner unpublished
Satureja thymbra[*]	P	1	Lamiaceae	Israel	Phrygana	2[†]	Potts et al. 2001
Silene vulgaris[‡]	P	4	Caryophyllaceae	Sweden	Temperate field	1	Pettersson 1991

[*] Pollinator effectiveness was only determined for a subset of the visiting taxa.

[†] Diversity and evenness indices were calculated for each site and averaged over sites. Other studies for which multiple sites were used did not publish results from each site, but summed visits over all sites.

[‡] Pollinator visitation rate is the number of individuals per plant per unit time. All others are based on number of visits per flower or inflorescence.

[§] Pollinator effectiveness and visitation data were collected at a site where the plant is non-native.

However, in terms of how plant reproduction is impacted by a loss of just one of those pollinators, it would be relatively specialized because a loss of one pollinator taxon might greatly reduce plant reproduction.

Pollinator evenness was calculated for all plant species by dividing Simpson's diversity index by pollinator richness:

$$E_{1/D} = \frac{1/D}{S}.$$

Estimating $E_{1/D}$ allows the examination of the two components of diversity (evenness and richness) separately (Smith and Wilson 1996). Evenness reaches a maximum of one when $S = 1/D$, and diversity declines for a given S when evenness declines. Because the taxonomic level at which pollinators were identified varied between studies, pollinators were grouped at the genus level when possible.

Diversity and evenness for studies that published visitation data from multiple sites was calculated for each site separately, then averaged to estimate the pollinator diversity contributing to a single population's reproduction. Visitation data for *Calathea, Coffea,* and *Ipomopsis* were published as a sum across multiple sites (Table 2.2), so estimates for these species overestimate diversity in any one population. When visitation data were collected for multiple years (only done for perennial species), data were summed across years to provide one total diversity estimate. For perennial plants, characterizing the diversity of the pollinating assemblage over multiple years more accurately estimates the diversity of pollinators affecting lifetime plant reproduction. Differences among studies in sampling intensity, as well as different ways of handling multiple years, multiple populations, and different taxonomic groupings do not affect the conclusions of this paper because our aim is not to understand the actual distribution of generalization, but to compare methods of estimating generalization (see Discussion). We note that annuals are underrepresented in this study so this may potentially bias our results.

Visitation vs. importance

To examine how including pollinator effectiveness alters estimates of pollinator diversity based only on visitation, pollinator diversity was calculated using both visitation and importance. Pollinator richness was determined for each plant species using visitation (visitation richness) and importance data (**effective richness**), where effective richness is the number of taxa actually pollinating a plant species. Effectiveness was not determined for 16-40% of the taxa visiting *Asclepias incarnata, Hormathophylla,* and *Ipomopsis* so these pollinator taxa were not included in comparisons of richness and evenness and richness and visitation. Leaving out the pollinator taxa for which effectiveness was not determined did not change evenness by more than 0.10, and thus resulted in little change in the relationship between richness and diversity. Because pollinator effectiveness was determined for less than half of the visiting taxa to *Satureja, Lithophragma,* and *Lavandula* and leaving out these visiting taxa resulted in increases in evenness greater than 0.10, these three studies were not included in comparisons of richness and importance diversity. However, because they obtained comprehensive visitation data, these studies were included in comparisons of visitation diversity and visitation richness.

Results

Pollinator diversity across 17 plant species

Visitation richness ranged from two to 45 genera (Table 2.3). Because importance diversity includes richness, evenness, and effectiveness of pollinators contributing to plant reproductive success, it is the most comprehensive estimate of generalization. To determine how well the most common measure of generalization, pollinator richness, predicts pollinator diversity as calculated using either visitation rates or importance values, visitation and importance diversity were each regressed on

Table 2.3. Estimates of richness, diversity, and evenness of each plant's pollinator assemblage calculated using visitation data versus importance data. Diversity and evenness were calculated using Simpson's diversity index ($1/D$) and $E_{1/D}$, respectively. Plant species are ordered by visitation richness. Data for *Citrullus, Hormathophylla, Satureja, Macromeria,* and *Lithophragma* are averaged over multiple populations, while *Ipomopsis, Calathea,* and *Coffea* are summed over multiple populations (Table 2.1). Importance data for *Satureja, Lithophragma, Hormathophylla,* and *Lavandula* are not included due to the lack of effectiveness data for the majority of the pollinating taxa.

	Visitation			Importance		
Plant Species	Richness	Diversity	Evenness	Richness	Diversity	Evenness
Ipomopsis aggregata	2	1.09	0.55	2	1.15	0.58
Dieffenbachia longispatha	2	1.90	0.95	2	1.75	0.88
Macromeria viridiflora	3	1.48	0.49	3	1.19	0.40
Prosopis velutina	5	4.39	0.88	5	2.89	0.58
Asclepias incarnata	6	2.14	0.36	6	2.99	0.50
Citrullus lanatus	6.33	2.46	0.39	6.33	2.90	0.46
Asclepias tuberosa	7	3.89	0.56	4	3.60	0.90
Calathea ovandensis	9	3.10	0.34	6	2.23	0.37
Coffea canephora	9	3.72	0.41	9	4.10	0.46
Heterotheca subaxillaris	10	4.43	0.44	10	4.74	0.47
Satureja thymbra	11	5.03	0.46			
Hormathophylla spinosa	12.7	2.5	0.2			
Lithophragma parviflora	13	1.52	0.12			
Silene vulgaris	13	5.60	0.43	10	6.20	0.62
Raphanus raphanistrum	15	3.90	0.26	14	3.91	0.28
Geranium thunbergii	29	12.48	0.43	24	6.46	0.27
Lavandula latifolia	45	7.51	0.17			

pollinator richness among species. An inherent correlation between richness and diversity exists because richness is included in diversity (DeBenedictis 1973; Stirling and Wilsey 2001). Still, richness alone explained just 57% of the variation in visitation diversity and 65% of the variation in importance diversity (Figure 2.1). The slope of the relationship between diversity and richness was much less than one due to a significant negative relationship between evenness and visitation richness (Figure 2.2). Furthermore, there were many changes in rank among plant species when richness was used as compared to visitation diversity, and these rank changes were due to differences in the evenness of pollinator assemblages (Table 2.3).

Visitation vs. importance

In roughly two-thirds of the plant species, and all of the more specialized ones (visitation richness < 7 taxa), all visiting taxa were effective pollinators. For the other five plant species, an average of three genera of visitors were not effective pollinators, representing 7-43% of the visiting taxa (Table 2.3). Richness, diversity, and evenness calculated using only visitation data were all highly significantly correlated with their corresponding estimates calculated using importance data (richness $r = 0.98$; diversity $r = 0.84$; evenness $r = 0.80$).

To determine how variation in pollinator importance is determined by effectiveness versus visitation, importance for each pollinator taxon was regressed on effectiveness and visitation separately for each plant species, and the resulting R^2 values were compared using a paired *t*-test. In most plant species, visitation rates were a far better predictor of pollinator importance than pollinator effectiveness (Table 2.4). Although there was more variance among pollinators in their visitation rates than in their effectiveness for more than half of the plant species included in this analysis (Table 2.4), the contribution of visitation and effectiveness to importance was not simply a function of the variance in each variable. Though visitation was the main determinant of

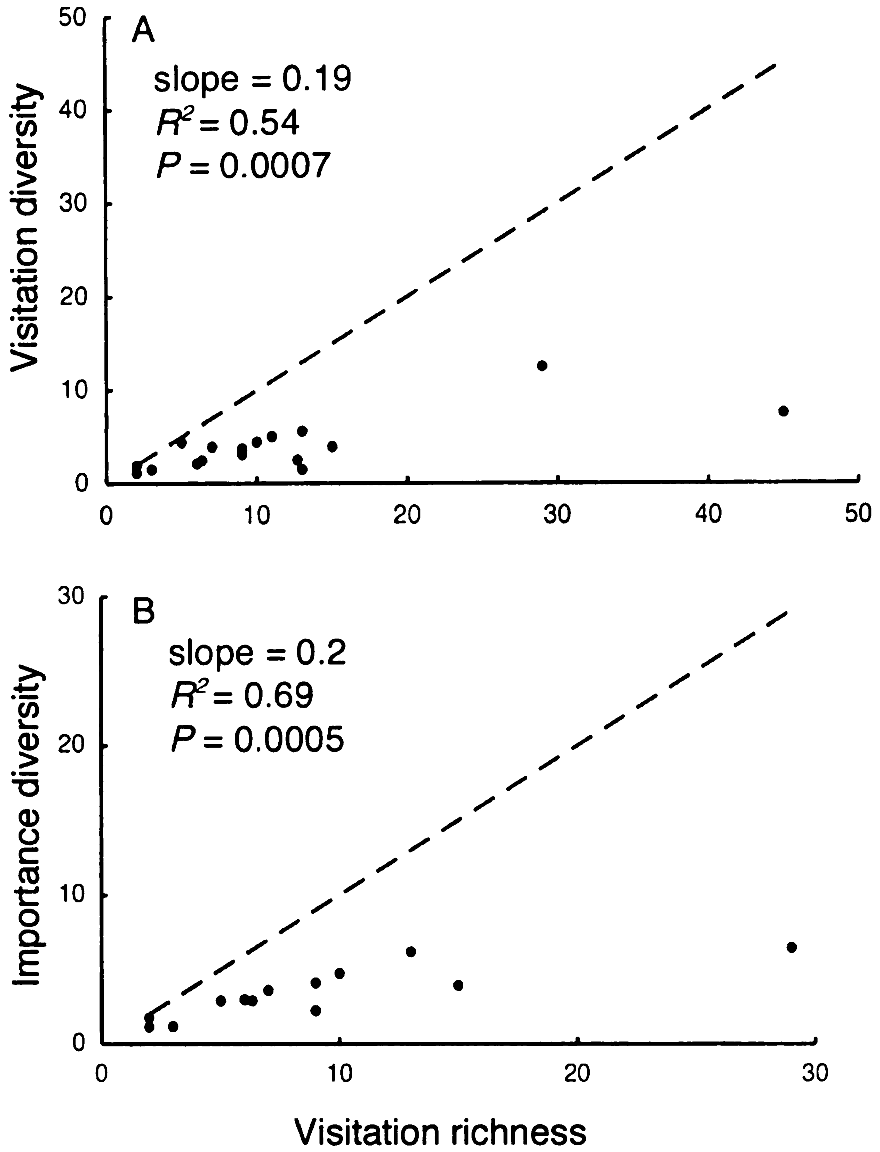

Figure 2.1. Regression of (a) visitation diversity and (b) importance diversity on visitation richness, where each point represents a different plant species. The dashed line represents the maximum diversity for each plant's pollinator assemblage if all pollinators were equal in their visitation rates (a) and importance (b); circles are the actual diversity estimates for each plant's pollinator assemblage. Values for (a) include indices for *Satureja, Lithophragma, Hormathophylla,* and *Lavandula* which are not included in (b) due to the lack of effectiveness data for the majority of the pollinating taxa.

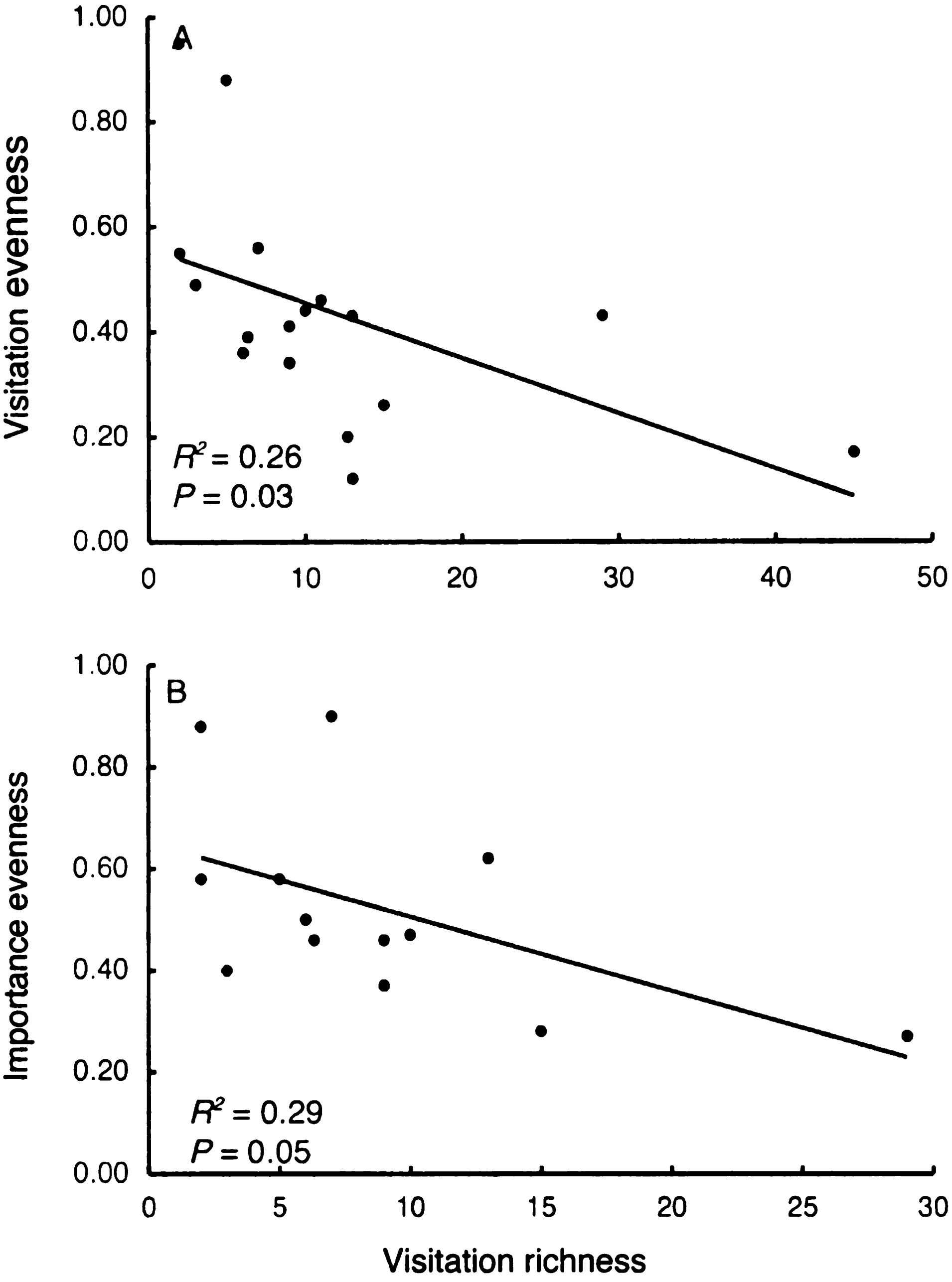

Figure 2.2. Regression of (A) visitation evenness and (B) importance evenness on visitation richness, where each point represents a different plant species. (A) includes indices for *Satureja, Lithophragma, Hormathophylla,* and *Lavandula* which are not included in (B) (see Figure 2.1).

Table 2.4. Variation in pollinator importance explained by effectiveness versus visitation and coefficients of variation of effectiveness and visitation for each plant. Visitation and effectiveness were regressed on importance separately; a multiple regression including both is overparameterized because the product of visitation and effectiveness is importance. Regressions could not be performed for *Dieffenbachia* and *Ipomopsis* because they were visited by only two pollinator genera. One average value for visitation, effectiveness, and importance was calculated for each pollinator of each plant species. Plant species are ordered by effectiveness R^2. Visitation explained a significantly greater proportion of variance in importance than did effectiveness (mean difference = 0.56; paired $t = -5.37$, $P < 0.0001$).

	R^2		CV	
Plant species	Visitation	Effectiveness	Visitation	Effectiveness
Silene vulgaris	0.68**	0.0003	1.20	1.50
Raphanus raphanistrum	0.94***	0.003	1.74	0.71
Satureja thymbra	0.78*	0.005	0.62	0.29
Heterotheca subaxillaris	0.86***	0.02	1.18	0.46
Hormathophylla spinosa	0.96**	0.02	1.13	0.21
Lithophragma parviflora	0.99***	0.06	1.77	0.60
Citrullus lanatus	0.40	0.07	1.50	0.43
Calathea ovandensis	0.81**	0.09	1.46	2.05
Geranium thunbergii	0.82***	0.11	0.43	1.05
Coffea canephora	0.99***	0.25	1.26	0.14
Lavandula latifolia	0.94***	0.26*	1.42	0.61
Asclepias tuberosa	0.83*	0.30	0.96	1.08
Asclepias incarnata	0.02	0.40	1.47	1.03
Prosopis velutina	0.63	0.81*	0.42	0.53
Macromeria viridiflora	0.99*	0.82	0.99	0.32

*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.0001$

importance overall, effectiveness proved to be more important in determining variation in pollinator importance than visitation rates for *A. incarnata* and *Prosopis* (Table 2.4).

Discussion

Due to the inherent correlation between richness and diversity, richness will always be a general surrogate for diversity. However, the present study revealed that estimates of pollinator diversity are only moderately well predicted by pollinator richness (Figure 2.1). The inability of richness measures alone to accurately predict pollinator diversity is further shown by the change in rank of several plants when richness versus diversity is used to estimate generalization. Increasing numbers of pollinator taxa resulted in a less even assemblage because many of the visiting taxa are rare, as noted previously by Herrera (1989). While pollinator richness provides some understanding of generalization in pollination systems, accounting for evenness is a much more accurate approach.

Understanding the diversity of pollinators can influence conservation and management decisions because plant reproduction and plant population growth may be greatly impacted by a reduction in pollinator availability (Kearns et al. 1998; Havens 1999; Larsen et al. 2005). For example, Ricketts (2004) found that, in a population with low bee richness, a decrease in honeybee visitation to coffee plants led to a reduction in crop yield. However, in a population with high bee richness, native bees compensated for a reduction in honeybee visitation to coffee plants, causing little reduction in reproduction--thus, a more rich or diverse pollinator assemblage might lead to more stable plant reproduction, despite losses in pollinator species. Whether richness or diversity estimates are more predictive of stability remains to be tested. Studies examining the relationship between pollinator diversity and plant reproduction in the face of shifting pollinator assemblages are greatly needed. Despite the widely held idea that pollinator effectiveness is crucial and should always be taken into account, the present study indicates that visitation rather than effectiveness is the main driving factor in determining pollinator importance. This was partly because the majority of visiting taxa were true pollinators, in contrast with the view that effective pollinators make up only a small fraction of floral visitors.

Previous studies on single plant species noted that pollinator visitation seems to play a larger role in pollinator importance than pollinator effectiveness (Motten et al. 1981; Olsen 1997), while other studies have suggested that effectiveness was important (Armbruster et al. 1989; Fenster et al. 2004). Past studies, however, did not quantify the value of visitation versus effectiveness. Similar to our findings, a recent meta-analysis (Vazquez et al. 2005) showed that visitation rates play a more important role in plant reproduction than effectiveness.

However, effectiveness did explain a substantial amount of the variation in pollinator importance in approximately one third of the plant species for which we could make this comparison. Still, visitation was a better predictor of importance for all species but *A. incarnata* and *Prosopis*, and only for *Prosopis* was the effectiveness significant (Table 2.4). It is interesting to note that effectiveness was important for both species of *Asclepias*, which have specialized modes of pollen removal/deposition. Pollinator effectiveness may be important for such species because many visitors may be unable to pollinate effectively. Due to the limited number of species included in our analysis, we could not address whether effectiveness is more important for species with specialized pollen removal/deposition mechanisms. Because we have no way of predicting for which plant species effectiveness will be important, pollination biologists should continue to measure effectiveness on the majority of pollinators whenever possible for a full understanding of how different pollinators contribute to a plant's reproductive success.

Thus far we have only addressed the ecological importance of measuring pollinator effectiveness. Many pollination studies examining pollinator effectiveness are motivated by the idea that effectiveness can provide insight into which pollinators are responsible for floral evolution (e.g., Grant and Grant 1965; Primack and Silander 1975; Schemske and Horvitz 1984; Herrera 1989; Fishbein and Venable 1996; Olsen 1997; Ivey et al. 2003). However, more efficient or abundant pollinators may not necessarily be those that are selecting on any given floral trait (Aigner 2001; Fenster et al. 2004; Aigner 2005). Without knowing how seed production from an important visitor is affected by variation in some floral trait, we cannot understand how a visitor influences the evolution of floral traits (Strauss et al. 2005). To date, few studies have measured selection by individual pollinator taxa in a generalized plant and have related this to pollinator effectiveness.

This study is not meant to provide a characterization of generalization in pollination systems because it is based on data sets gathered with different levels of sampling effort and with different numbers of populations. Future studies characterizing pollinator generalization of several species should take into account differences in sampling intensity since diversity indices are sensitive to sampling effort (Ollerton and Cranmer 2002). Herrera (2005) suggests using rarefaction to account for differences in sampling, although we were unable to use such a technique due to insufficient published data on sampling regime. In addition, because of the difficulty in estimating effectiveness for a large number of pollinator taxa, the plants included in this study may be biased toward specialization. What this study does suggest is that diversity indices should be incorporated into any study addressing pollinator generalization rather than simply estimating pollinator richness. Our study also indicates that pollinator effectiveness may not be as important as it is commonly believed to be, although more studies on plant species with high visitation richness are certainly needed.

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CHAPTER 3

VISITATION, EFFECTIVENESS, AND EFFICIENCY OF 15 GENERA OF VISITORS TO WILD RADISH, *RAPHANUS RAPHANISTRUM* (BRASSICACEAE)

Abstract

Quantifying pollinator effectiveness has been advocated by pollination biologists for decades because pollinators differ in their effectiveness at removing and depositing pollen. However, effectiveness is rarely estimated directly as seed set resulting from a single visit by the majority of taxa visiting generalist plants. Furthermore, pollinators often vary temporally in their visitation rates, which can cause temporal variation in selection on floral traits. In this study we quantify how visitors to wild radish, *Raphanus raphanistrum*, differ in their effectiveness as pollinators by quantifying the number of pollen grains removed and the number of seeds set during a single visit. We calculate each pollinator's importance to plant reproduction as the product of visitation rate and single visit seed set. This study was conducted across two years to examine temporal variation in the pollinator assemblage. Finally, to begin to understand mechanisms influencing differences in pollinator effectiveness we regressed pollinator body size on pollen removal and seed set effectiveness. We found that pollinators do differ in pollen removal and seed set effectiveness as well as in their visitation rates, but that pollinator importance is primarily determined by visitation rates rather than effectiveness. In contrast to similar studies over just two years, we found little variation in the composition of the pollinator assemblage across the two years of this study, suggesting that pollinator-mediated selection may sometimes be consistent across years in this generalist plant. Larger pollinators were more effective than small pollinators at setting seed, but pollen removal effectiveness may be more influenced by foraging behavior than size.

Introduction

Previous studies have found that many pollinator species often contribute to a plant's reproduction during a given year (Herrera 1987; Fishbein and Venable 1996;

Olsen 1997; Kandori 2002; Ivey et al. 2003), although we do not know the relative frequency of generalization in plant-pollination systems in nature (Waser et al. 1996; Johnson and Steiner 2000; Vazquez and Aizen 2003). Most studies of pollinator generalization have measured only the number of visiting taxa. However, all visitors may not be equal in either their visitation rates or their ability to transfer pollen (pollinator effectiveness), and some plant visitors do not pollinate at all (Spears 1983; Schemske and Horvitz 1984; Armbruster et al. 1989; Fishbein and Venable 1996). Therefore, pollination biologists have pointed out the need to characterize the relative importance of each pollinator when studying the degree of generalization of a plant's pollinator assemblage (Johnson and Steiner 2000; Fenster et al. 2004), where importance values incorporate both the quantity (visitation rates) and quality (effectiveness) of each pollinator taxon (Lindsey 1984; Olsen 1997). We define pollinator importance as the total number of seeds set by each pollinating taxon relative to the total number of seeds produced during the study (Kandori 2002).

Because characterizing effectiveness for a diverse assemblage of visitors is difficult, importance data exist for only a small number of generalist plants. Pollination biologists estimate effectiveness using many different methods. For example, studies have quantified effectiveness at removing and/or depositing pollen (Herrera 1987; Pettersson 1991; Fishbein and Venable 1996; Ivey et al. 2003), pollen load on pollinators (Lindsey 1984; Sugden 1986; Talavera et al. 2001; Moeller 2005), and probability of contacting stigmas and anthers (Lindsey 1984; Sugden 1986; Armbruster 1988). Seed set is rarely used in measuring effectiveness (but see Schemske and Horvitz 1984; Thompson and Pellmyr 1992; Olsen 1997; Kandori 2002; Wiggam and Ferguson 2005) despite the fact that the more common estimates of pollinator effectiveness may not accurately predict a pollinator's contribution to plant reproduction (Wilson and Thomson 1991; Fishbein and Venable 1996). Combining estimates of pollen removal effectiveness with pollen deposition effectiveness (or with seed set effectiveness) can provide an estimate of pollinator efficiency, defined as the number of pollen grains deposited or seeds set per pollen grain removed (Galen and Stanton 1989; Harder and Thomson 1989; Young and Stanton 1990; Conner et al. 1995).

Although many studies have documented differences in pollinator effectiveness among pollinators, the reason for such differences is not well understood. A few studies have found that insects foraging for pollen are often less efficient than those foraging for nectar (Wilson and Thomson 1991; Conner et al. 1995). Differences in visit duration among pollinators have been implicated in influencing pollinator effectiveness, where visit duration has been found to be both positively related to pollinator efficiency or effectiveness (Fishbein and Venable 1996; Ivey et al. 2003), and negatively related to effectiveness (Boyd 2004). Morphological aspects of pollinators can also contribute to differences in efficiency, such as tongue length (Schemske and Horvitz 1984) and body size (Kandori 2002), although few studies have examined how morphological differences among pollinators influence their effectiveness.

In addition to differences in effectiveness among pollinators, plants may experience variation in the pollinator assemblage from year to year because environmental fluctuations lead to fluctuations in the population dynamics of pollinator species (Herrera 1988; Fleming et al. 2001). Variation in visitation frequency can alter selection on floral traits from year to year (Schemske and Horvitz 1989), which can hinder adaptation of floral traits to any one pollinator (Herrera 1988; Pettersson 1991). Furthermore, models examining when plants should specialize on a subset of the pollinating assemblage show that increased temporal variation in pollinator assemblages decreases the likelihood of specialization (Waser et al. 1996).

This study measures the relative importance of 15 genera of insect visitors to *Raphanus raphanistrum* (wild radish). For each visiting taxon, we determined 1) relative visitation rates in two years, 2) pollinator effectiveness (using both number of seeds set and number of pollen grains removed during a single visit), 3) pollinator importance (the proportion of the total number of seeds set during the season by each pollinator), and 4) pollinator efficiency (number of seeds produced per pollen grain removed). To begin to understand mechanisms behind differences in pollinator effectiveness, we examined the relationship between pollinator body mass and effectiveness.

Methods

Study system

Raphanus raphanistrum is a hermaphroditic, annual herb that is native to Europe but has subsequently become a naturalized and prevalent agricultural weed on six continents (Holm et al. 1997). *Raphanus raphanistrum* is self-incompatible (Sampson 1964), relying entirely on insect pollinators for plant reproduction. *Raphanus raphanistrum* is visited by many different pollinator genera, spanning 3 insect orders--Lepidoptera, Diptera, and Hymenoptera (Kay 1978; Kay 1982; Conner and Rush 1996). Visitors range in size from small sweat bees (e.g., *Dialictus* and *Halictus*) and syrphid flies (e.g., *Toxomerus*) to much larger honeybees (*Apis mellifera*), bumblebees (*Bombus*), syrphid flies (*Eristalis*), and cabbage butterflies (*Pieris rapae*) (Conner and Rush 1996). In *R. raphanistrum*, most small bees and all syrphid flies collect or feed only on pollen (Conner and Rush 1996), butterflies feed solely on nectar, and large bees feed on both (Rush et al. 1995).

Seed set effectiveness

In 2002, plants were grown in 15 cm pots in a pollinator-free greenhouse and transported in a covered vehicle to old fields lacking *R. raphanistrum* at the Kellogg Biological Station in Hickory Corners, Michigan. These old fields were surrounded by farm fields, the principal habitat of *R. raphanistrum* in North America, and thus were likely to contain pollinators similar to those pollinating 'natural' populations. In order to attract pollinators and provide pollen for seed siring, a five-meter diameter circular array of 15 plants was placed in the field and exposed to pollinators for five minutes, while focal plants were left in the covered vehicle to prevent visitation. After five minutes, one unvisited focal plant was placed in the center of the array. As soon as a focal plant was placed in the field it was continuously observed. After each visit to a flower on the focal plant, the pollinator was identified to at least the genus level, and a numbered straw was placed over the pistil to prevent further visitation. This was repeated for up to 20 flowers per focal plant, and up to 10 focal plants per day for 40 days throughout the flowering season (June-September). At the end of each day all plants were returned to the greenhouse, straws were removed, and each flower was tagged with a unique code corresponding to the code recorded for that single visit. Flowers were allowed to set seed and all fruits were collected and the total number of seeds produced per fruit was determined. A total of 1315 seeds were produced from 2314 single visits.

A one-way ANOVA was used to test for differences between orders and genera of pollinators of *R. raphanistrum* in the number of seeds set during a single visit. Proportional pollinator importance for each pollinator genus was calculated as the proportion of the total number of seeds set in this experiment by each pollinator, which is equivalent to the product of the number of visits and per visit seed production (Kandori 2002).

Pollen removal effectiveness

In 2001, plants were grown in the greenhouse as described above, and unvisited plants were transported to the same old fields at the Kellogg Biological Station, one of which contained a large experimental population of *R. raphanistrum*. One plant was placed in the field at a time and continuously observed for visitation. After a pollinator visited a flower, anthers from three long and two short stamens (wild radish has four long and two short stamens) were removed from the visited flower and from the nearest unvisited flower on the same stalk and placed in separate clean vials. The identity of the pollinator was recorded to the genus level when possible. This was repeated over several flowers per plant on up to 15 plants per day for 44 days throughout the flowering season (June-October), providing a total of 470 single visits. Pollen grains were counted using a

Coulter Counter (model Z_{BI}, Coulter Electronics, Hialeah, FL; for details see Rush et al. 1995). The number of pollen grains removed from anthers was estimated as the difference between the number of grains on the unvisited anthers and the number of grains remaining on the visited anthers (Harder 1990; Young and Stanton 1990; Rush et al. 1995). Adjacent *R. raphanistrum* flowers have similar pollen counts--75% of the variation in pollen number is between plants rather than within a plant (Rush et al. 1995). Due to small sample sizes for each genus, pollinators were divided into five groups based on size and taxonomy: large bees (*Bombus* and *Apis*), small bees (*Colletes, Ceratina, Dialictus, Augochlorella*), Lepidoptera (*Thymelicus* and *Pieris*), larger syrphid flies (*Eristalis, Syrphus, Syritta*), and small syrphid flies (*Toxomerus*). Genera within each group did not differ significantly in pollen removal. A one-way ANOVA was used to test for differences in pollen removal at both the order and the group level.

To determine if pollen removal is a good predictor of seed set effectiveness, we averaged seed set effectiveness for the five taxon groups used for pollen removal and regressed seed set on pollen removal. To estimate pollinator efficiency for each pollinator, we divided the average number of seeds set by the average number of pollen grains removed in single visit. Finally, to test whether body size predicts seed set effectiveness, we regressed both pollen removal and seed set effectiveness based on the five functional groups on body size and regressed seed set effectiveness of the 15 genera of pollinators on body size. Two to five individuals per insect genus were weighed after being air dried for at least six months. Dry body mass was averaged over all weighed individuals.

Pollinator visitation across years

Visitation of pollinator taxa in 2001 was compared to visitation in 2002, where visitation is based on the proportion of single visits made by each pollinator during the 2001 pollen removal study and the 2002 seed set effectiveness study. Because some pollinators were not identified to the genus level in 2001, some taxa were grouped based on size, resulting in nine pollinator groups (Table 3.1). Taxa from 2002 were put in these same nine groups and Spearman's rank-sum correlation coefficients of visitation in 2001 and 2002 were calculated. JMP version 5.0.1.2 (SAS 2003) was used for all analyses.

Results

Seed set effectiveness

In 2002, *R. raphanistrum* was visited by 15 different genera (Figure 3.1). Although we were unable to identify all visitors to the species level, a survey of *R. raphanistrum* pollinators in Illinois in 1993 and 1994 (J.K. Conner, unpub.) along with our own identifications during this study indicates that there were approximately 11 species in the genus *Dialictus*; two species in the genus *Halictus*; two species in the genus *Bombus*; three species in the genus *Toxomerus*; one species in each of the genera *Syritta, Ceratina, Augochlorella, Colletes, Apis, Thymelicus, Pieris, Allograpta, Sphaerophoria,* and *Syrphus*; and two species in the genus *Eristalis.* Species within each genus are similar in terms of their morphology and behavior, and thus are not likely to differ in efficiency. *Halictus, Dialictus,* and *Augochlorella* are in the family Halictidae, *Ceratina*, *Bombus,* and *Apis* are in the family Apidae, and *Colletes* is in Colletidae. *Thymelicus* is in the family Hesperiidae, *Pieris* is in Pieridae, and all flies are in the family Syrphidae.

Sweat bees in the genus *Dialictus* were the most frequent visitors, followed by the syrphid flies *Toxomerus*, *Syritta*, and *Eristalis*. These four genera made 81% of all visits to *R. raphanistrum* and each of the other 11 pollinator genera accounted for less than 5% of the visits (Figure 3.1A).

Fourteen of the 15 genera that visited *R. raphanistrum* were effective pollinators, with an average seed set greater than zero (Figure 3.1B). Allograpta (N=17 visits) was the only taxon that did not effect any seed set during the experiment. Of the 14 effective pollinators, seed set effectiveness varied by more than an order of magnitude, from an

Table 3.1. Percentage of visits made to *Raphanus raphanistrum* by nine pollinator groups in 2001 and 2002. 'Small bees' include *Dialictus, Ceratina, Colletes*, and *Halictus*; and 'Medium syrphid flies' include *Syritta* and *Syrphus*.

	% Visits	
Pollinator	2001	2002
Small bees	40.7	52.5
Apis mellifera	1.7	1.9
Bombus	1.3	0.6
Thymelicus	0.4	1.0
Pieris rapae	7.7	0.9
Toxomerus	28.1	21.1
Medium syrphid flies	11.9	14.1
Allograpta	3.2	0.8
Eristalis	4.9	7.3

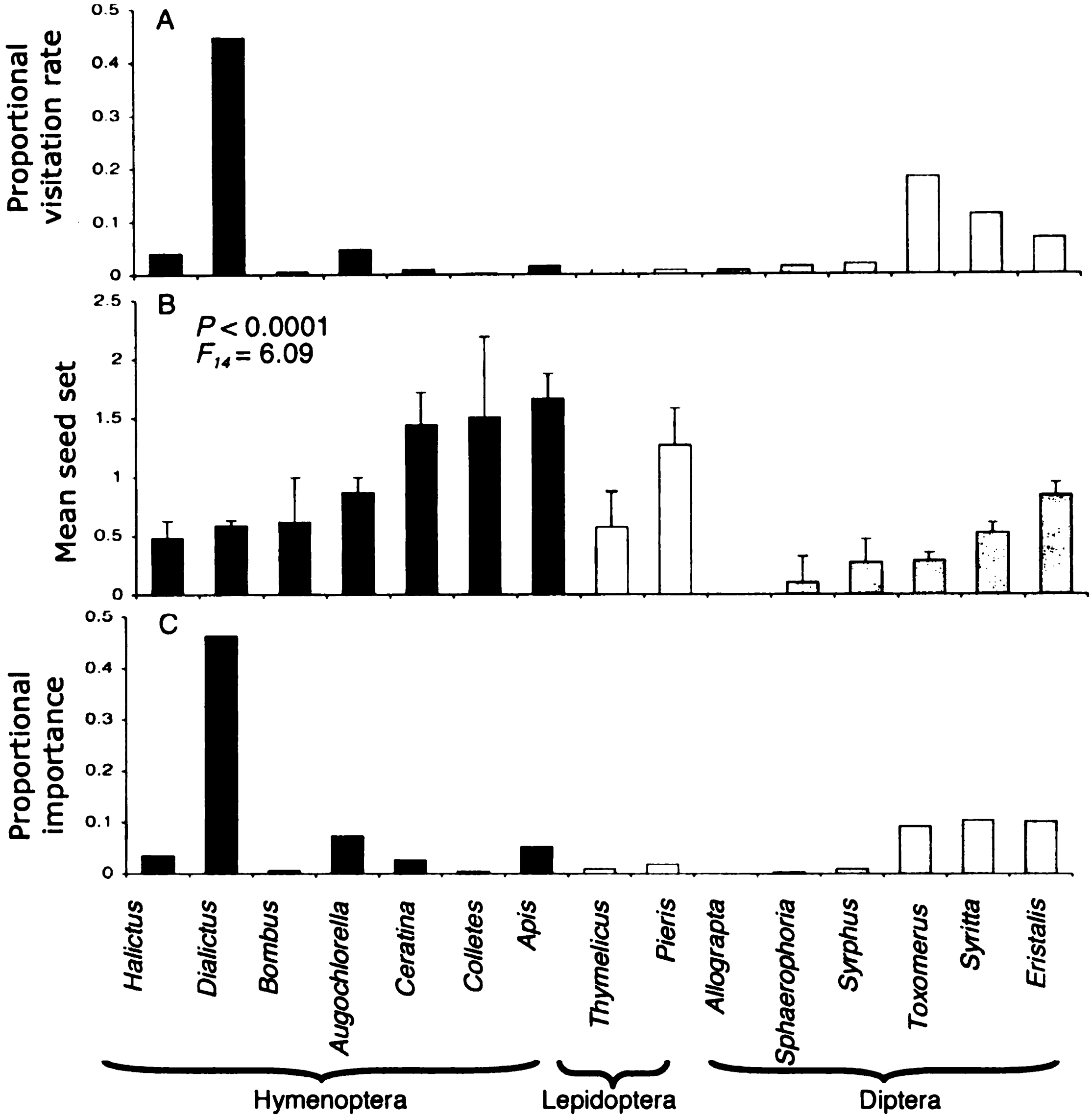

Figure 3.1. Pollinator (A) visitation, (B) effectiveness, and (C) importance for each pollinator genus of *R. raphanistrum*, where effectiveness is the mean (+ 1 SE) number of seeds set during a single visit, visitation is the proportion of total visits made in 2002, and importance is the proportion of total number of seeds produced, equivalent to the product of visitation and effectiveness. Genera are grouped and shaded by insect order and arranged by increasing effectiveness within orders.

average of 0.10 seeds per visit (*Sphaeorphoria*) to 1.66 seeds per visit (*Apis*); (Figure 3.1B). Despite the significant differences in effectiveness, pollinator importance was determined primarily by differences in visitation rates (Figure 3.1). There was no relationship between a pollinator's visitation rate and its effectiveness ($r^2 = 0.04$, $P = 0.48$).

The three orders of pollinators, Hymenoptera, Lepidoptera, and Diptera, differed significantly in their seed set effectiveness (Figure 3.2A), with Hymenoptera visits resulting in significantly higher seed set than Diptera visits. Lepidoptera effectiveness was higher than the other two orders, but not significantly so due to small sample size.

Pollen removal vs. seed set effectiveness

All five groups of pollinators removed pollen during a single visit, but pollinators did not differ in the number of pollen grains removed from short stamen anthers ($F = 0.47$, $P = 0.76$). Pollinators did differ in the number of grains removed from long stamen anthers (Fig 3A), and in the total number of pollen grains removed when long and short stamen anthers were combined ($F = 2.41$, $P = 0.05$). *Toxomerus* removed the least amount of pollen, and small bees and large syrphid flies removed the most. Pollinators differed at the order level in the number of pollen grains they removed from long stamen anthers with Hymenoptera removing the most, Diptera next, and Lepidoptera the least (Figure 3.2B). When seed set effectiveness was analyzed using the same five pollinator groups, *Toxomerus* had the lowest seed set effectiveness, similar to its low pollen removal, but in contrast to the pollen removal results, small bees and large syrphid flies had low seed set effectiveness and large bees the highest (Figure 3.3B). Thus, pollen removal effectiveness was not a good predictor of seed set effectiveness ($r^2 = 0.04$, $P = 0.76$).

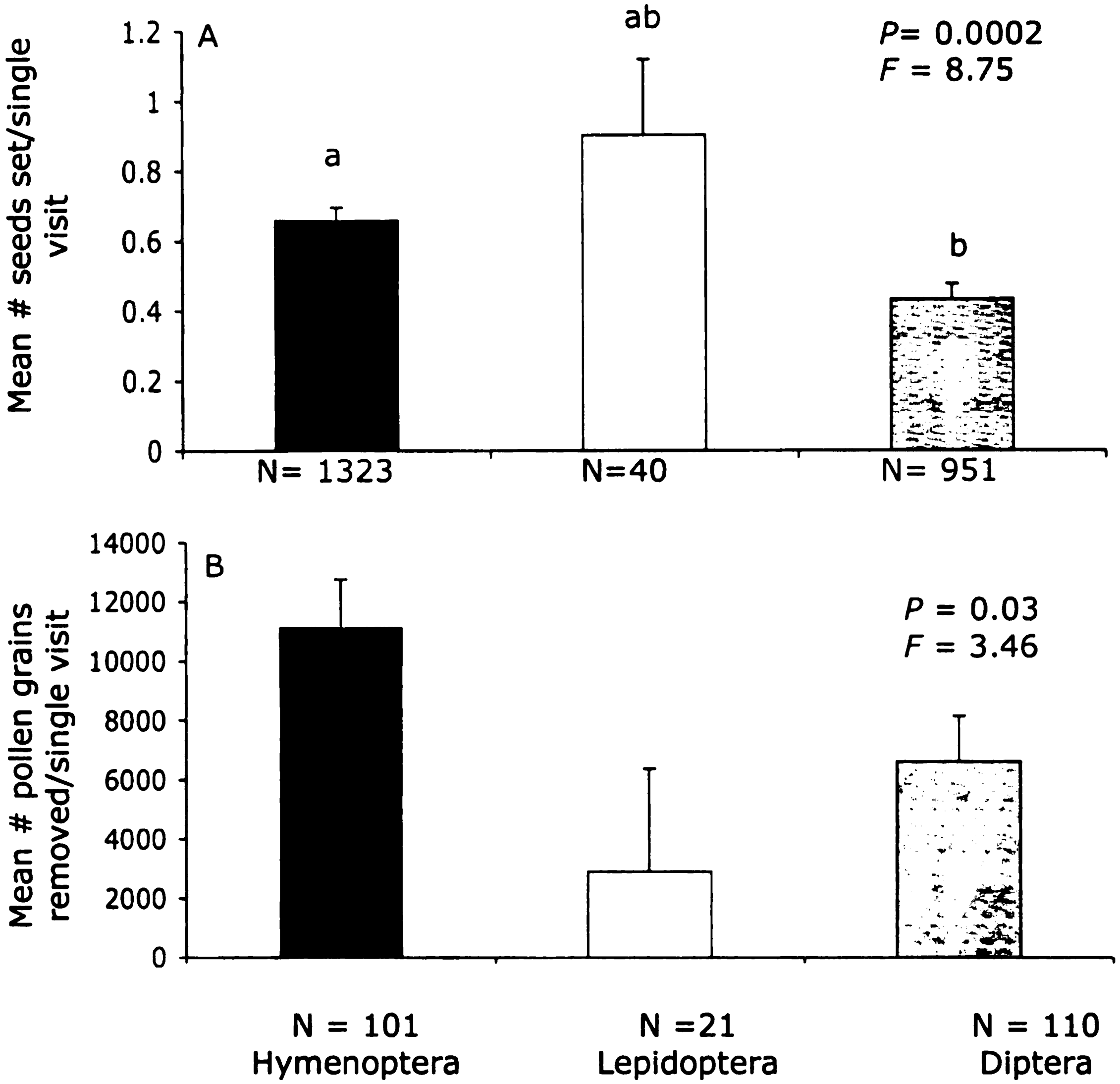

Figure 3.2. Pollinator efficiency for insect orders. (A) Mean (+ 1 SE) number of seeds set and (B) mean (+1 SE) number of pollen grains removed from long stamen anthers during a single visit for each order pollinating *Raphanus raphanistrum*. Orders that do not share a letter in common are significantly different at $P \leq 0.05$ according to Tukey's HSD test. None of the pairwise differences were significant for (B)

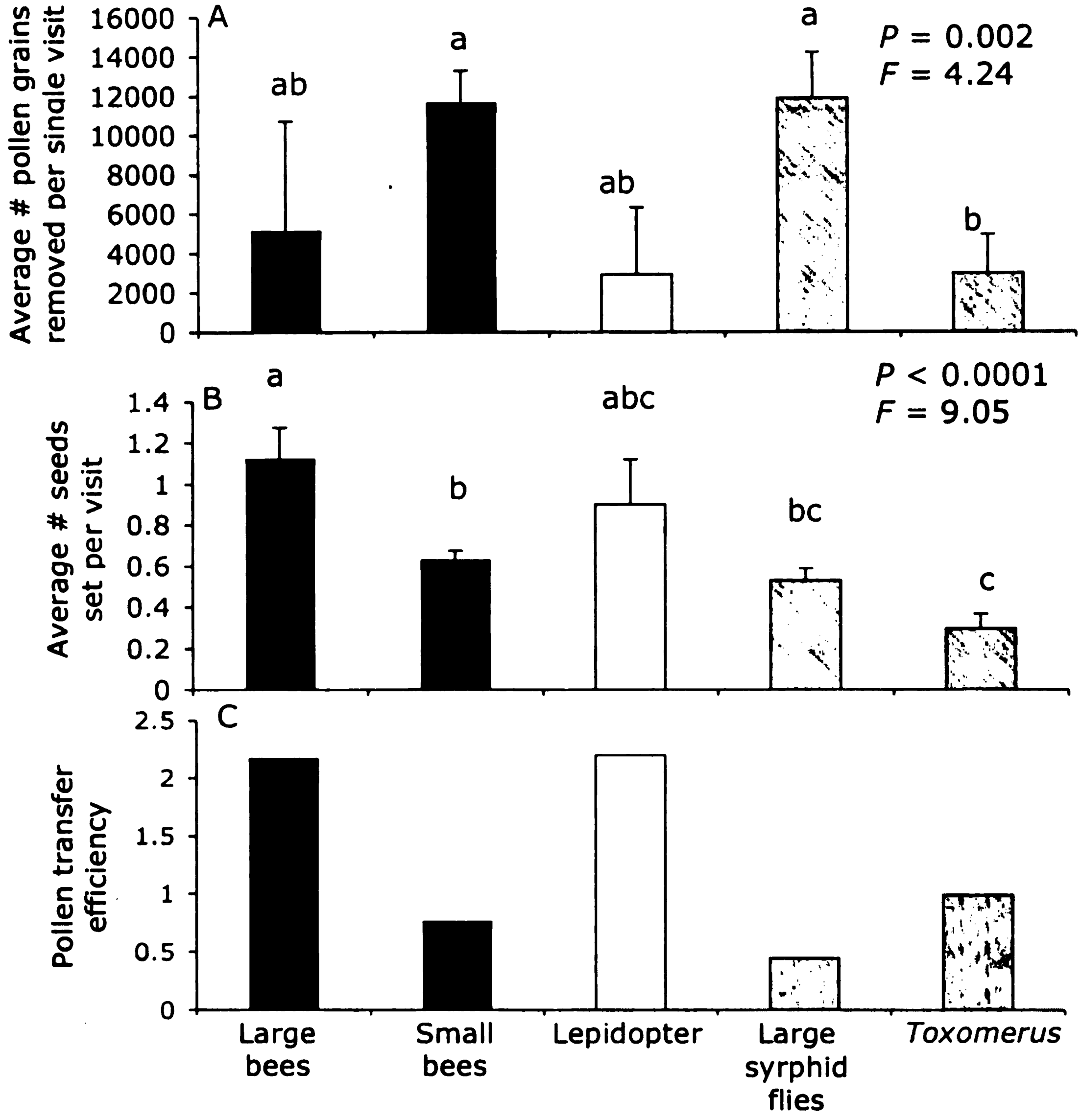

Figure 3.3. Pollinator efficiency for five groups of pollinators. (A) Pollen removal effectiveness (# pollen grains removed from long stamen anthers during a single visit); (B) pollinator seed set effectiveness (C) pollen transfer efficiency (number of seeds set per pollen grain removed x10^{-4}). 'Large bees' include *Apis* and *Bombus*; 'Lepidoptera' include *Thymelicus* and *Pieris*; 'Small bees' include *Augochlorella, Dialictus, Ceratina, Colletes*, and *Halictus*; 'Large syrphid flies' include *Allograpta, Eristalis, Syritta,* and *Syrphus.* Orders are color coded as in Figure 3.1. Genera that do not share a letter in common are significantly different at $P \leq 0.05$ according to Tukey's HSD.

Table 3.2. Mean body mass of *Raphanus raphanistrum* visitors (1 SE). N=number of insects weighed for each genus.

Genus	Order	Larger groups	N	Body mass (mg)
Eristalis	Diptera	Large syrphid fly	5	40.8 (7.7)
Syrphus	Diptera	Large syrphid fly	3	8.7 (1.6)
Allograpta	Diptera	Large syrphid fly	3	5.9 (0.6)
Syritta	Diptera	Large syrphid fly	3	3.4 (0.1)
Sphaerophoria	Diptera	Large syrphid fly	3	3.6 (1.0)
Toxomerus	Diptera	*Toxomerus*	3	1.2 (0.4)
Bombus	Hymenoptera	Large bee	3	41.0 (10.2)
Apis	Hymenoptera	Large bee	3	30.0 (2.3)
Halictus	Hymenoptera	Small bee	3	10.2 (1.5)
Augochlorella	Hymenoptera	Small bee	3	5.2 (1.6)
Ceratina	Hymenoptera	Small bee	3	4.0 (1.4)
Dialictus	Hymenoptera	Small bee	3	2.4 (0.4)
Pieris	Lepidoptera	Lepidoptera	2	25.7 (12.8)
Thymelicus	Lepidoptera	Lepidoptera	2	18.9 (6.7)

Body size vs. effectiveness

Visitors to *R. raphanistrum* ranged in size by more than an order of magnitude, varying from 1.4 mg (Toxomerus) to 40 mg (Bombus) (Table 3.2). There was not a significant relationship between pollinator body mass and seed set effectiveness when all 15 pollinators were included in the analysis (r^2=0.17, *P*=0.15). However, when one possible outlier, *Ceratina,* was removed from the analysis, there was a significant positive relationship between mass and effectiveness (Figure 3.4A). *Ceratina* was also an outlier in terms of its behavior of foraging only on nectar. Although this bee was similar in size to other halictids (Table 3.2), its foraging behavior was similar to that of the large honeybee, *Apis* (H. Sahli, pers. obs.). When pollinators were grouped into the five categories used to analyze pollen removal effectiveness, there was a strong relationship between body size and the number of seeds set during a single visit (Figure 3.4B), but body size did not predict pollen removal (Figure 3.4C).

Pollinator visitation rates

Visitation rates of different pollinator taxa did not differ temporally across 2001 and 2002 (Table 3.1), as the rank of visitation frequency for the nine pollinator groups was consistent across years (r_s = 0.77, *P* = 0.01). In both years, small bees were the most common visitor followed by *Toxomerus* and medium syrphid flies. Because the majority (60%) of single visits in 2001 were made in a field adjacent to a large experimental population of wild radish, the similarity of visitation across years also indicates that visitors recorded in this study are likely to be representative of those in natural populations of wild radish in the area.

Discussion

Although we found that *R. raphanistrum* pollinators differed greatly in their effectiveness and efficiency, pollinator importance was determined primarily by

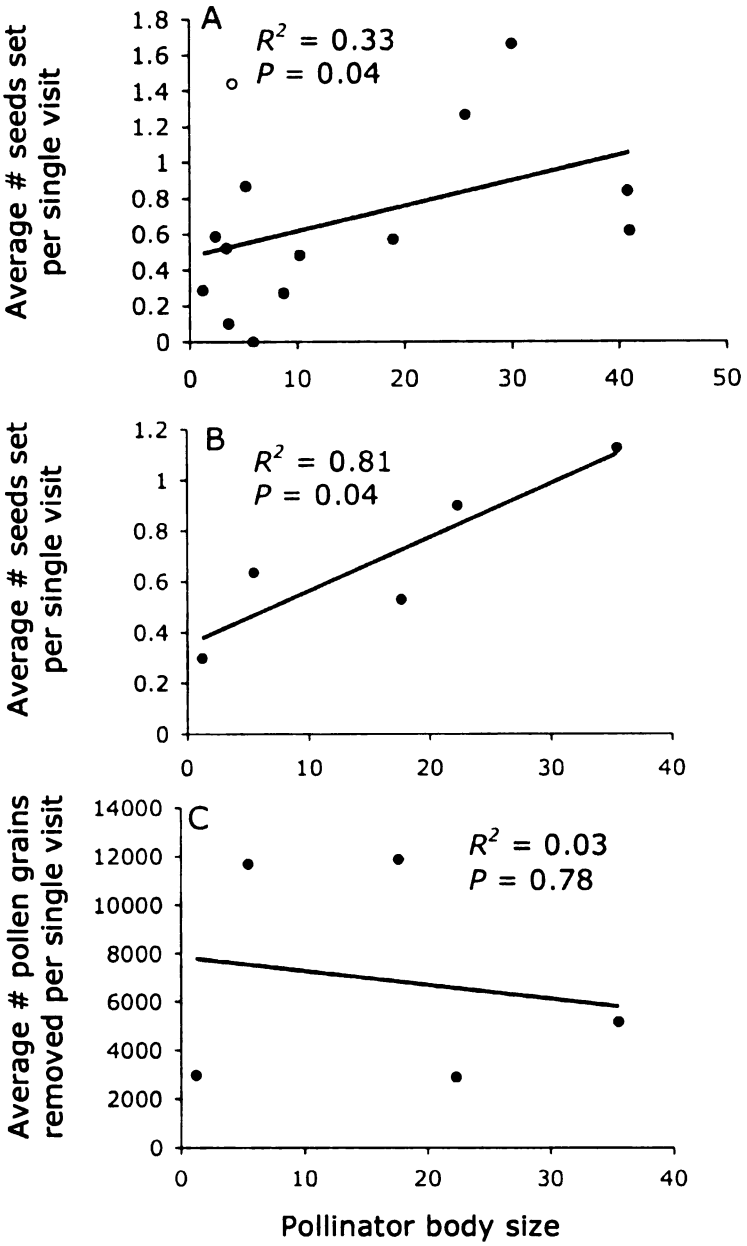

Figure 3.4. Regressions of measures of effectiveness on body size. (A) Seed set effectiveness for each pollinating genus, (B) seed set effectiveness for the five pollinator groups (see Figure 3.3), and (C) pollen removal effectiveness. Fitted line and r^2 value presented in (A) do not include the outlier, *Ceratina* (open circle).

visitation frequency rather than pollinator effectiveness, a common result found across plant species (Vazquez et al. 2005; Sahli and Conner 2006). Despite the superior efficiency of large bees and Lepidoptera, the less efficient pollinators were the most important to plant reproduction simply because they were the most frequent visitors to *R. raphanistrum* during our study. This result highlights the importance of characterizing visitation rates of many visitors to plant species in order to determine which visitors are likely to be important for reproductive success. Measuring pollinator effectiveness and efficiency may not be as important and may actually obscure our understanding of pollinator importance if examined without visitation rates. However, in some populations and some species, effectiveness may prove important, especially when there is little variation among pollinators in visitation rates (Vazquez et al. 2005). Furthermore, effectiveness, when coupled with studies measuring natural selection by different pollinators and visitation rates, may tell us about past and current pollinator-mediated selection. For instance, there may be current selection on floral traits to make pollinators more effective at depositing pollen on stigmas, and past selection may have resulted in other pollinators being currently more efficient at deposition.

Visitation determined most of the variation in pollinator importance because visitation to wild radish was extremely uneven. The small proportion (26%) of pollinators responsible for 81% of the reproduction for *R. raphanistrum* indicates that this plant is not extremely generalized, relying on only four genera for most of its reproduction. Studies on other plant species visited by a large number of taxa have also found that a small percentage of the visitors are responsible for roughly 80-90% of total plant reproduction (e.g., Lindsey 1984; Devall and Thien 1989; Herrera 1989; Gomez and Zamora 1999), whereas other studies have shown some plants to have more even pollinator assemblages (e.g., Pettersson 1991; Kandori 2002; Ivey et al. 2003).

Although only a minority of the visiting taxa were responsible most of plant reproduction during the two years of our study, it is unknown whether low visitation rates of other taxa were due to low densities of those taxa in the population we studied, competition with other plants for pollinators, or competition among pollinator taxa. Thus, a reduction in *Dialictus* or *Toxomerus* visits, or a reduction in co-occurring flowering plants, may increase visitation by previously less frequent taxa. For example, Ricketts (2004) found that seed set in coffee populations visited by many pollinator taxa was buffered against a decrease in the dominant honeybee because previously infrequent visitors increased in visitation rates with the loss of the dominant taxon. Therefore, the high number of taxa that are effective pollinators of *R. raphanistrum* may buffer this plant against shifts in pollinator assemblages across time and space, perhaps contributing to its great success as a global weedy colonizer.

Results from this study also showed that differences in pollinator effectiveness can partially be explained by size, where larger taxa are more effective pollinators in terms of their ability to effect seed set. Kandori (2002) found the same relationship in *Geranium*. One exception to this trend in our study was *Ceratina*, a small, non-social bee that was an extremely effective pollinator. This pollinator was one of the few small pollinators that foraged for nectar, plunging its entire body into the tube of the flower in order to reach the nectar and possibly contacting the stigma more often. In fact, this species was never observed collecting pollen (H. Sahli, pers. obs.). The nectar feeding behavior exhibited by this small bee is one possible explanation for its rather high effectiveness, as compared to the effectiveness of similar sized pollinators, which foraged primarily on pollen, and thus perhaps mainly contacted the anthers.

While body size can predict a pollinator's effectiveness at setting seeds, it is not predictive of a pollinator's ability to remove pollen. Because most of the pollinators of *R. raphanistrum* are primarily foraging on pollen, behavioral differences among pollinators in how they actively collect pollen, which are unrelated to size, may have the biggest impact on pollen removal. The differences between pollinators in their pollen removal effectiveness and in their efficiency at transferring pollen were largely due to differences in feeding behaviors. Large bees and Lepidoptera removed the least pollen during a single visit, probably because they both feed primarily on the nectar of *R. raphanistrum*. Their small pollen removal, coupled with their large size, resulted in a high pollinator efficiency, indicating that they do not need to remove much pollen in order to transfer pollen to a receptive stigma. Conversely, small bees and syrphid flies primarily feed on or collect pollen while visiting *R. raphanistrum*, which obviously results in the inefficient transfer of pollen grains from anthers to stigmas. Thus, our study indicates that pollinator effectiveness and efficiency is a function of both behavior and morphology.

There was surprisingly little temporal variation in the pollinator composition of *R. raphanistrum* during the two years of our study. Temporal variation in pollinator assemblages is often thought to be quite pronounced and can be responsible for changing selection pressure from year to year if taxa differ in their selection on traits. Indeed, other studies measuring temporal visitation, even on *R. raphanistrum*, have documented annual changes in pollinator assemblages where the most frequent visitor taxa vary annually (Herrera 1988; Schemske and Horvitz 1988; Pettersson 1991; Rush et al. 1995; Fishbein and Venable 1996; Mahy et al. 1998; Kandori 2002). Many of these past studies were also carried out over only two years, yet they still found striking differences in the composition of the pollinator assemblage. Therefore, our findings are atypical in showing that pollinator assemblages were quite constant across two years at our field sites. Our finding suggests that, given pollinator-mediated selection on wild radish (Conner et al. 1996a; Conner et al. 1996b; Morgan and Conner 2001; Conner et al. 2003), selection may be maintained across years, enabling a consistent response to selection and, thus, adaptation to pollinators. This goes against a common view that temporal variation prevents generalist plants from being adapted to any one pollinator (Pettersson 1991; Waser et al. 1996; Gomez and Zamora 1999).

CHAPTER 4

SELECTION BY SEVEN POLLINATOR GENERA ON FLORAL TRAITS IN WILD RADISH (*RAPHANUS RAPHANISTRUM*) THROUGH BOTH MALE AND FEMALE FITNESS

Abstract

While trade-offs in resource use are often thought to play a critical role in the evolution of specialized species, the evolutionary importance of trade-offs in generalists is not well understood. Trade-offs can lead to specialization during constant resource availability, or may lead to a generalist that is not strongly adapted to any one resource if resources vary. However, if trade-offs are weak or non-existent, an organism may evolve to use many resources efficiently. In this study, we examined how different pollinators influence the evolution of floral traits in a generalist plant, wild radish (*Raphanus raphanistrum*), by measuring selection on floral traits through both male and female fitness by the seven most abundant pollinator genera. Plants that had been artificially selection to increase variation in anther exsertion and stamen dimorphism, two traits exhibiting little variation in natural populations of wild radish, were used in this study. Overall, we found little evidence that pollinators differed in their selection on floral traits. In fact, flower size was under similar selection pressure by different pollinators, and anther exsertion and stamen dimorphism each experienced significant selection by only one pollinator. We also found that the magnitude of selection was stronger through female fitness than through male fitness on the traits we studied, and selection through female fitness occurred primarily on attraction traits rather than traits affecting pollen transfer. In order to understand total selection on a trait when pollinators are present in different abundances, we calculated an average selection gradient for each trait, weighted by each pollinator's importance to reproduction as measured in the field. Variation in visitation rates among pollinator taxa caused total selection gradients to be similar to those of the most important pollinator, the sweat bee *Dialictus*. Thus, although wild

radish is able to use many insects as effective pollinators, it may also be adapting to the most common pollinator, without much trade-off in the use of other pollinators.

Introduction

Interactions between organisms and their resources play a critical role in the evolution and divergence of species. Many ecological and evolutionary factors contribute to the continuum in resource use seen in nature (Bernays and Graham 1988; Thompson 1994; McPeek 1996), leading to some organisms being extremely specialized, using only one resource, while other organisms are extremely generalized, using resources in proportion to their availability in nature (Thompson 1994). The forces driving the evolution and maintenance of generalist and specialist lifestyles are not well understood. There are three non-mutually exclusive ways in which an ecological generalist lifestyle may be maintained over time. First, spatial and temporal resource heterogeneity may prevent adaptations that allow an organism to adapt to a subset of its resources (Futuyma and Moreno 1988; Herrera 1988; Thompson 1994). Adaptation in a heterogeneous environment may be prevented if there is a trade-off in adapting to different environments, whereby an adaptation that is beneficial for the use of one resource is deleterious for use of another (e.g. Levins 1968; Gould 1979; Via 1991; Reboud and Bell 1997; Cooper and Lenski 2000; Cooper et al. 2001). I refer to this as the trade-off hypothesis because selection on a trait by one resource opposes that of another resource (Figure 4.1A). In an extreme example of a trade-off, opposing selection gradients could lead to no net selection on a trait when both resources are present in equal abundance.

However, evidence for fitness trade-offs in different resource environments is rarely found (e.g. Bennett et al. 1992; Reboud and Bell 1997; Kassen and Bell 1998; Weaver et al. 1999; Fry 2001). A second way in which a generalist lifestyle may be maintained is if one trait is an adaptation for many resources, which I refer to as the common adaptive peaks hypothesis. A common adaptive peak may exist if the optimal

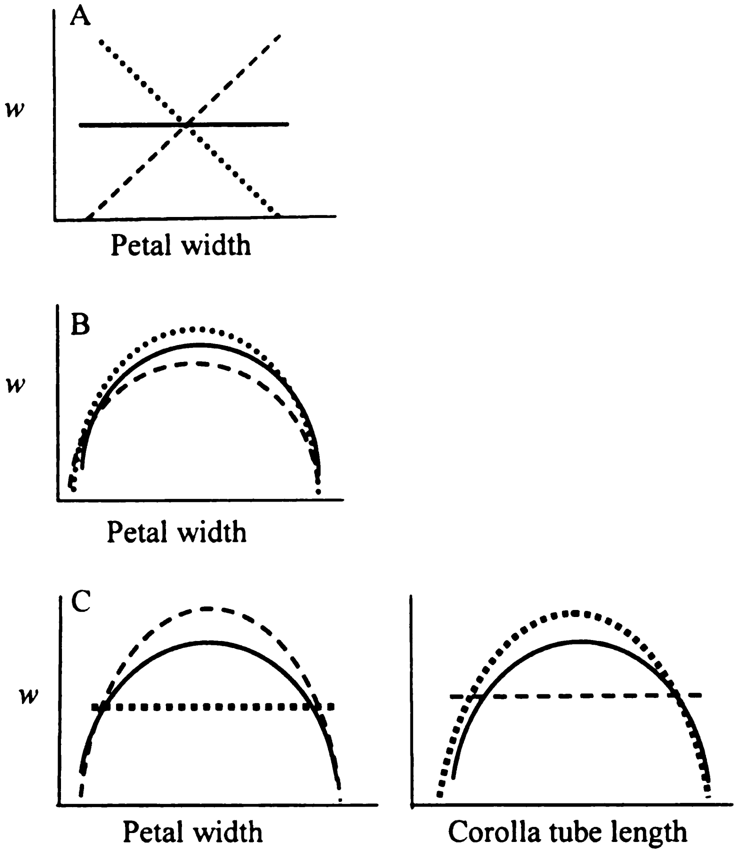

Figure 4.1. Hypothetical fitness functions expected for (A) trade-off hypothesis, (B) multiple adaptive peaks hypothesis, and (C) trait specialization hypothesis. Dotted lines represent fitness functions generated by pollinator (1), dashed lines represent pollinator (2), and solid lines are the average fitness functions. Relative fitness (*w*) is on the y-axes and floral traits are on the x-axes.

phenotype for using one resource is similar to that for using another resource. Thus, selection on a trait by different resources may be similar (Figure 4.1B). Evidence for the common adaptive peaks hypothesis has been found in some selection studies on *E. coli*, where fitness in some novel environments increased with adaptation of lines grown in a single environment (Travisano and Lenski 1996; Cooper et al. 2001). Therefore, the adaptive peaks in both the selected and the novel environments must have been similar. Finally, an organism may contain *morphological specializations*, or traits that are adapted for a subset of an organism's resources. For instance, the generalist cabbage looper caterpillar has different adaptations that allow it to overcome defense mechanisms of different host plants (Dussourd and Denno 1994; Berenbaum 1995; Dussourd 1997). Similarly, floral mobility in *Impatiens* flowers may be a morphological specialization in response to selection by only hummingbirds, and not bumblebees (Hurlbert et al. 1996). Thus, a generalist may contain different traits that are morphological specializations for different resources, allowing it to be well adapted to many resources at once. This could occur if different resources select on different traits (Figure 4.1C).

One area of specialization that has received much recent attention is the degree to which plants specialize on pollinators (Ollerton 1996; Waser et al. 1996; Johnson and Steiner 2000). Pollinators are an important resource for reproduction in many angiosperms (Tepedino 1979); therefore, the same processes that cause specialization of any organism to a subset of its resources should, likewise, cause plants to specialize on pollinators. While many studies have measured spatio-temporal variability in pollinator visitation (Waser et al. 1996 and references therein) and pollinator effectiveness of different pollinators (Schemske and Horvitz 1984; Herrera 1987; Herrera 1989; Stanton et al. 1991; Gomez and Zamora 1999), empirical studies measuring selection by different pollinators in generalist plant species are lacking. Determining the relative importance of the above three scenarios can improve our understanding of trait specialization in generalized organisms.

Furthermore, since the majority of plant species are hermaphroditic (Yampolsky and Yampolsky 1922), selection can be acting on traits through both male and female fitness. The question of whether selection on floral traits is stronger through male versus female fertility has been discussed for almost three decades. Because seed set is thought to be limited strongly by resources, rather than pollinators, the prevailing view has been that pollinator-mediated selection should be stronger through male than female fitness due to larger variance in male fitness than female fitness (Charnov 1979; Willson 1979; Charnov 1982). However, seed production in many plants has been found to be pollen limited as some time or in some populations (reviewed in Burd 1994; Ashman et al. 2004), suggesting that selection on floral traits through female fitness can be important. The paucity of studies examining selection through male fertility using seed siring success to estimate male fitness have prevented the field from empirically addressing to whether selection is often stronger through male than female function. By measuring selection through both male and female fitness we can not only estimate total selection on floral traits, but can also test whether selection is stronger through male and female fitness.

In this study, we examined floral adaptation in a generalist plant, wild radish (*Raphanus raphanistrum*), by measuring selection on floral traits through both male and female fitness by the seven most important pollinator genera. We addressed three questions: (1) What roles do trade-offs, common adaptive peaks, and morphological specializations play in maintaining ecological generalization? (2) How does variation in pollinator visitation and effectiveness influence the strength of selection exerted by each pollinator? (3) Does the strength of selection through male and female function differ for different traits and different pollinators? To estimate selection by different pollinators, we used a combination of cage experiments and natural pollination in the field. To our knowledge, this is the first empirical study demonstrating how selection by more than two pollinators determines total selection on morphological traits in a generalist plant, and is one of the few studies measuring selection by multiple agents of selection in any generalist organism. Furthermore, it is one of the few studies measuring total selection on a trait through both male and female fitness in a hermaphrodite.

Methods

Study System

Wild radish (*Raphanus raphanistrum*) is a self-incompatible, hermaphroditic, annual herb that is native to Europe but has subsequently become a naturalized and important agricultural weed on six continents (Hulten and Fries 1986; Holm et al. 1997). *Raphanus raphanistrum* is effectively pollinated by at least 14 pollinator genera (Chapter 3) that span 3 insect orders -- Lepidoptera, Diptera, and Hymenoptera (Kay 1976; Conner and Rush 1996). Pollinators from these orders range in size from small sweat bees (*Dialictus spp.*) and syrphid flies (*Toxomerus spp.*) to honeybees (*Apis mellifera*), bumblebees (*Bombus spp.*), and cabbage butterflies (*Pieris rapae*) (Conner and Rush 1996). In *R. raphanistrum*, most small bees and all syrphid flies collect or feed only on pollen (Conner and Rush 1996), butterflies feed solely on nectar, and large bees feed on both (Rush et al. 1995). Because wild radish is pollinated by insects differing in size and behavior, it is a likely candidate for experiencing differential selection from different selective agents.

Floral Traits

Wild radish is a member of the Brassicaceae family and exhibits the family diagnostic tetradynamous stamen condition with two short and four long stamens. The length difference between the long stamen and the short stamen will be referred to as stamen dimorphism, and is calculated in this paper as the difference between the long and the short filament lengths (Figure 4.2). Stamen dimorphism displays little variation in natural populations due to a high correlation between the short and long stamen lengths (Conner and Via 1993). Similarly strong correlations are found between long filament

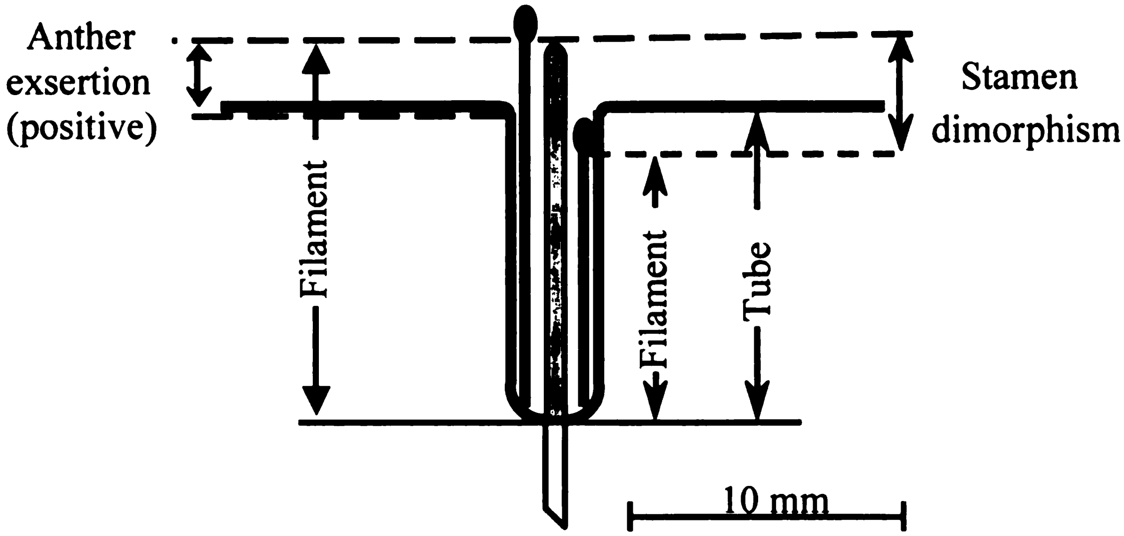

Figure 4.2. Lateral cross-section of a wild radish flower showing anther exsertion and stamen dimorphism. Redrawn from Conner *et.al.* (1995)

length and corolla tube length (Conner and Via 1993), which leads to very little within population variation in anther exsertion, or the placement of the anther relative to the opening of the flower (Figure 4.2). Anther exsertion is calculated as the difference between the long filament length and the corolla tube length. Because many pollinators of wild radish are primarily pollen feeders, the placement of the anthers relative to one another and relative to the opening of the flower, may influence pollinator behavior and body position, thus influencing the efficiency of pollen removal and deposition.

Previous studies have detected natural selection on both anther exsertion and stamen dimorphism through male fitness by the entire pollinator assemblage (Morgan and Conner 2001; Conner et al. 2003), but have not separated out selection by individual pollinator taxa.

In addition to studying selection on anther exsertion and stamen dimorphism, we examined selection on flower number and flower size, two traits that increase visitation rates in many species (e.g. Cruzan et al. 1988; Young and Stanton 1990; Campbell et al. 1991; Eckhart 1991). Furthermore, pollinators have been found to differ in their selection on flower size (Conner and Rush 1996; Galen 1996). In this study, flower size was calculated as the first principal component of six floral traits-petal length, petal width, long filament length, short filament length, corolla tube length, and pistil length (see Conner and Via 1993 for details).

Origin of plants used for the experiment

Since little variation in anther exsertion and stamen dimorphism is found in natural populations, all plants used in this experiment are from lines artificially selected to increase variation in anther exsertion and stamen dimorphism over 7 and 5 generations, respectively. Artificially increasing variation increases the power to detect selection on traits (Schluter 1988), as well as helps test whether populations in nature are at a fitness peak (Grafen 1988). Plants from each of six lines were selected to either increase anther exsertion, decrease anther exsertion, or decrease stamen dimorphism (two replicates of each). Two control lines were maintained over the same number of generations but were mated randomly, thus experiencing minimal selection (Conner and Karoly, unpub). Variance in anther exsertion after artificial selection ($\sigma^2 = 0.87$) was more than twice that in the control lines ($\sigma^2 = 0.41$), and variance in stamen dimorphism after artificial selection ($\sigma^2 = 0.42$) was almost twice that of the control population ($\sigma^2 = 0.25$).

2001 Single taxon days

As part of a separate experiment measuring total selection on floral traits in nature without separating selection by different pollinator taxa, three artificial arrays of 24 plants each were constructed by growing eight plants from each of the anther exertion artificial selection lines (8 high exsertion, 8 low exsertion, 8 control). Array plants were placed in a square grid in an old field at Kellogg Biological Station in Hickory Corners, Michigan, with plants from each selection line regularly interspersed in the grid. Each array was placed in the field on several single days throughout the growing season and plants were returned to the greenhouse at the end of each day and allowed to set seed. Each day an array was placed in the field, one flower from each plant was removed and photographed, and the six floral traits listed above were measured using NIH Object Image. Pollinator observations on each plant were carried out in order to characterize visitation rates of insect visitors. On two days during the experiment, the majority of the total visits were made by a single pollinator taxon, either the small sweat bee, *Dialictus* (98% of all visitors), or the small syrphid fly, *Toxomerus* (89%). We measured selection during these two days in order to estimate selection by each pollinator. Tissue from each array plant was collected and 11% of all seeds produced by *Toxomerus* visits (230 seeds total) and 16% of all seeds produced by *Dialictus* visits (123 seeds total) during each of the two days were germinated to collect leaf and bud tissue for subsequent paternity analysis.

2002 Field single-visit study

An equal number of seeds from each artificial selection line (high exsertion, low exsertion, low dimorphism, and 2 control lines) were planted in 25 cm pots in the greenhouse and fertilized with 1 1/2 tsp Osmocote Plus 15-9-12 fertilizer (Scotts-Sierra, Marysville, OH). Six artificial arrays of 15 plants each (3 from each of the 5 lines) were constructed, choosing plants based on their anther exsertion or stamen dimorphism to maximize the variance of each trait in each array, and to minimize differences between arrays in the trait means.

One array was removed from the greenhouse per day and placed next to an old field succession plot in the Kellogg Biological Station's LTER site in Hickory Corners, Michigan. The agricultural setting is similar to the environment of many wild radish populations, yet lacks wild radish, making paternity analysis possible for seeds produced during the experiment. Arrays were rotated throughout the summer with each array spending a total of 3-8 days in the field. Plants were placed one meter apart in a circular array, with plants from each of the five lines arranged in a regular fashion (i.e., high exsertion, low dimorphism, control exsertion, low exsertion, control dimorphism). The 15 plants in the array served as pollen donors; these are the plants for which selection through male fitness was estimated. After pollinators began visiting plants in the array, a single unvisited focal plant, the pollen recipient, was placed in the center of the circle, equidistant to each pollen donor. The pollen recipient was observed until a visit to a flower occurred. Pollen recipients are the plants for which selection through female fitness was measured.

After a single visit to the pollen recipient, the identity of the pollinator was recorded or the pollinator was caught and labeled for later identification. A straw labeled with a unique code was then placed over the flower to prevent further visitation, and the unique code, along with the pollinator's identity, was recorded. Single visits to unvisited flowers were permitted for up to 20 flowers per pollen recipient. The pollen recipient was then removed to prevent further visitation. Up to 10 pollen recipients were used each day and all array plants and pollen recipients were returned to the greenhouse at the end of the day.

Each day an array was placed in the field, one flower was removed from each array plant and focal plant and photographed, and floral traits (short filament length, long filament length, corolla tube length, pistil length, and petal length and width) were measured using NIH Object Image. Anther exsertion and stamen dimorphism were calculated as described above. The total number of flowers on each array plant was counted each day the array was in the field. A total of 87 different pollen recipients were exposed during 40 days throughout the summer, resulting in 2563 single visits and 1437 seeds.

We estimated selection through both male and female fitness by the four most abundant pollinator genera in 2002: the small syrphid fly, *Toxomerus*, the medium syrphid fly, *Syritta*, the large syrphid fly, *Eristalis*, and the small sweat bee, *Dialictus*. These four taxa made 81.3% of the total visits and were responsible for 76% of all seeds produced in 2002 (Table 4.1). Although we grouped taxa at the genus level, all species within each genera are similar in size and behavior (H. Sahli, pers. obs.) and are, thus, not likely to differ in their selection. Furthermore, *Syritta* was comprised of only one species, *Eristalis* of only two species, and *Toxomerus* of only three species. *Dialictus* was likely comprised of up to 11 different species (Chapter 3), but these species were all extremely similar in morphology and behavior, preventing identification to the species level in the field. All seeds resulting from visits made by each of the above four pollinators were germinated and leaves and flower buds were collected for the paternity analysis in order to determine the relative number of seeds sired by each male in each array (male fitness).

Enclosure experiment

There are three taxa that are important visitors in other populations of wild radish, but were uncommon during the 2002 field experiment: bumblebees (*Bombus*), honeybees (*Apis mellifera*), and cabbage butterflies (*Pieris rapae*) (Kay 1976; Kay 1978; Stanton et al. 1986). We also wished to test how the presence of multiple pollinators together might alter selection by each pollinator individually. Therefore, to estimate selection by these three pollinators separately and in combination, we constructed three 6' x 15' x 15' outdoor enclosures at the Kellogg Biological Station. Each cage contained either a honeybee hive, a hive of *Bombus impatiens* (Koppert Biological Systems; Romulus, MI), or wild caught cabbage butterflies. To measure selection when all three were present, the bumblebee hive and cabbage butterflies were temporarily moved into the cage with the honeybee hive.

Three artificial arrays of 20 plants each were constructed, for a total of 60 plants. Five plants from each of the lines (high exsertion, low exsertion, low dimorphism, and control) were chosen for each array. Plants were grown in the greenhouse as described above and left in the pollinator-free greenhouse when not in use. Before an array was exposed to a pollinator treatment, one flower from each plant was removed and photographed for later measurement, and the total number of open flowers per plant was counted. All open flowers were marked with colored tape for subsequent identification of the pollinator responsible for fruit production. Array plants were then placed in a cage in a 4 x 5 square, with plants from each line interspersed in a regular fashion. Once an array was placed in a cage, plants were observed for a 10-minute period and the number of visits per plant was recorded. When plants received an average of 10 visits per flower, the average daily visitation rate in nature (Sahli, unpub. data), the array was removed from the cage, returned to the greenhouse and allowed to set seed. This was repeated for each array across all four pollination treatments, with each array being exposed to bumblebees, honeybees, and cabbage butterflies for three days each (with the exception

Table 4.1. Proportion of visits made and of the total number of seeds effected by each pollinator genus during the 2002 single visit study. Taxa in bold are those for which selection was estimated using data from the single visits.

Taxon	% visits	% total seeds effected by each pollinator
Apis	1.8	5.2
Bombus	0.6	0.6
Augochlorella	4.8	7.4
Halictus	4.1	3.5
Colletes	0.2	0.5
Ceratina	1.1	2.7
Dialictus	**44.9**	**46.2**
Pieris	0.8	1.8
Thymelicus	0.9	0.9
Eristalis	**6.8**	**10.2**
Syritta	**11.3**	**10.4**
Sphaerophoria	1.7	0.3
Syrphus	2.1	1.0
Allograpta	0.8	0
Toxomerus	**18.3**	**9.3**

of two days of exposure to bumblebees for one array), and each array being exposed twice to the treatment of all three pollinators combined. After each bout of exposure to pollinators, arrays were left in the pollinator-free greenhouse for at least five days, after which time stigmas were no longer receptive. Fruits were collected after maturation (approximately 3 weeks) and the pollinator treatment was recorded for all fruits produced. Leaf tissue was collected from each array plant for later genotyping. 288-313 seeds produced from each pollinator treatment were planted and leaf and flower bud tissue was collected for paternity analysis.

Abundance of each pollinator genus in the combined pollinator treatment was manipulated in an attempt to minimize differences in abundance among pollinators. However, *Apis* always made the majority of the visits (55%), *Pieris* 30%, and *Bombus* 15%.

Paternity Analysis

Total genomic DNA was extracted from each array plant and each offspring using QBIOGENE'S FastDNA Kit and the FastPrep Instrument (Carlsbad, CA) following the kit protocol. Individuals were genotyped at eight microsatellite loci derived from Brassica: Bn26a, Bn35d, Brms 005, Na10-H06, Na12-E05, Na14-E08, Ra1-H08, and Ra2-E11 (UK CropNet Brassica database; http://ukcrop.net/brassica.html). Microsatellites were PCR amplified in 10-μl reactions consisting of: 1.0μL each of 10x buffer and 25mM $MgCl_2$, 0.25 μL of 2mM dNTPs, 7.05 μL of water, 0.1 μL of *Taq* polymerase, 0.15 μL of the 10 μM primers, with a fluorescently labeled forward primer, and 0.3μL (~6ng) of DNA. The PCR program consisted of one initial denaturing phase at 94°C for 10 minutes, followed by 25 cycles, each consisting of 94°C for one minute, annealing at 50°- 61°C (depending on the primer) for 45 seconds, and an extension at 72°C for 1 minute. The 25 cycles were followed by an additional elongation step of 72°C for 10 minutes. Microsatellite samples were run on 6% polyacrylamide gels, with 8 μl Temed and 400 μl 10% APS added to each gel. Bands were visualized on FMBIO II

Multiview scanner (Hitachi Software Engineering Co. Ltd., San Francisco, CA). Alleles were scored using FMBIO Analysis 8.0 (Hitachi Software Engineering 1991-1999) and placed in bins using Allelogram 1.2 (Manaster 1998). Because genotyping at these eight microsatellite loci gave us 95% exclusion probability rather than total exclusion probability, we conclusively assign each offspring to only one father. Therefore, we used the fractional method to assign paternity fractionally, with the fraction of paternity proportional to the likelihood of paternity (following Devlin et al. 1988). An alternative method to estimating male fertility when multiple fathers are likely is to assign paternity based on the most-likely father. However, this alternative method consistently overestimates male fitness of homozygous individuals (Devlin et al. 1988), thus is not preferred.

Estimating Selection Gradients

Standardized selection gradients (Lande and Arnold 1983) were calculated by regressing relative fitness on the floral traits after standardizing the traits within an array to mean = 0 and variance = 1. The model for estimating selection through male and female fitness was: relative fitness = anther exsertion + anther exsertion^2 + stamen dimorphism + stamen dimorphism^2 + number of open flowers + flower size + flower size^2 (but see below for exceptions), with the models differing only in terms of the estimate of fitness--either relative fractional paternity (male fitness) or relative number of seeds produced (female fitness). Fitness was relativized within arrays by dividing each individual's fitness by the array mean fitness. The squared term for flower number was not included in the model because there is no hypothesis for stabilizing selection on flower number and because sample sizes were modest (Table 4.2).

In the 2001 single taxon day analysis, selection on stamen dimorphism was not measured because no plants from the dimorphism artificial selection lines were used and sample sizes were low (Table 4.2). In the 2002 single-visit study, the model used to estimate selection through female fitness included the total number of single visits made

Table 4.2. Sample sizes for estimating selection through male and female fitness for each pollinator taxon and the number of offspring genotyped per potential father.

Taxon	# males	# females	# offspring genotyped per potential father
Dialictus[1]	75	62	1.48
Dialictus[2]	24	24	5.13
Toxomerus[1]	45	63	0.44
Toxomerus[2]	24	24	9.54
Eristalis[1]	45	26	1.78
Syritta[1]	45	38	1.58
Bombus[3]	60	60	5.17
Apis[3]	60	60	5.10
Pieris[3]	60	60	4.8
Apis + *Pieris* + *Bombus*[3]	60	60	5.22

[1] 2002 single visit experiment

[2] 2001 days when arrays were visited primarily by one taxon

[3] 2003 cage experiment

to each focal plant. Including number of visits in the model accounts for differences in seed production due to differences in the number of days a focal plant was exposed to pollinators and in the amount of time per day a focal plant was allowed to be visited. Thus, differences in female fitness due to visitation rates were eliminated from the analysis. Because flower size and flower number are traits likely to affect visitation rates, these two traits were not included in the model for selection through female fitness.

Linear estimates of selection were estimated from a model that did not include the squared terms (Lande and Arnold 1983), and 95% confidence intervals for all selection gradients were obtained by bootstrapping the original data 10,000 times. In each bootstrap iteration, observations were randomly sampled with replacement and selection gradients were calculated. Two-tailed p-values were determined as the proportion of the number of selection gradients above or below zero (for negative and positive selection gradients, respectively) multiplied by two (Crowley 1992; Legendre and Legendre 1998). Regression analyses and bootstrapping were done in SAS (9.1).

Total selection on floral traits was estimated by averaging selection through male and female fitness (Morgan 1992). Confidence intervals on the total selection gradients were estimated by averaging the standard errors from the regression on male and female fitness, and multiplying by two. Finally, a weighted average selection gradient and standard error was obtained for each trait, weighting gradients and standard errors by the proportion of total seeds effected by each pollinator.

Selection through male versus female fitness

To test for differences in the magnitude of selection through male versus female fertility, we performed a paired t-test on the absolute values of selection gradients estimated through male and female fitness for each trait. Although traits are correlated, and thus non-independent, selection gradients are already corrected for correlations among traits. Furthermore, correlations among traits were generally low (range of absolute value: 0.02-0.60)

Results

Selection on anther exsertion and stamen dimorphism

Dialictus exerted significant stabilizing selection on anther exsertion through both male and female fitness, *Bombus* and *Apis* both selected to increase anther exsertion through male fitness, and *Eristalis* selected to decrease anther exsertion through female fitness (Table 4.3). However, when selection through male and female fitness were averaged only *Dialictus* significantly selected on anther exsertion, exerting stabilizing selection (Table 4.3, Figure 4.3A).

Bombus and *Toxomerus* exerted stabilizing selection on stamen dimorphism through male and female fitness (respectively), *Apis* selected for decreased dimorphism through male fitness, and *Eristalis* selected for decreased dimorphism through female fitness (Table 4.4). However, *Apis* was the only pollinator to significantly select on stamen dimorphism when selection through both male and female fitness were averaged, where the selection gradient had a significant negative linear term and a marginally significant positive quadratic term (Table 4.4, Figure 4.3b). This indicates a decline in fitness as stamen dimorphism increases. Confidence intervals for all pollinators overlapped, indicating no significant differences among pollinators in their selection.

Selection on flower size

Bombus selected to decrease flower size through male fitness, but exerted stabilizing selection on flower size through female fitness (Table 4.5). *Dialictus* exerted stabilizing selection on flower size through female fitness, and *Bombus, Apis*, and *Pieris* combined exerted stabilizing selection on flower size through both male and female fitness. Upon averaging selection through male and female fitness, *Dialictus* and *Bombus*, *Apis,* and *Pieris* combined exerted stabilizing selection on flower size (Figure 4.3, Table 4.5). Neither *Bombus, Apis*, nor *Pieris* exerted significant selection on flower size individually, and flower size selection gradients for all three pollinators were similar (Table 4.5)

Table 4.3. Standardized linear (β) and quadratic (γ) selection gradients for anther exsertion by different pollinators. β estimates represent directional selection, and γ estimates represent curvature of the line. Total selection is the average of selection through male and female fitness. Values in bold are significant at $P \leq 0.10$; exact P values could not be determined for total selection gradients. *BAP* = *Bombus* + *Apis* + *Pieris*.

	Male fitness		Female fitness		Total Selection	
Pollinator	β	γ	β	γ	β	γ
Dialictus[1]	0.07	-0.06	**-0.32**	-0.16	-0.13	-0.11
	(-0.09, 0.23)	(-0.36, 0.20)	(-0.68, 0.01)	(-0.60, 0.30)	(-0.35, 0.10)	(-0.43, 0.21)
Dialictus[2]	-0.02	**-0.42***	-0.09	**-0.58***	-0.06	**-0.50***
	(-0.26, 0.22)	(-0.92, -0.08)	(-0.47, 0.32)	(-1.28, -0.06)	(-0.39, 0.28)	(-0.94, -0.06)
Toxomerus[1]	-0.1	0.12	0.1	0.20	0.00	0.16
	(-0.75, 0.46)	(-0.54, 1.18)	(-0.30, 0.58)	(-0.36, 1.06)	(-0.55, 0.55)	(-0.60, 0.92)
Toxomerus[2]	0.25	-0.16	0.1	0.10	0.18	-0.03
	(-0.18, 0.69)	(-1.14, 0.76)	(-0.14, 0.32)	(-0.30, 0.50)	(-0.16, 0.51)	(-0.51, 0.45)
Eristalis[1]	-0.06	-0.06	**-0.52***	-0.06	-0.29	-0.06
	(-0.34, 0.26)	(-0.36, 0.38)	(-1.07, -0.03)	(-1.4, 1.02)	(-0.7, 0.12)	(-0.49, 0.37)
Syritta[1]	-0.21	0.36	0.09	-0.40	-0.06	-0.02
	(-0.49, 0.05)	(-0.18, 1.04)	(-0.44, 0.82)	(-1.44, 0.64)	(-0.43, 0.31)	(-0.48, 0.44)
Bombus[3]	**0.14***	**0.20**	-0.03	0.001	0.06	0.10
	(0.01, 0.28)	(-0.04, 0.38)	(-0.15, 0.09)	(-0.26, 0.22)	(-0.11, 0.22)	(-0.16, 0.36)
Apis[3]	**0.13**	-0.08	-0.02	0.06	0.06	-0.01
	(-0.003, 0.25)	(-0.28, 0.12)	(-0.15, 0.12)	(-0.18, 0.34)	(-0.08, 0.19)	(-0.23, 0.21)
Pieris[3]	0.08	-0.04	**-0.14**	0.08	-0.03	0.02
	(-0.07, 0.22)	(-0.32, 0.18)	(-0.32, 0.02)	(-0.18, 0.30)	(-0.19, 0.13)	(-0.26, 0.30)
BAP[3]	-0.03	0.04	-0.06	0.10	-0.05	0.07
	(-0.16, 0.09)	(-0.22, 0.30)	(-0.17, 0.07)	(-0.12, 0.32)	(-0.11, 0.02)	(-0.17, 0.31)

* $P \leq 0.05$; ** $P \leq 0.01$

[1] Selection gradients estimated in the 2002 single visit experiment

[2] Selection gradients estimated in the 2001 days when arrays were visited primarily by one genus

[3] Selection gradients estimated in the 2003 cage experiment

[†] Gradient shown is only that through male fitness

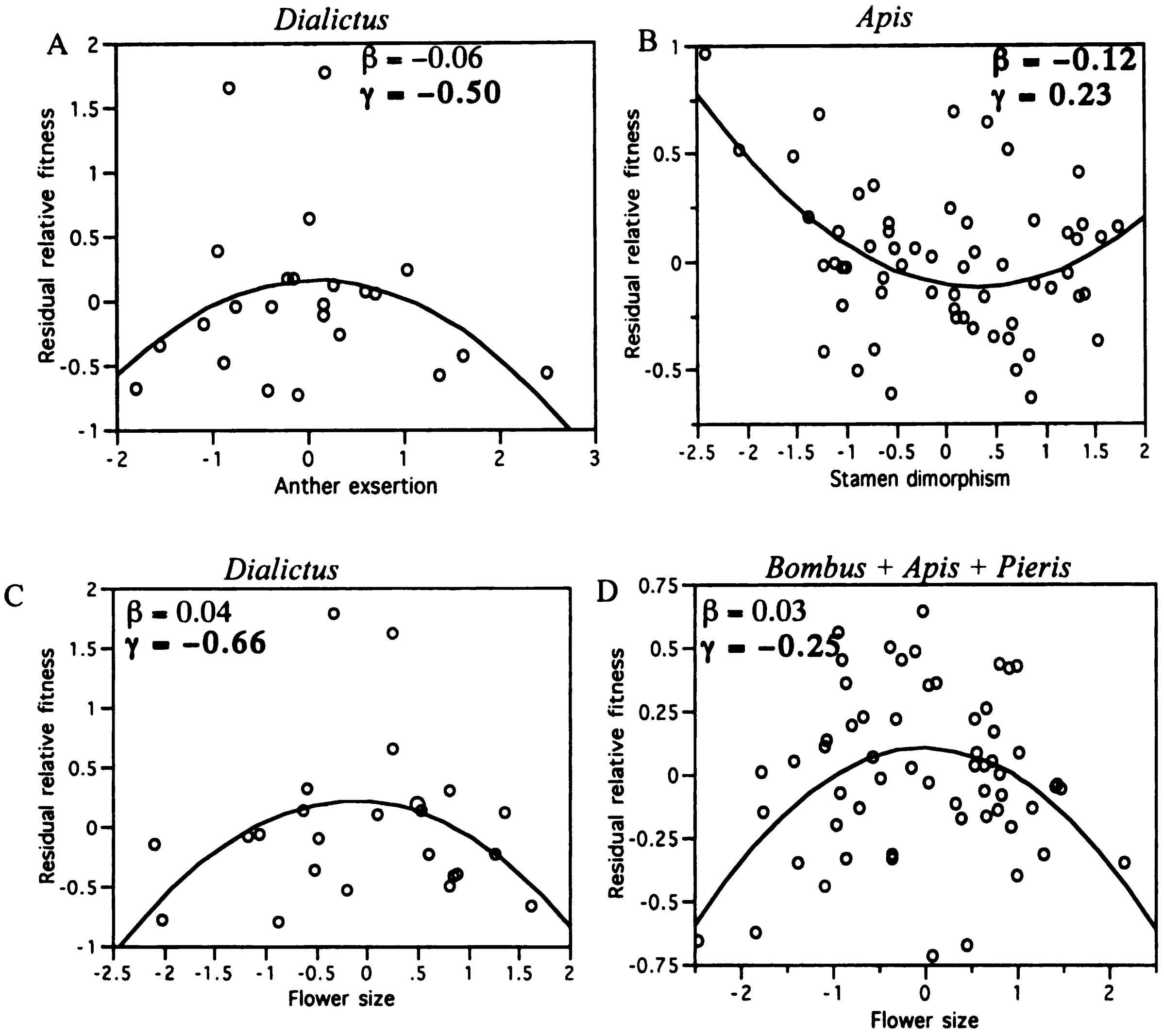

Figure 4.3. Selection through total (male and female) fitness by (A) *Dialictus* on anther exsertion, (B) *Apis* on stamen dimorphism, (C) *Dialictus* on flower size, and (D) *Apis, Bombus,* and *Pieris* combined on flower size. Standardized trait values are shown on the x-axis. Gradients significant at $P \leq 0.05$ are in bold.

Table 4.4. Standardized linear (β) and quadratic (γ) selection gradients for stamen dimorphism by different pollinators (95% CIs). β estimates represent directional selection, and γ estimates represent curvature of the line. *BAP* = *Bombus* + *Apis* + *Pieris*. Total selection is the average of selection through male and female fitness. Values in bold are significant at $P \leq 0.10$; exact P values could not be determined for total selection gradients.

Pollinator	Male fitness		Female fitness		Total Selection	
	β	γ	β	γ	β	γ
Dialictus[1]	0.02 (-0.11, 0.15)	0.004 (-0.20, 0.18)	0.16 (-0.09, 0.39)	-0.24 (-0.68, 0.14)	0.09 (-0.13, 0.31)	-0.12 (-0.48, 0.24)
Toxomerus[1]	-0.07 (-0.54, 0.46)	0.54 (-0.74, 1.52)	0.15 (-0.16, 0.47)	**-0.48*** (-1.46, -0.06)	0.04 (-0.50, 0.58)	0.03 (-0.79, 0.85)
Eristalis[1]	0.16 (-0.11, 0.48)	0.10 (-0.30, 0.56)	**-0.53*** (-1.18, -0.07)	-0.06 (-1.8, 1.34)	-0.18 (-0.60, 0.22)	0.02 (-0.41, 0.45)
Syritta[1]	-0.13 (-0.36, 0.10)	-0.10 (-0.48, 0.28)	-0.35 (-1.16, 0.27)	0.68 (-0.54, 2.36)	-0.24 (-0.61, 0.13)	0.29 (-0.25, 0.83)
Bombus[2]	0.02 (-0.12, 0.18)	**-0.22** (-0.46, 0.008)	-0.005 (-0.14, 0.16)	0.08 (-0.16, 0.30)	0.007 (-0.15, 0.17)	-0.07 (-0.33, 0.19)
Apis[2]	**-0.14*** (-0.25, -0.02)	**0.14** (-0.04, 0.38)	-0.10 (-0.28, 0.07)	**0.32** (-0.04, 0.56)	**-0.12*** (-0.24, 0)	**0.23*** (0.01, 0.45)
Pieris[2]	0.01 (-0.10, 0.13)	-0.004 (-0.18, 0.16)	0.09 (-0.05, 0.26)	0.04 (-0.22, 0.30)	0.05 (-0.1, 0.2)	0.02 (-0.22, 0.26)
BAP[2]	0.03 (-0.10, 0.16)	0.0004 (-0.18, 0.20)	-0.03 (-0.15, 0.08)	**0.22**** (0.06, 0.42)	0.00 (-0.07, 0.07)	0.11 (-0.05, 0.27)

* $P \leq 0.05$; ** $P \leq 0.01$

[1] Selection gradients estimated in the 2002 single visit experiment

[2] Selection gradients estimated in the 2003 cage experiment

Selection on flower number

Plants with fewer flowers open at a time sired more offspring when pollinated by *Eristalis* (Table 4.6), but *Toxomerus*, *Apis*, and *Bombus*, and the treatment with all three pollinators all selected to increase floral display through female fitness. Selection by the above pollinators to increase floral display remained significant in the total estimate of selection on flower number (Table 4.6, Figure 4.4). However, because selection by *Eristalis* on flower number through female fitness was not measured, we do not know if the selection to decrease flower number would be present in the total selection gradient.

Selection through male vs. female fertility

Overall, the magnitude of selection was greater through female fertility (0.19) than through male fertility (0.11) (paired *t*-ratio, 3.50, $P = 0.001$; N =54). Because female fertility in the single visit studies is completely pollen limited, and thus does not come reflect selection through female fitness under natural visitation rates, we tested for differences in the magnitude of selection with the single visit studies excluded and still found selection through female fitness to be higher than that through male fitness (paired $t = -2.17$, $P = 0.04$, N = 38). Selection gradients estimated through male and female fitness were not correlated ($R = 0.08$, $P = 0.56$).

To test if selection through male and female fitness differed more for traits influencing attraction (i.e., flower number and flower size) as opposed to pollinator efficiency traits (anther exsertion and stamen dimorphism), we performed paired *t*-tests as above on each subset of traits. When all studies were included in the analysis, selection through female fitness was greater than that through male fitness for traits affecting attraction (mean = 0.20 vs. 0.08; paired $t = 2.60$; $P = 0.02$; $N = 18$) and for traits affecting efficiency (mean 0.19 vs. 0.12; paired $t = 2.40$; $P = 0.02$; $N = 36$). However, when single visit studies were removed from the analysis, selection through female fitness was still significantly greater through female fitness than through male fitness for attraction traits (mean 0.19 vs. 0.08; paired $t = 2.60$; $P = 0.02$; $N = 18$), but the magnitude of selection on

Table 4.5. Standardized linear (β) and quadratic (γ) selection gradients for flower size by different pollinators. Dashed lines indicate selection was not measured. Total selection is the average of selection through male and female fitness. Values in bold are significant at $P \leq 0.06$; exact P values could not be determined for total selection gradients. *BAP* = *Bombus* + *Apis* + *Pieris*.

Pollinator	Male fitness		Female fitness		Total Selection	
	β	γ	β	γ	β	γ
Dialictus^1	-0.002	0.02	--	--	-0.002†	0.02†
	(-0.12, 0.11)	(-0.18, 0.24)			(-0.12, 0.11)	(-0.18, 0.24)
Dialictus^2	0.13	-0.38	-0.06	**-0.94****	0.04	**-0.66***
	(-0.13, 0.42)	(-0.94, 0.16)	(-0.42, 0.30)	(-1.86, -0.28)	(-0.31, 0.38)	(-1.2, -0.12)
Toxomerus^1	0.1	0.04	--	--	0.10†	0.04†
	(-0.40, 0.54)	(-0.80, 0.80)			(-0.40, 0.54)	(-0.80, 0.80)
Toxomerus^2	-0.02	0.02	0.12	-0.16	0.05	-0.07
	(-0.49, 0.46)	(-1.02, 1.1)	(-0.22, 0.44)	(-0.80, 0.48)	(-0.28, 0.38)	(-0.69, 0.55)
Eristalis^1	0.19	0.28	--	--	0.19†	0.28†
	(-0.15, 0.53)	(-0.16, 0.78)			(-0.15, 0.53)	(-0.16, 0.78)
Syritta^1	0.07	0.02	--	--	0.07†	0.02†
	(-0.17, 0.33)	(-0.44, 0.60)			(-0.17, 0.33)	(-0.44, 0.60)
Bombus^3	**-0.17****	**0.16***	0.04	**-0.24***	-0.07	-0.04
	(-0.29, -0.07)	(-0.006, 0.36)	(-0.14, 0.18)	(-0.44, -0.02)	(-0.22, 0.09)	(-0.30, 0.22)
Apis^3	0.02	0.01	0.04	-0.12	0.03	-0.05
	(-0.09, 0.13)	(-0.20, 0.26)	(-0.10, 0.18)	(-0.36, 0.14)	(-0.09, 0.15)	(-0.27, 0.17)
Pieris^3	0.003	0.02	-0.02	0.01	-0.009	0.01
	(-0.1, 0.11)	(-0.16, 0.20)	(-0.19, 0.13)	(-0.28, 0.38)	(-0.15, 0.13)	(-0.23, 0.26)
BAP^3	0.06	**-0.24****	0.006	**-0.26****	0.03	**-0.25***
	(-0.06, 0.17)	(-0.44, -0.06)	(-0.14, 0.15)	(-0.46, -0.08)	(-0.03, 0.09)	(-0.45, -0.05)

* $P \leq 0.05$; ** $P \leq 0.01$

[1] Selection gradients estimated in the 2002 single visit experiment

[2] Selection gradients estimated in the 2001 days when arrays were visited primarily by one genus

[3] Selection gradients estimated in the 2003 cage experiment

[†] Gradient shown is only that through male fitness

Table 4.6. Standardized linear (β) selection gradients for flower number by different pollinators. Dashed lines indicate selection was not measured. Values in bold are significant at $P \leq 0.10$; exact P values could not be determined for total selection gradients. *BAP = Bombus + Apis + Pieris.*

Pollinator	Male fitness	Female fitness	Total Selection
Dialictus[1]	-0.05 (-0.18, 0.07)	--	-0.05[†] (-0.18, 0.07)
Dialictus[2]	-0.05 (-0.33, 0.26)	-0.17 (-0.64, 0.28)	-0.11 (-0.44, 0.22)
Toxomerus[1]	0.15 (-0.20, 0.67)	--	0.15[†] (-0.20, 0.67)
Toxomerus[2]	0.02 (-0.40, 0.39)	**0.58**** (0.20, 0.84)	**0.30*** (0.04, 0.50)
Eristalis[1]	**-0.28*** (-0.54, -0.04)	--	**-0.28**[†]* (-0.54, -0.04)
Syritta[1]	0.02 (-0.21, 0.24)	--	0.02[†] (-0.21, 0.24)
Bombus[3]	0.04 (-0.1, 0.19)	**0.25**** (0.10, 0.41)	0.15 (-0.005, 0.30)
Apis[3]	0.06 (-0.05, 0.17)	**0.21**** (0.05, 0.36)	**0.14*** (0.02, 0.26)
Pieris[3]	-0.05 (-0.15, 0.06)	**0.16** (-0.03, 0.34)	0.06 (-0.9, 0.20)
BAP[3]	0.07 (-0.05, 0.18)	**0.16*** (0.03.0.29)	**0.12*** (0.06, 0.18)

* $P \leq 0.05$; ** $P \leq 0.01$

[1] Selection gradients estimated in the 2002 single visit experiment

[2] Selection gradients estimated in the 2001 days when arrays were visited primarily by one genus

[3] Selection gradients estimated in the 2003 cage experiment

[†] Gradient shown is only that through male fitness

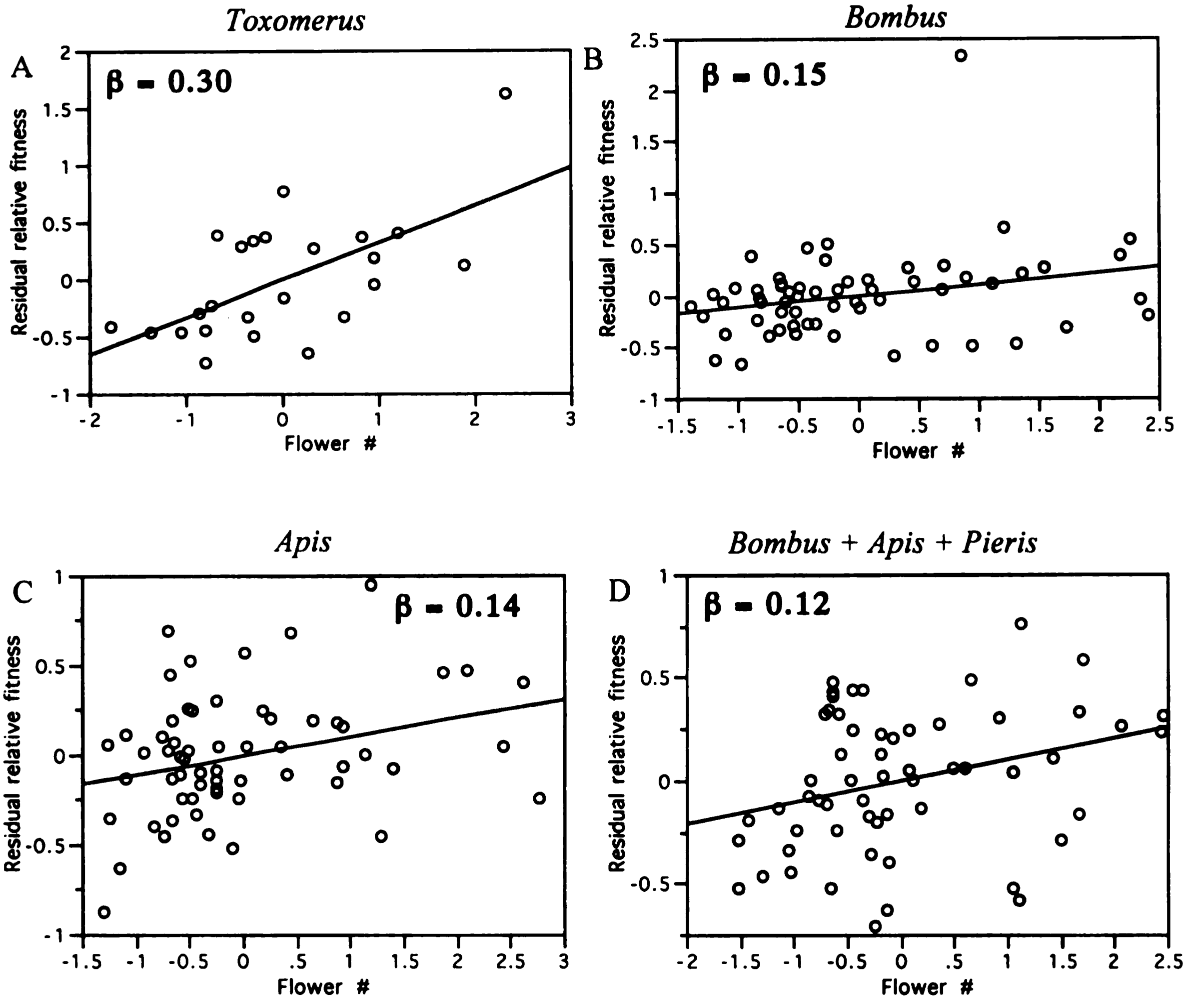

Figure 4.4. Selection on flower size through total fitness (male and female) by (A) *Toxomerus*, (B) *Bombus*, (C) *Apis*, and (D) *Apis, Bombus*, and *Pieris* combined. Standardized trait values are shown on the x-axis.

efficiency traits was the same through both male and female fertility (mean = 0.11; paired $t = 0.18$; $P = 0.86$; $N = 20$).

Average selection on traits

Since selection by *Dialictus* and *Toxomerus* was estimated using two different methods, we estimated the weighted average selection gradient for each trait twice, once using the single visit selection gradients for *Toxomerus* and *Dialictus*, and once using the single taxon day selection gradients for *Toxomerus* and *Dialictus*. Because visits by *Dialictus* cause 46% of all seeds set (Table 4.1), the weighted selection gradients for each trait are similar to the selection exerted by *Dialictus* alone. Thus, when the weighted selection gradient was calculated using the selection gradients estimated from the single visits, selection gradients for all traits were close to zero (Table 4.7). However, when weighted selection gradients were calculated using the 2001 single taxon day results, the γ terms for both anther exsertion and flower size were large and negative, and the β terms were close to zero, suggesting stabilizing selection on both of these traits. However, due to large variance around these estimates, they were not significantly different from zero (Table 4.7).

To compare differences in the average selection gradient when *Bombus*, *Apis,* and *Pieris* were all combined (*BAP*) with the weighted average of the individual selection gradients for each pollinator, selection by each pollinator alone was weighted by the proportion of total visits each taxon made in the *BAP* treatment (see Methods). Weighted selection gradients were similar to those in the *BAP* treatment for all traits except flower size (Table 4.8). However, confidence intervals around the weighted average selection gradient for flower size overlapped those of *BAP*, preventing us from detecting a significant non-additive effect of selection by each pollinator alone versus when all three pollinators were combined.

Table 4.7. Total linear (β) and quadratic (γ) selection gradients for each trait estimated by weighting each taxon's total selection gradient (Table 7) by its proportional importance to reproduction. Squared terms are the quadratic estimates of selection. 95% confidence intervals in parentheses were calculated as two standard errors from the mean selection gradient, where standard errors are the weighted average standard error for each trait.

Trait	$\beta^{\dagger}$	$\gamma^{\dagger}$	$\beta^{\ddagger}$	$\gamma^{\ddagger}$
Anther	-0.11	-0.05	-0.05	-0.29
exsertion	(-0.40, 0.18)	(-0.44, 0.34)	(-0.33. 0.28)	(-0.71, 0.14)
Stamen	-0.004	-0.01	--	--
dimorphism	(-0.29, 0.28)	(-0.44, 0.42)		
	0.04	0.05	0.06	-0.34
Flower size	(-0.16, 0.25)	(-0.13, 0.23)	(-0.25, 0.37)	(-0.78, 0.11)
	-0.03	--	-0.05	--
# open flowers	(-0.22, 0.15)		(-0.32, 0.23)	

[†] Gradients estimated from the 2001 one taxon days are not included

[‡] Gradients for *Toxomerus* and *Dialictus* are based on the single taxon day analysis rather than the single visit analysis. Because stamen dimorphism was not included in the analyses for *Toxomerus* and *Dialictus* during the single taxon days, a weighted average was not calculated for this trait.

Table 4.8. Average total selection gradients when *Apis*, *Bombus*, and *Pieris* (*ABP*) are all present and weighted average selection gradients estimated by multiplying each pollinator's selection gradient by its visitation rates in the environment with all pollinators combined. Values in bold are significant based on 95% confidence intervals.

Trait	Total selection for *BAP*		Weighted average selection for *BAP*	
	β	γ	β	γ
Anther exsertion	-0.05 (-0.11, 0.02)	0.07 (-0.17, 0.31)	0.03 (-0.11, 0.17)	0.02 (-0.23, 0.26)
Stamen dimorphism	0.00 (-0.07, 0.07)	0.11 (-0.05, 0.27)	-0.06 (-0.18, 0.09)	0.12 (-0.11, 0.35)
Flower size	0.03 (-0.03, 0.09)	**-0.25** (-0.45, -0.05)	0.005 (-0.13, 0.13)	-0.03 (-0.26, 0.20)
# open flowers	**0.12** (0.06, 0.18)	--	0.11 (-0.02, 0.24)	--

Discussion

Support for trade-offs

Although trade-offs were commonly found in our study when selection gradients through male and female fitness were examined separately, a trade-off in pollinators' total selection on floral traits was only found on one occasion: selection on flower number. The only trade-off in selection on flower number occurred in selection by the large syrphid fly, *Eristalis* versus the large bees and the small syrphid fly, *Toxomerus*. Furthermore, since we could not measure selection on flower number by *Eristalis* through female fitness, we do not know if total selection on the trait would still be negative. Thus, there may be no trade-off in adaptation of flower number to different pollinators.

In general, greater flower number is thought to increase fitness because it is not only correlated with total ovule and pollen number, but floral display also attracts pollinators (Willson and Rathcke 1974; Cruzan et al. 1988; Eckhart 1991; Conner and Rush 1996). The negative directional selection through male fitness exerted by *Eristalis* could either be due to the large syrphid flies avoiding plants with many flowers, or due to them removing or transferring less pollen from plants with more flowers. It is difficult to understand why a pollinator would avoid plants with more flowers unless these plants were already being visited by other pollinators, in which case selection by one pollinator would be altered by the presence of other pollinators. We were unable to test for interactions among *Eristalis* and other pollinators in this experiment to determine if selection by *Eristalis* is dependent upon the presence or absence of other pollinators.

Support for common adaptive peaks and morphological specializations

The fact that we only detected significant selection by *Dialictus* on anther exsertion, and by *Apis* on stamen dimorphism suggests that only a subset of the pollinators are actually selecting on floral traits. This indicates the possibility of morphological specializations where all pollinators but one do not select on the traits. However, because confidence intervals around the selection gradients measured for other pollinators overlap those of *Dialictus* and *Apis*, we cannot rule out the possibility that pollinators share a common adaptive peak. We can conclude that most of the significant selection exerted by individual pollinators does not oppose that of other pollinators, indicating an ability to adapt to one pollinator without a trade-off in adaptation to another pollinator. However, the overlap in confidence intervals of selection gradients among pollinators could also be due to low statistical power to estimate selection. Sample sizes were relatively similar among pollinators, so this is unlikely to fully explain our result of significant selection by only a subset of the pollinators.

Stabilizing selection on flower size by both *Dialictus* and by the three pollinators *Bombus, Apis*, and *Pieris* combined does suggest a common adaptive peak among these four pollinators. However, it is interesting that none of the three larger pollinators selected on flower size by themselves, but only in combination was selection detected. While we could not detect a significant difference in the expected selection on flower size when total selection on a trait is purely additive (i.e., the weighted average selection gradient) and the actual selection when all three pollinators were present, the results are suggestive of an interaction, although the variance in our weighted average selection gradient prevents us from drawing a firm conclusion.

Selection through male vs. female function

A recent review of 7 studies measuring selection on attraction traits through both male and female fitness (Ashman and Morgan 2005) found no significant difference between gender functions in the magnitude of selection. Our findings, however, show that selection on traits is stronger through female fertility than through male fertility. Our estimates of selection through female fertility in the single visit studies are likely stronger than expected under natural pollination conditions since seed set was extremely pollen limited rather than resource limited. However, our finding of stronger selection through female fitness than male fitness was upheld when the single visit studies were excluded from the analysis. Still, the few times in which plants were exposed to pollinators, combined with the fact that they were grown in pots with ample fertilizer suggests that the plants used in all of our experiments were not likely to be as resource limited as those in natural populations, indicating that our plants were likely more pollen-limited than under natural field conditions. Thus, our findings of stronger selection through female than male fitness might not have been upheld in a natural population. Furthermore, error rates in estimating male fitness are likely to be higher than those in estimating female fitness, reducing our power to detect selection through male fitness relative to that through female fitness.

Although selection was stronger through female fitness, this pattern was largely driven by differences in traits influencing attraction rather than traits influencing efficiency of pollen transfer. Including the single visit studies, where female fertility was strongly pollen limited, allowed us to detect strong selection on efficiency traits through female fertility, whereas in experiments where plants were not as heavily pollen limited there was no difference between selection on efficiency traits through male or female fertility. This finding shows the context dependence of selection through female function, where selection is stronger through female function when plants are pollen limited, a conclusion that has been supported in previous studies (Ashman and Morgan 2005). Results from our study do not support the common view that selection is stronger through male than through female fertility, and clearly more studies measuring selection through both male and female fitness on more plant species are needed.

The impact of pollinator importance on total selection

If pollinators differ in how they select on a trait, differences in pollinator frequency can alter the total selection on a trait because total selection will be weighted more heavily by the most important pollinator. The large variance among pollinators of *R. raphanistrum* in their importance to plant reproduction (Chapter 3), which is caused mainly be variation in visitation rates, and differences among pollinators in the selection they exert indicates that total selection on a trait is dependent upon the relative visitation frequencies of each taxon. When a weighted average selection gradient was calculated through total fitness, we found that selection on traits primarily reflected selection by the most important pollinator, *Dialictus*. Therefore, although multiple pollinators are present in plant populations and differ in their selection on traits, if one pollinator dominates and selects on floral traits, our findings indicate the population will adapt to the most common pollinator, supporting Stebbins' principle of specialization on the most important pollinator (Stebbins 1970). In this case, wild radish may be ecologically generalized, using many pollinators as resources, but evolutionarily specialized, adapting to only a subset of these resources. Two lines of evidence suggest that selection on flower size and anther exsertion is occurring in the presence of multiple pollinators. Conner *et al.* (2003) also found stabilizing selection on both anther exsertion and flower size in 1992 when they estimated selection on planted arrays under natural pollination. Furthermore, Conner *et. al.* (unpub.) found stabilizing selection on anther exsertion in a similar study in 2001. Results from this study, as well as those from previous studies measuring selection by the entire pollinator community, indicate that the stabilizing selection on anther exsertion detected in nature, and the little variation in anther exsertion in natural populations, may be due to stabilizing selection exerted by *Dialictus*. The stabilizing selection on anther exsertion is equivalent to correlational selection on filament and tube length, and thus may be responsible for the very strong correlations between these traits (Conner and Via 1993; Conner 2002). Additionally, temporal or spatial shifts in the dominant pollinator could lead to differences in the total selection on these traits.

Conclusions

Given previous findings of stabilizing selection on anther exsertion when all pollinators are present, and findings from this study suggesting that *Dialictus* is the only pollinator exerting stabilizing selection on anther exsertion, the simplest explanation is that selection by the dominant pollinator, *Dialictus*, may be influencing evolution of anther exsertion in populations of wild radish in the eastern United States. This study suggests that, although plants may be visited by many pollinator genera, generalists may be able to use multiple resources while still adapting to a subset of these resources. This study also emphasizes that the strength and direction of selection on floral traits is strongly dependent on the local abundance of each pollinator taxon in the community and its selection pressure.

CHAPTER 5

THE CONTRIBUTION OF GENETIC DRIFT AND DIVERGENT SELECTION TO PATTERNS OF POPULATION DIFFERENTIATION IN THE GLOBALLY DISTRIBUTED WEED, WILD RADISH (*RAPHANUS RAPHANISTRUM*)

Abstract

Weedy species with large distributions may face strong selection pressure to adapt to new environments, leading to genetic differentiation among populations. However, genetic drift can also produce differentiation. To test whether natural selection has contributed to trait divergence, we compared differentiation at presumptively neutral loci to differentiation of floral and phenological traits of wild radish (*Raphanus raphanistrum*) across populations on three continents distributed throughout its current range. We sampled eight populations occurring in agricultural settings of subspecies *raphanistrum* and one population of a non-weedy, native subspecies, *maritimus*, to examine how agricultural populations differed from the non-agricultural population, as well as testing for differences in traits in the introduced populations (non-Mediterranean) versus those in native populations (Mediterranean). By comparing estimates of Q_{ST} and F_{ST}, we found that most floral traits had not diverged across populations, but that flowering time and ovule number have experienced strong divergent selection across populations, with ovule number differing between subspecies, and flowering time differing between Mediterranean and non-Mediterranean populations. Our results suggest that, despite the colonization and persistence of wild radish on three continents, selection on the traits we studied did not differ among the introduced populations. The evolution of flowering time may have been the most important adaptation allowing the spread of this major agricultural weed. Adaptation to different pollinators does not seem to have been important in influencing reproductive success in new environments, perhaps due to the ability of wild radish to use many insect genera as effective pollinators.

Introduction

Organisms colonizing new environments likely face environmental conditions to which they are not well adapted. Thus, the colonization ability and persistence of organisms colonizing new habitats could be strongly influenced by the population's ability to adapt to new abiotic and biotic conditions (Blossey and Notzold 1995; Ellstrand and Schierenbeck 2000). Alternatively, species may be pre-adapted to new environmental conditions, or may experience more benign environments in their introduced locations if there is an absence of competitors or predators (Elton 1958; Crawley 1987). However, the fact that almost all of the examples we have of rapid adaptation involve the establishment of populations in a novel environment (Reznick and Ghalambor 2001 and references therein) suggests that evolution has played a role in the success of colonizations.

While divergent selection can create locally adapted populations, founder effects, drift, and migration among populations can all prevent local adaptation from occurring depending on the strength of selection, population size, and the amount of gene flow between populations. Furthermore, drift or founder effects can also create patterns of differentiation similar to those created by selection (Wright 1931; Lande 1976; Lynch 1990). One approach to determining the relative importance of drift and natural selection in their contribution to population differentiation compares differentiation at presumptively neutral loci (F_{ST}) with differentiation of quantitative traits (Q_{ST}) (Lande 1992; Spitze 1993). Since F_{ST} at truly neutral loci represents differentiation due to mutation and drift in the face of migration (Wright 1951), if $Q_{ST} = F_{ST}$, then we cannot rule out that neutral processes alone have contributed to differentiation in the trait. However, if $Q_{ST} > F_{ST}$ for a given trait, this suggests that natural selection has led to divergence of the populations. Finally, a finding of $Q_{ST} < F_{ST}$ indicates that convergent selection has prevented populations from diverging due to drift (Lande 1992; Spitze 1993; Whitlock 1999; McKay and Latta 2002). Comparisons of F_{ST} and Q_{ST} are especially useful when studying local adaptation in many populations because making reciprocal transplants among multiple populations is very difficult.

While comparisons of F_{ST} and Q_{ST} have been made for many species, few have studied widely distributed organisms that have recently colonized new habitats. Koskinen et al. (2002) found remarkably rapid divergence in life-history traits of grayling in recently introduced populations in Norway with no migration between populations. Their findings indicate that local adaptation can be quite rapid in newly introduced organisms, despite low effective population sizes. However, similar studies on recently introduced populations across a large geographic area have not been done.

The success of widely occurring weedy and invasive species in new environments implies that selective pressures are similar across environments, that organisms exhibit much plasticity in their ability to survive in different environments (Marshall and Jain 1968; Rice and Mack 1991; Parker et al. 2003), or that species are able to rapidly adapt to new habitats (Baker and Stebbins 1965; Baker 1974; Lee 2002). Flowering plants that are introduced to new habitats not only must survive under potentially different environmental conditions, but they must also be successfully pollinated by potentially new pollinator species, particularly if they are outcrossing annuals. A lack of effective pollinators in the introduced environment and the ability to adapt to new pollinators could impact a species' ability to persist and spread (Parker 1997; Richardson et al. 2000).

One such widespread, introduced species is wild radish, (*Raphanus raphanistrum*). Thought to be native to the Mediterranean basin (Hulten and Fries 1986; Holm et al. 1997), wild radish has successfully colonized a variety of locations, leading to its naturalization on all continents except Antarctica (Holm et al. 1997). Not only has wild radish colonized these new areas, it has also become a major agricultural weed, causing yield losses in a variety of crops in North America (Webster and MacDonald 2000; Warwick and Francis 2005), Europe (Bostrom et al. 2003), and Australia (Streibig et al. 1989; Cousens et al. 2001). Due to the self-incompatibility system of wild radish

(Sampson 1964) and its annual to biennial habit, this species relies completely on pollination by insects for reproduction. Therefore, the successful reproduction of this species in its introduced locations indicates that it has been able to use pollinators in its new habitats to effect reproduction. With the combined differences in abiotic factors such as temperature and water availability, as well as differences in pollinator assemblages across continents, selection is likely to have played a role in the evolution of morphological and phenological traits of wild radish across both its native and introduced habitats, leading to locally adapted populations. Furthermore, pollinator-mediated selection has been found to be acting in North America on specific floral traits of wild radish such as anther exsertion, stamen dimorphism, and flower size (Conner et al. 1996a; Conner et al. 1996b; Morgan and Conner 2001; Conner et al. 2003). Therefore, floral traits are likely candidates for experiencing divergent selection pressures across a wide geographic scale.

In this study we focus on differences of floral and phenological traits of wild radish across populations distributed throughout its current range and the genetic relatedness of these widespread populations. Specifically, we determine (1) how much genetic differentiation in floral and phenological traits among populations of wild radish has occurred by partitioning the amount of additive genetic variation within versus between populations (Q_{ST}), (2) how genetically different are populations at putatively neutral loci (F_{ST}), and (3) whether populations in closer proximity to one another are more genetically similar than those farther apart (isolation by distance). Results from our study reveal not only how non-native plants are able to persist and thrive in new locations in a short time period, but also address the origins of introduced wild radish populations.

Methods

Study System

Wild radish is a self-incompatible herb that is visited by at least 15 different genera of pollinators in three orders, Hymenoptera, Diptera, and Lepidoptera (Sahli and

Conner, unpub, Kay 1976; Kay 1982; Conner and Rush 1996). There are four recognized subspecies of *R. raphanistrum*, subspecies *maritimus, landra, raphanistrum*, and *rostratus* (Warwick and Francis 2005). Subspecies *raphanistrum* is commonly found growing among agricultural crops and is the only subspecies found in North America and Australia (Warwick and Francis 2005). *Raphanus raphanistrum* was first mentioned as an introduced weed in the eastern United States in the 1820s and 1830s (Torrey and Gray 1838), and is dispersed to other locations as a contaminant of grain seed (Woolcock and Cousens 2000). Although wild radish is most often described as an annual to biennial herb, subspecies *landra* and *maritimus* have been described as perennial in Europe (Chater 1993).

Estimating Q_{ST}

Seeds of subspecies *raphanistrum* were collected from eight different populations located on three continents: Kalamazoo, MI, USA (KM); Binghamton, NY, USA (NY); Aura, Finland (AF); Masku, Finland (MF); Madrid, Spain (MS); Westonia, Australia (WA); Cowra, Australia (CA); and Naracoorte, Australia (NA). All of the above populations were growing amidst grain crops, with the exception of MS, which was growing along the edge of a grain field, and MF, which was growing along a roadside. One population of subspecies *maritimus* was collected in the sand dunes behind the Atlantic beaches of Santander province in Spain (SS) (Table 5.1).

Parental Measurements

In 2003, one seed from each of 50 maternal plants per population was planted in a 10 cm pot in MetroMix 360 potting soil (Scotts-Sierra, Marysville, OH) in the greenhouse at Kellogg Biological Station. We added ¼ tsp of Osmocote Plus 15-9-12 fertilizer (Scotts-Sierra, Marysville, OH) twelve days after seeds were planted. Pots were arranged in 50 blocks with each block containing one plant from each of the nine populations.

Table 5.1. Location of populations, the number of families per population used to estimate $Q_{ST,}$ and the number of parental generation individuals used to estimate F_{ST}.

Pop	Location	Continent	Lat./Long.	NQ_{ST}	NF_{ST}
WA	Westonia, Australia	Australia	31°23' S /118°32' E	22	28
NA	Naracoorte, Australia	Australia	36°96' S /140°73' E	39	31
CA	Cowra, Australia	Australia	31°18' S /152°20' E	24	29
AF	Aura, Finland	Europe	60°36' N / 22°34' E	36	30
MF	Masku, Finland	Europe	60°34' N / 22°6' E	27	30
SS	Noja. Spain	Europe	43°29' N / 3°31' W	16	16
MS	Madrid, Spain	Europe	40°26' N / 3°42' W	25	25
KM	Kalamazoo,Michigan USA	North America	42°16' N / 85°35' W	23	30
NY	Binghamton, New York USA	North America	42°6' N / 75°54' W	30	30

Plants within each population were randomly assigned to a block to eliminate systematic environmental differences between populations. For each seed planted, germination time, time to first flower, and height of first flower was recorded. The third flower from each plant was removed and photographed and the number of ovules in each flower was counted by gently pressing the pistil between two glass slides to view the ovules. Six floral traits were measured from the digital images of flowers using NIH Object Image (2.12; Vischer 2004): petal length and width, corolla tube length, length of one short and one long filament, and pistil length (Conner and Via 1993). Three composite traits were calculated from the above measurements: anther exsertion (long filament length minus corolla tube length), stamen dimorphism (long filament length minus short filament length), and flower size (first principal component of the above six traits). Because most plants from the SS population had not flowered after four months, any plants that had not flowered were placed in an environmental chamber with temperatures and day lengths simulating those typical of a winter in Spain. Over a period of four weeks the temperature was dropped to 55° F during the day and 46° F at night, and day length was shortened to 10 hours. Temperature and day length was dropped by 6° F and two hours, respectively, each week. Plants were left at 55° F at a day length of 10 hours for 74 days, after which time the temperature and day length were increased gradually to 72° F and 15 hours of light, again over a period of four weeks. Plants that eventually flowered were measured as described above.

After all of the above measurements were taken, plants were randomly mated within each population, with each plant serving as both a mother and a father. Using this mating design, 16-39 full sib families per population were generated (Table 5.1). Leaf and bud tissue was collected from each plant and stored in an ultracold freezer (-80° C) for later estimation of F_{ST} (see below).

Offspring Measurements

Four seeds from each family were planted in 156 blocks. Seeds within each population were randomly assigned to a block to eliminate environmental differences between populations, with only one plant per population per block. Due to differences in the number of families per population, some blocks did not contain a plant from every population. All of the above measurements were recorded for these offspring. Plants which had not flowered after approximately three months were put through the same temperature and day length treatments as described above, this time in the greenhouse. Plants were left at 55° F at a day length of 10 hours for 30 days before increasing temperature and day length to 72° F and 15 hours.

For each of the above traits, between population variance was determined as the variance among population means, and additive genetic variance (V_A) within populations was calculated using parent and offspring data using the program nf6.p in Quercus (Shaw 1991). Q_{ST} was calculated as:

$$Q_{ST} = \sigma^2_{g(b)} / (\sigma^2_{g(b)} + 2\sigma^2_{g(w)}),$$

as defined by Spitze (1993), where $\sigma^2_{g(b)}$ is the genetic variance between populations and $\sigma^2_{g(w)}$ is V_A within populations, averaged over populations. 95% confidence limits on Q_{ST} estimates were obtained using parametric bootstrapping in Quercus; 1000 data sets were randomly generated using the mean and standard deviation from the observed data and Q_{ST} was calculated for each data set to obtain 95% confidence intervals. Heritability (h^2) was calculated for all traits in all nine populations as V_A/V_P, where V_A was determined using the program nf3.p in Quercus, and V_P is the phenotypic variance within a population. Heritability averaged over all populations for each trait was regressed on the Q_{ST} estimate for the trait to test whether h^2 predicts trait divergence among populations.

Estimating F_{ST}

Total genomic DNA was extracted from 16-30 individuals per population (Table 5.1) using Qbiogene's FastDNA Kit and the FastPrep Instrument (Carlsbad, CA) following the kit protocol. Individuals were genotyped at eight microsatellite loci derived from Brassica: Bn26a, Bn35d, Brms 005, Na10-H06, Na12-E05, Na14-E08, Ra1-H08, and Ra2-E11 (UK CropNet Brassica database; http://ukcrop.net/brassica.html). Microsatellites were PCR amplified in 10-μl reactions as described in Chapter 3. and microsatellite samples were run on 6% polyacrylamide gels, with 8 μl Temed and 400 μl 10% APS added to each gel. Bands were visualized on FMBIO II Multiview scanner (Hitachi Software Engineering Co. Ltd., San Francisco, CA). Alleles were scored using FMBIO Analysis 8.0 (Hitachi Software Engineering 1991-1999) and placed in bins using Allelogram 1.2 (Manaster 1998). Wright's (1951) F_{ST} quantifies differentiation among populations due to drift and mutation, and was calculated in terms of variance of allele frequencies:

$$F_{\text{ST}} = \sigma^2_p \,/\, p(1\text{-}p)$$

where σ^2_p is among population variance in the allele frequency, and p is the average frequency of the allele across populations (Weir and Cockerham 1984). F_{ST} was calculated in FSTAT 2.9.3.2 (Goudet 1995) and 95% confidence intervals were determined by bootstrapping over loci. Q_{ST} was considered to be statistically different from F_{ST} when 95% confidence intervals of Q_{ST} did not overlap point estimates F_{ST}. FSTAT was used to test for heterozygote deficit by permuting alleles among individuals within samples and using F_{IS} as a statistic for comparison. A test for isolation by distance was performed by computing a regression of F_{ST} estimates on geographic distances and using Mantel permutations to test for independence between geographic and genetic differences in the ISOLDE option in GENEPOP (Raymond and Rousset 1995).

Three different estimates of Q_{ST} and F_{ST} were made, one with all nine populations included, one with only the eight populations of subspecies *raphanistrum*, and one with only the seven non-native *raphanistrum* populations. The last analysis was made because the Mediterranean populations (both MS and SS) flowered much later than the other seven populations. For this reason, measurements made on the Spanish populations were not done in a common environment with the other populations. Because conditions in a greenhouse are not constant over time, we cannot rule out the possibility that differences between the Spanish populations and the remaining populations are due to environmental differences rather than genetic differences. Furthermore, we were interested in testing whether the non-native populations have diverged from one another.

Results

Population differentiation based on microsatellite markers

With the exception of two loci in SS, all loci in all populations were polymorphic (Table 5.2). Based on the fixation index, F_{IS}, four populations had a heterozygote deficit: the Australian populations (WA, NA, CA) and one Mediterranean population (MS; Table 5.2). Several alleles were unique to particular populations: NA, MF, SS, and CA each had one unique allele, KM and WA each had two, and MS had four.

Overall F_{ST} values ranged from 0.11 (among non-Mediterranean *raphanistrum* populations), to 0.18 (among all nine populations). F_{ST} values calculated from each locus were relatively similar (Table 5.3), suggesting that these microsatellite loci were neutral or nearly so. Pairwise F_{ST} values ranged from zero differentiation between the two Finland populations to large differentiation ($F_{ST} = 0.50$) between the two Mediterranean populations (Table 5.4). Furthermore, F_{ST} values between the two Mediterranean populations were higher than all other pairwise comparisons between the Mediterranean populations and other populations, with MS being most similar to NY and SS being most

Table 5.2. Number of alleles per locus and the fixation index (F_{IS}) for each population.

Pop	WA	CA	NA	AF	MF	SS	MS	KM	NY	Avg. F_{IS}
Bn35d	6	6	5	6	6	2	3	8	7	
Brms-005	10	9	7	6	9	4	8	11	6	
Na14-E08	6	6	4	6	4	4	6	5	6	
Ra2-E11	8	5	6	3	4	1	5	4	7	
Ra1-H08	4	5	4	5	5	1	6	4	5	
Na12-E05	9	8	5	3	5	3	6	6	5	
Na10-H06	4	4	4	3	4	2	3	4	4	
Bn26a	6	6	7	3	4	5	5	5	5	
F_{IS}	0.09*	0.17*	0.11*	0.08	0.03	-0.08	0.14*	0.05	0.04	0.02
Total # alleles	53	49	42	35	41	22	42	47	45.04	

* significantly different from zero at 5% level

Table 5.3. F_{ST} values for each locus in each analysis (95% confidence intervals)

Locus	F_{ST}		
	All populations	All subspecies. *raphanistrum* populations	All non-Mediterranean populations
Bn35d	0.16	0.15	0.09
Brms005	0.14	0.11	0.11
Na14E08	0.16	0.14	0.15
Ra2E11	0.26	0.18	0.11
Ra1H08	0.10	0.07	0.03
Na12E05	0.13	0.11	0.09
Na10H06	0.23	0.15	0.13
Bn26a	0.23	0.21	0.19
Mean	0.18 (0.14, 0.21)	0.14 (0.11, 0.17)	0.11 (0.09, 0.14)

Table 5.4. Pairwise F_{ST} values for the nine populations.

	CA	NA	AF	MF	SS	MS	KM	NY
WA	0.17	0.13	0.14	0.13	0.31	0.26	0.11	0.12
CA		0.15	0.16	0.14	0.34	0.25	0.14	0.14
NA			0.14	0.14	0.34	0.24	0.11	0.13
AF				-0.01†	0.38	0.27	0.07	0.09
MF					0.34	0.26	0.06	0.08
SS						0.50	0.34	0.34
MS							0.19	0.15
KM								0.04

†not significantly greater than zero; all other F_{ST} values are significant at $P < 0.05$. (Significance determined by permutation tests).

similar to WA (Table 5.4). There was no relationship between genetic differences and geographic distance ($P = 0.58$).

Quantitative traits

All floral traits except flower size were heritable in most populations; however, flowering time, germination time, and height of first flower generally did not have significant heritabilities. Over all traits and populations, heritability ranged from -0.03 - 0.80 (Table 5.5). Non-normality of traits prevented the model from converging in several cases (Table 5.5). Q_{ST} values ranged from 0 (flower size, Figure 5.1C) to 0.92 (time to flower, Figure 5.1B), with an average Q_{ST} estimate of 0.32 among all nine populations, 0.26 among all subspecies *raphanistrum*, and 0.14 among only the non-Mediterranean populations. Although average Q_{ST} values were larger than F_{ST} values for all three comparisons, few of the individual Q_{ST} estimates were significantly larger than F_{ST} (Figure 5.1).

Q_{ST} estimates for petal length and petal width, ovule number, time to first flower, and height of first flower generally had the highest values of Q_{ST}, but some of these estimates were greatly influenced by one or both of the Spanish populations (Figure 5.1). Q_{ST} for ovule number was significantly larger than F_{ST} when all nine populations were included, with the *maritimus* population having fewer ovules than any of the *raphanistrum* populations (Figure 5.2A). However, Q_{ST} did not differ from F_{ST} once the *maritimus* population was removed (Figure 5.1B,C).

The Q_{ST} estimate for flowering time was significantly larger than F_{ST} when the *maritimus* population was removed (Figure 5.1B), but was not significantly greater than F_{ST} when all populations were included (Figure 5.1A) and among the non-Mediterranean populations (Figure 5.1C). Examination of the population mean flowering times shows that the two Mediterranean populations flowered much later than the other seven populations, with SS flowering later than MS (Figure 5.2B). The large confidence limits around our estimate of Q_{ST} for flowering time when all nine populations are included is

Table 5.5. Narrow-sense heritability (h^2) estimates for each trait in each of the nine populations of *R. raphanistrum* calculated as V_A/V_P. V_A was measured using restricted maximum likelihood analysis in Quercus. Heritabilities are in bold when 95% confidence intervals based on twice the standard deviation of V_A did not encompass zero. Dashes indicate the model to estimate V_A did not converge.

Trait	WA	CA	NA	AF	MF	KM	NY	MS	SS
Flower size	0.23	0.11	0.16	--	**0.38**	0.17	0.29	0.08	0.39
Petal length	**0.18**	0.05	**0.12**	**0.20**	**0.36**	0.01	**0.23**	-0.03	**0.08**
Petal width	**0.32**	**0.19**	**0.51**	**0.19**	**0.27**	**0.59**	**0.14**	**0.17**	**0.04**
Pistil length	**0.38**	**0.17**	**0.41**	--	**0.70**	**0.55**	**0.35**	**0.27**	**0.26**
Anther exsertion	**0.16**	**0.20**	**0.35**	**0.40**	**0.32**	**0.32**	**0.03**	**0.38**	**0.59**
Stamen dimorphism	**0.25**	**0.36**	**0.15**	**0.61**	-0.01	**0.72**	**0.49**	**0.32**	--
Ovule #	**0.44**	**0.17**	**0.38**	**0.41**	**0.41**	**0.49**	**0.30**	**0.33**	0.10
Germination time	0.04	0.06	0.23	**0.28**	0.13	1.37	0.13	0.09	0.18
Flower time	0.40	0.38	0.66	**0.28**	0.25	--	0.52	0.09	--
Flower height	0.34	0.47	0.76	0.28	0.26	0.27	0.54	0.31	0.80

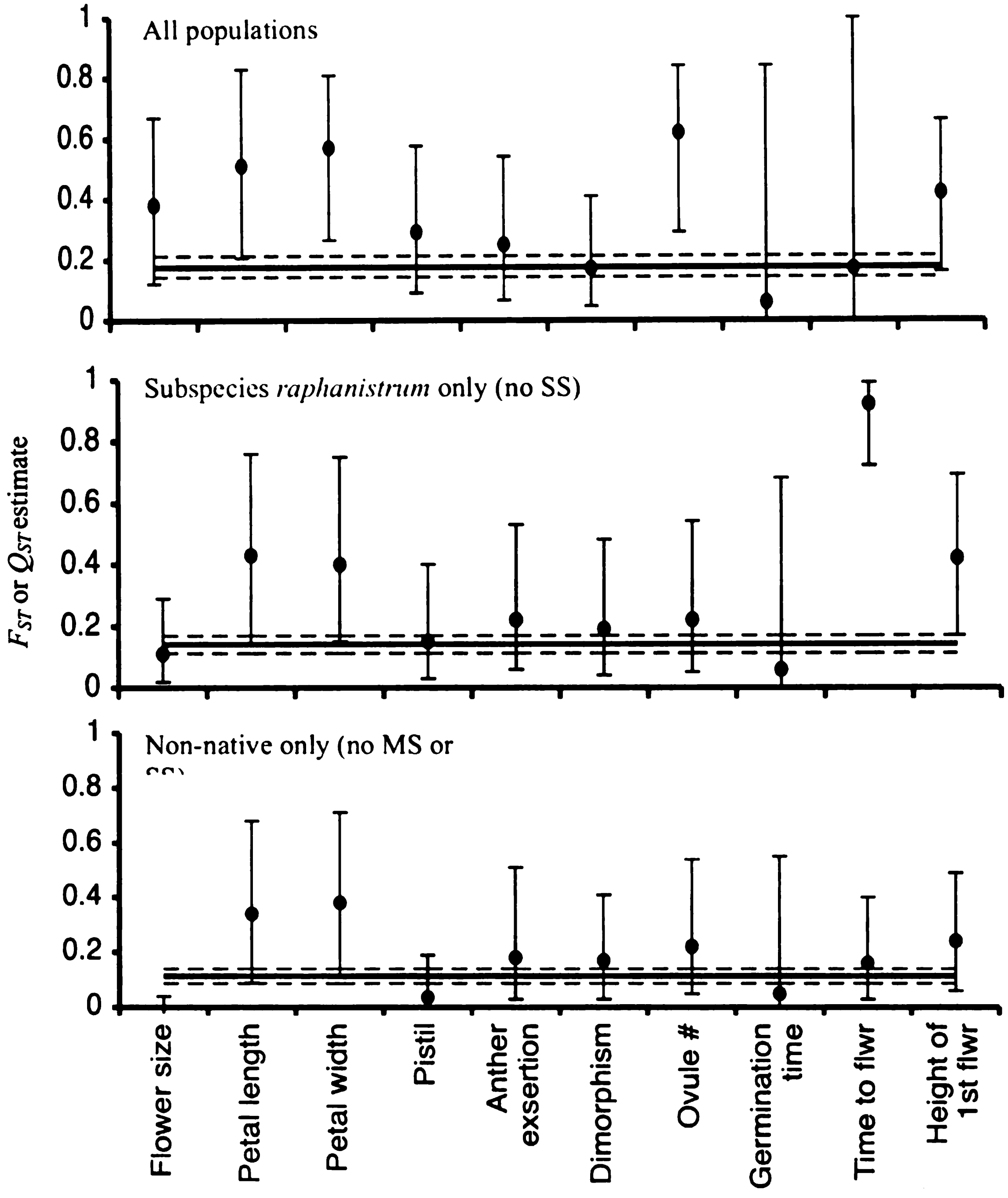

Figure 5.1. F_{ST} and Q_{ST} estimates for several traits. (A) F_{ST}/Q_{ST} among all nine populations, (B) among subspecies ***raphanistrum*** (no SS), and (C) among ***raphanistrum*** populations in the non-native range (no MS or CS). The horizontal line is the F_{ST} estimate with the dashed lines indicating 95% confidence limits around the estimate. Bars around the Q_{ST} estimates are 95% confidence limits based on a parametric bootstrap.

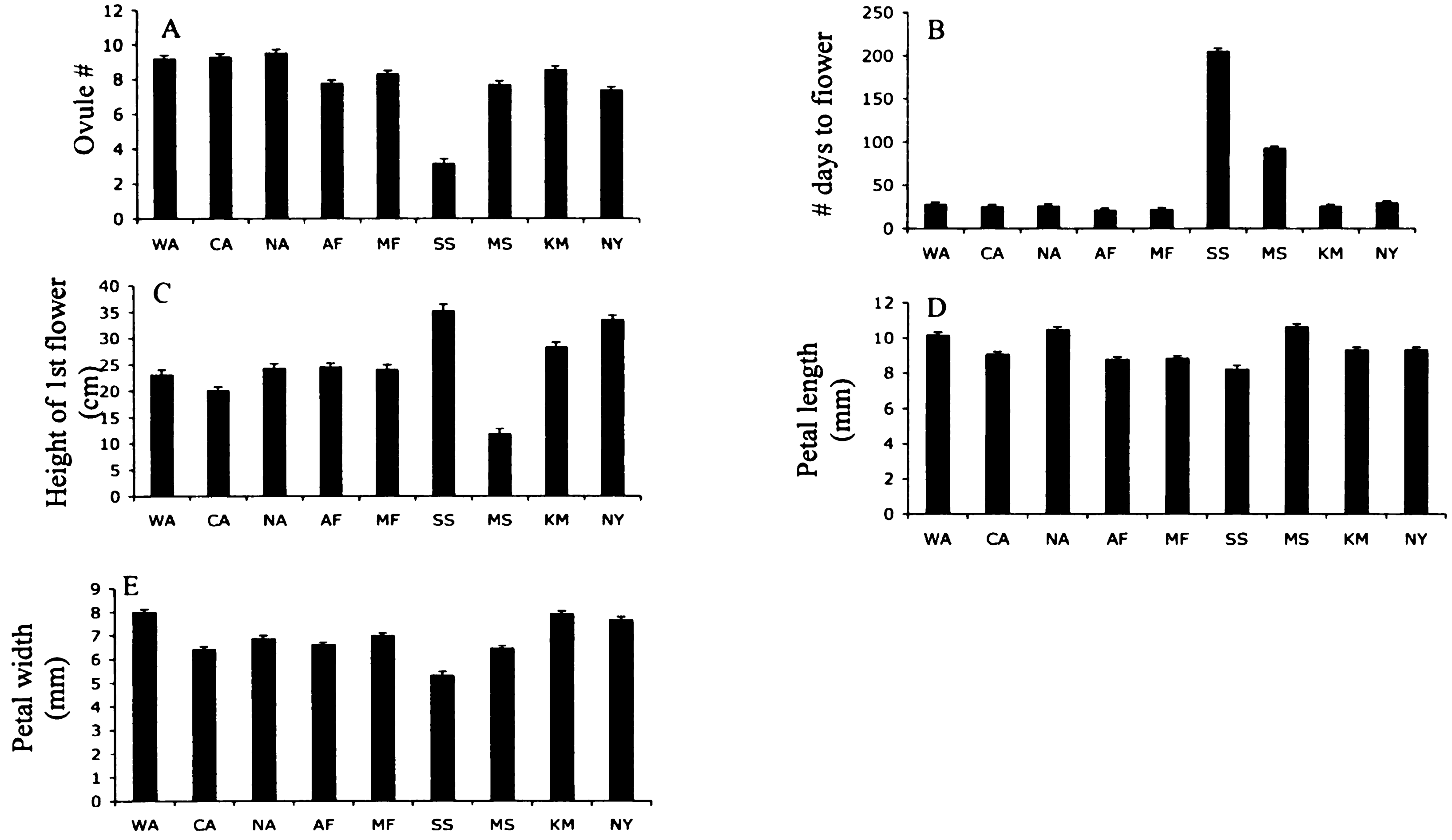

Figure 5.2. Population mean (+ 1 S.E.) (A) ovule number (B) flowering time, (C) height of first flower, (D) petal length, and (E) petal width for each population.

evidence of our inability to obtain precise estimates of Q_{ST} for this trait. This is likely due to the extreme non-normality of flowering time (Shapiro-Wilks test for goodness of fit, $P < 0.0001$), which no transformation improved. The SS population was more extreme in its distribution of flowering time, with individuals flowering from 35 days to 300 days, which might have prevented accurate estimates of Q_{ST} when it was included in the analysis.

Q_{ST} for height of the first flower was marginally significantly larger than F_{ST} among the eight *raphanistrum* populations. The two Mediterranean populations differed in the height of their first flower-- SS had the tallest flower stalks and MS had the shortest flower stalks among all populations (Figure 5.2C). Finally, Q_{ST} for both petal length and width were marginally significantly greater than F_{ST} in all three analyses, with petal width being significantly greater than F_{ST} in the comparison among all nine populations (Figure 5.1A). MS, NA, and WA had the longest petals (Figure 5.2D), whereas KM, NY, and WA had the widest petals (Figure 5.2D). SS had both the shortest and most narrow petals. Q_{ST} for flower size was significantly less than F_{ST} among the non-Mediterranean populations (Figure 5.1C). Heritability was not predictive of a trait's past divergence among populations, as the regression of h^2 on Q_{ST} was not significant (Figure 5.3).

Discussion

To our knowledge, this is the first study to compare F_{ST} and Q_{ST} among populations distributed across the globe. However, despite the large geographic variation among the populations we sampled, we found little evidence for differentiation in floral traits, even among those traits that we know are under selection in natural populations such as anther exsertion, stamen dimorphism, and flower size (Conner et al. 1996a; Conner et al. 1996b; Morgan and Conner 2001; Conner et al. 2003). The lack of significant divergence in most floral traits is not due to a lack of genetic variation for

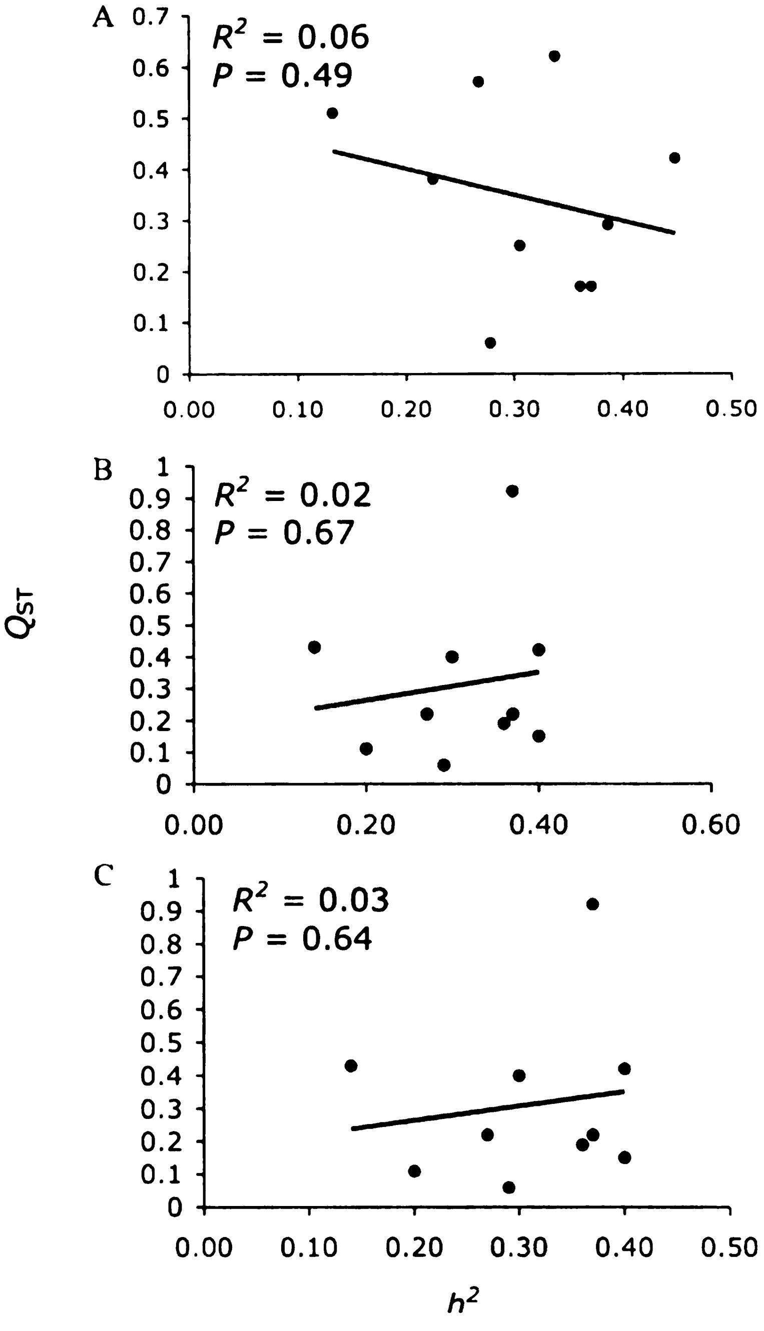

Figure 5.3. Regression of the average narrow sense heritability (h^2) across populations on Q_{ST} estimates, where each point is one trait. Regression is based on (A) all nine populations, (B) all subspecies *raphanistrum* populations (no SS), and (C) all subspecies *raphanistrum* populations in the non-native region (no SS or MS).

selection to act on. In fact, the floral traits we measured were highly heritable in most populations. Findings from this study indicate that, although *R. raphanistrum* has a global distribution and relies completely on pollinators for reproduction, pollinator-mediated selection does not differ strongly across populations. A previous study has shown that almost all of the visiting genera of North American populations are effective pollinators, although genera do differ in their effectiveness (Chapter 3). We have also found that only a subset of the pollinating genera significantly select on floral traits such as anther exsertion, stamen dimorphism, and flower size (Chapter 4.). Thus, selection must not be strongly different in these populations, preventing strong divergence due to natural selection in the approximately 200 generations that have occurred since the introduction to non-native regions. The success of wild radish in introduced regions may be largely due to similar pollinator assemblages across continents. Our findings suggest that *R. raphanistrum* has not needed to adapt to local pollinator assemblages despite colonizing distant locations, which is one possible reason why it is a successful weed despite being dependent on pollination. Additionally, it seems that petal length and width are the only two floral traits that have diverged due to selection, possibly due to differences in selection by different pollinators. Petal size has been shown to influence visitation rates (e.g. Clements and Long 1923; Bell 1985; Young and Stanton 1990; Conner and Rush 1996 Galen 1989; Eckhart 1991). Although most studies have found larger corollas to increase visitation rates, Galen (1989) found selection on corolla size to be pollinator specific--bumblebees selected for larger corollas whereas the rest of the pollinator assemblage did not select on corolla size. Differences in selection by different pollinators, combined with differences in pollinators across populations could result in the observed floral divergence in petal size. Unfortunately, little is known about the pollinators in the populations we studied, but we do know that pollinator genera are similar in North America, Britain, and Finland (Kay 1976; Kay 1982; K. Lehtila, pers.

com.); that is, syrphid flies, bumblebees, honeybees, cabbage butterflies, and small sweat bees are the major pollinators in the above locations.

Few studies comparing F_{ST} and Q_{ST} have examined divergence in floral traits, but those that have found that continuous morphological traits had Q_{ST} values similar to F_{ST} (Podolsky and Holtsford 1995; Widen et al. 2002; Jorgensen et al. 2006), but that categorical traits relating to floral coloration usually had estimates of Q_{ST} much larger than F_{ST} (Podolsky and Holtsford 1995; Streisfeld and Kohn 2005; Jorgensen et al. 2006). Interestingly, the one continuous floral trait that had a Q_{ST} estimate larger than F_{ST} in a previous study was petal width in *Clarkia* (Podolsky and Holtsford 1995), suggesting that perhaps petal size is under stronger divergent selection by different pollinators than other traits. Although more studies on divergence of floral traits need to be done, the current research suggests that floral morphology has not diverged as rapidly as other traits.

While *R. raphanistrum* has been successful at using pollinator assemblages in its introduced habitat without altering its floral traits considerably, there seems to have been strong selection on flowering time and ovule number. Although we only included one population of subspecies *maritimus* in our study, ovule number is one of the characteristics separating *maritimus* from *raphanistrum* (Warwick and Francis 2005), with *maritimus* producing 2-4 seeds per fruit and *raphanistrum* producing 4-11 seeds per fruit (Jalas et al. 1996). Our study shows that this difference in ovule number between subspecies is due to divergent selection rather than drift alone. Low ovule number may correspond to a change in life history from perennial to annual, where perennials produce few seeds each year, but can flower over several years, whereas an annual life history would select for increased investment in seed production. Adapting to an agricultural habitat where plants are harvested annually could cause strong selection on ovule number if reproduction is forced to occur within a single year. A comparative study of F_{ST} and Q_{ST} in another widespread agricultural weed, *Senecio vulgaris,* found that allocation to reproduction was increased among agricultural habitats relative to ruderal habitats over a short distance (Steinger et al. 2002), emphasizing the divergent selection pressure on traits influencing allocation to reproduction in these different habitats.

Raphanus raphanistrum has experienced divergent selection in one other life history trait, flowering time. Populations in the Mediterranean experienced delayed flowering and individuals within these populations often required a cold treatment in order to induce flowering. Although Q_{ST} for flowering time was not different from F_{ST} when all nine populations were analyzed, this was likely due to the non-normality of the data. The comparison of flowering time among only the *raphanistrum* populations showed that non-Mediterranean populations have evolved to flower earlier, bolting as soon as a few large leaves have developed. In disturbed habitats such as agricultural fields which are plowed and tilled, late flowering plants may have no chance to reproduce, thus selecting for earlier flowering. However, in undisturbed environments with mild winter conditions, delayed reproduction should be favored as it is thought to provide a longer growing season, allowing greater reproductive output (Jones 1971). Although both Mediterranean populations experienced delayed flowering relative to non-native populations, the agricultural *raphanistrum* population flowered earlier than the coastal *maritimus* population, which could be due to the *raphanistrum* population experiencing selection for earlier reproduction due to its ephemeral habitat. It appears that the evolution of earlier flowering time may have permitted a rapid expansion and colonization of *R. raphanistrum* populations, allowing it to thrive throughout the globe.

Flowering time is quite variable in many species, and has been studied in other members of the Brassicaceae family. For example, the *Frigida* gene that controls flowering time in *Arabidopsis thaliana* has experienced strong divergence even within short geographic distances, and flowering time was the only trait for which Q_{ST} was significantly greater than F_{ST} in a similar comparative study on *A. thaliana* (Le Corre 2005). Other studies have found flowering time to have diverged along latitudinal gradients (Karhu et al. 1996; Jaramillo-Correa et al. 2001; Griffith and Watson 2005), and have shown the evolution of flowering time to be essential for range expansion into more northern latitudes (Griffith and Watson 2006).

Despite the similarities in flowering time between the two Mediterranean populations, the two subspecies occurring in the Mediterranean exhibited large genetic differences, with each Mediterranean population being more related to other populations outside of the Mediterranean than they were to one another. This large genetic difference indicates that some barrier to gene flow exists between these two populations within 340km of one another. More study is needed to examine the causes of reproductive isolation in these two subspecies. It is also unclear as to why selection differs among the two Mediterranean populations in petal size and height of the flowering stalk. Future study on environmental differences and pollinator differences across coastal versus agricultural landscapes in the Mediterranean would be well directed.

Although the populations we sampled were distributed throughout the globe, our study suggests that the agricultural environment is similar enough across populations that gene flow and genetic drift have overcome any divergent selection that may have been acting on the majority of the traits we examined. Alternatively, divergent selection could be acting on floral traits across populations, but it produces differentiation similar to that produced by the combined effects of drift, mutation, and migration. Our findings are particularly interesting when compared with previous comparisons of F_{ST} and Q_{ST} which, in general, have found most traits to have diverged more than neutral loci, indicating that divergent selection is rampant and strong enough to overcome the neutral effects of drift and migration (reviewed in Merila and Crnokrak 2001; McKay and Latta 2002). The contrast between our findings and previous findings is especially interesting given the much larger geographic area that we sampled compared with previous studies. However, because all but one of our populations were from an agricultural setting, environmental conditions may have been more similar than if populations were occurring in a more natural environment. Furthermore, the movement of wild radish seeds through contaminated grain crops likely increases gene flow relative to populations not under human influence.

Another major difference between our study and the vast majority of previous studies is that we estimated Q_{ST} based on additive genetic variance, whereas most previous studies have used total genetic variance (but see Petit et al. 2001; Koskinen et al. 2002; Widen et al. 2002; Palo et al. 2003). Including non-additive variance in estimates of Q_{ST} could cause a bias, although the direction of the bias is not well understood. Epistatic variance should decrease Q_{ST} estimates (Whitlock 1999; Lopez-Fanjul 2002), but dominance variance could increase or decrease Q_{ST} (Whitlock 1999; Lopez-Fanjul et al. 2003). Furthermore, the method used to determine confidence intervals around Q_{ST} could also bias conclusions. While a parametric bootstrap like that used in our study or a Bayesian approach produce the most accurate estimate of the confidence in the Q_{ST} estimate, the nonparametric bootstrap, one of the most common methods, has been found to underestimate the 95% confidence intervals (Morgan et al. 2005; O'Hara and Merila 2005). At this point it is unclear whether the estimation of confidence intervals and the use of total genetic variance have overestimated Q_{ST} differences from F_{ST} in previous studies.

Finally, our focus on floral traits may have prevented us from detecting many Q_{ST} estimates exceeding F_{ST} if most divergent selection occurs in life history traits rather than morphological traits. It is interesting that two of the three life history traits we looked at, flowering time and ovule number, both had Q_{ST} estimates exceeding F_{ST}. Q_{ST} estimates of more morphological and life-history traits would allow us to further test whether life history traits have, indeed, diverged more than morphological traits. However, previous studies have found life history traits to be less divergent than morphological traits (Merila and Crnokrak 2001). Merila and Crnokrak (2001) suggest that the reason for such little differentiation in life history traits of past studies may be due to the large amount of non-additive genetic variation contributing to life-history traits as opposed to morphological traits (Crnokrak and Roff 1995). If the inclusion of non-additive genetic variance causes a downward bias in Q_{ST}, past studies may have underestimated differences in life history traits relative to morphological traits. Future studies comparing divergence in life history traits morphological traits using additive genetic variance are clearly needed.

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Summary

Research from my dissertation falls into three main categories: 1) the characterization of generalization in plant-pollination systems, 2) the importance of different pollinators in influencing floral adaptation in a generalist, and 3) the importance of floral and phenological traits for adaptation to new environments. Chapters 2 and 3 demonstrated the importance of incorporating differences in visitation rates among pollinators to determine how generalized a plant is. Chapter 4 revealed the complexity of selection by different pollinators and their overall effect on floral adaptation. While generalists may experience trade-offs in adapting to a subset of their pollinator assemblage, it appears that these trade-offs are not common. Furthermore, it appears that only a small subset of the pollinators of wild radish are actually exerting selection on many of the floral traits. Thus, by evolving floral traits that are adapted to a subset of the pollinator assemblage, but that are still able to produce seeds when less abundant pollinators visit, wild radish seems to have evolved to use a large assemblage of pollinators without much cost. One consequence of wild radish's ability to successfully use so many different pollinators has been its ability to colonize and persist over a large geographic area. Chapter 5 demonstrated that floral traits have not become locally adapted in different populations, but that traits such as flowering time and ovule number have diverged among populations.

Future directions

This is the first study examining how selection by more than two pollinators influences floral adaptation in generalist species. Clearly, similar studies on other generalist species are needed. Furthermore, studies examining ecological interactions among pollinators and their effects on selection by individual pollinators will also be important for understanding adaptation in the face of multiple agents of selection. Selection on floral traits of wild radish has been found to vary temporally (Conner et al. 2003), but shifts in the most important pollinator alone may not be enough to explain these differences in total selection. For instance, in 1992 and 1993, the small sweat bee, *Dialictus* was the dominant pollinator of a studied population of wild radish (Conner et al. 1996a). In 1992, stabilizing selection on anther exsertion and flower size was found in this population, which was similar to the selection exerted by *Dialictus* alone (Conner et al. 2003 and Chapter 4). However, in 1993, there was selection to increase flower size and no significant selection on anther exsertion (Conner et al. 2003). An interaction among pollinators is one explanation for differences in selection across years despite the presence of the same dominant pollinator. Interactions may result in non-additive, or diffuse evolution, where selection by one pollinator changes in the presence of another pollinator (Iwao and Rausher 1997; Strauss et al. 2005).

Diffuse selection on resistance and tolerance in plants by different herbivores has been found in several studies (e.g., Pilson 1996; Juenger and Bergelson 1998; Stinchcombe and Rausher 2001), but has not been examined in pollination. Selection on flower size in the cage experiments containing honeybees, bumblebees, and cabbage butterflies (Chapter 4) may have been greater than the additive effects of selection by each pollinator alone, although we could not detect a significant difference among these selection gradients. Similar studies manipulating selection by pollinators individually and in combination with other pollinators would further our understanding of pollinator interactions. A more complex design measuring selection in the presence of only two pollinators (i.e., *Bombus* + *Apis*, *Bombus* + *Pieris*, *Apis* + *Pieris*) could help explain whether pollinators are interacting in their effects on selection and whether the presence of all three pollinators imposes additional interactions not present in the two-way interactions. Replicates of each pollination environment could improve our ability to test for interactions among pollinators.

Results from Chapter 5, which indicate little divergence in floral traits among populations separated by large geographic distances, are unexpected given the finding that some pollinators differ in their selection on floral traits, and that only a subset of pollinators are actually selecting on floral traits. However, we do not currently know the composition of pollinators in the nine populations included in the study. We do know that the pollinating community among the New York and Michigan populations are similar (Conner et al. 1996a; Chapter 3), and populations from Finland are pollinated by the same general group of pollinators (i.e., syrphid flies, bumblebees, honeybees, *Pieris*, and small bees; K. Lehtila, pers. comm.). However, the genera of some of the syrphid flies differ, as do the species of many of the others. A survey of the pollinating insects in different populations of wild radish could help to explain the reasons behind the little divergence found in floral traits across these populations.

In addition, the evolution of weediness in wild radish is an area that would benefit from further study. First, the phylogenetic relationships among subspecies of wild radish would help determine how these different subspecies evolved and which is most similar to the common ancestor. My dissertation only examined one population from subspecies *maritimus* and did not include subspecies *landra* and subspecies *rostratus*. Furthermore, their status as different subspecies needs to be tested more thoroughly by doing crosses among subspecies to compare relative degrees of post-mating pre-zygotic reproductive isolation. Comparisons of the pollinating assemblages and habitat type of each subspecies, as well as gene flow among subspecies would enable us to understand the forces driving reproductive isolation among subspecies. Finally, reciprocal transplants among populations in the introduced and native range coupled with estimates of selection on floral and vegetative traits among different populations would allow us to explicitly test how present-day selection differs across populations, as well as the degree of local adaptation.

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