THE ECOLOGY AND EVOLUTION OF WIND POLLINATION

by

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ABSTRACT

The evolution of wind pollination (anemophily) has occurred at least 65 times in the flowering plants and over 10% of angiosperm species are wind pollinated. However the pollination and mating of anemophily species is poorly understood, particularly in comparison with animal-pollinated species. My thesis employs a range of approaches and tools to examine the evolution and ecology of wind pollination. These include comparative analyses, theoretical modeling, field and glasshouse experiments, the use of genetic markers and quantitative genetics. Experimental studies on diverse taxa were used to address questions concerned with the efficacy of outcrossing mechanisms, the ecological and demographic context of pollination and mating, and the plasticity of sex allocation.

Comparative analyses indicated that wind pollination is correlated with unisexual flowers, reduced ovule number, small unshowy flowers, an absence of nectar, and open habitats. These analyses also demonstrated that anemophily originates more often in lineages with unisexual flowers. This suggests that wind pollination evolves in diclinous taxa as a mechanism of reproductive assurance because autonomous selfing is mechanically precluded. Empirical data on stigmatic pollen loads in 19 anemophilous species challenge the widespread assumption that anemophilous plants commonly have uniovulate flowers because
they capture few pollen grains. Further, a model based on floral costs and the aerodynamics of pollen capture demonstrated that when flowers are inexpensive it is optimal to produce many flowers each with few ovules, because this allows more efficient sampling of the airstream.

Manipulative field experiments on seven Carex species indicated that neither monoecy nor protogyny, two putative outcrossing mechanisms, are effective at limiting selfing. Based on these results I suggest that geitonogamy can provide reproductive assurance in anemophilous species with unisexual flowers. Field experiments and the application of sex-specific markers in Rumex nivalis revealed that the local neighbourhood of maternal plants affects pollination intensity and progeny sex ratios. Finally, I demonstrated that plant density in Ambrosia artemisiifolia affects stigmatic pollen loads but not outcrossing rates. Through a quantitative genetics experiment in A. artemisiifolia, I detected significant genetic variation for plasticity in sex allocation, potentially enabling adaptive adjustment of sex allocation to local environmental conditions.
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Evolution of wind pollination

The evolution of wind pollination (anemophily) from animal pollination represents one of the major evolutionary transitions in the flowering plants. Approximately 10% of angiosperm species rely on wind for the transport of pollen between plants and anemophily has originated at least 65 times in diverse animal-pollinated lineages (Linder 1998). The evolution of wind pollination is a puzzling phenomenon because it is commonly viewed as a wasteful and random process at the mercy of unpredictable environmental conditions. Haldane (1932) commented on the paradox of evolving wind pollination:

‘A plant of an insect-pollinated species which did not attract insects, even if it secured fertilization by accidental wind-blown pollen would probably fail to fertilise any of its fellows, insects having carried pollen to them before any grains from it were blown to them’

Haldane 1932, p. 69.

Why should plants that are pollinated by animals abandon their pollinators in favour of the wind? This is one of the questions that I address in this thesis.

The evolution of wind pollination is thought to occur when environmental conditions render biotic pollination less advantageous (Regal 1982; Cox 1991). For example, a decline in pollinator abundance or changes in the abiotic environment limiting pollinator activity have been invoked to explain why wind pollination has evolved in particular taxa (Berry & Calvo 1989; Weller et al. 1998; Goodwillie 1999). This implies that when pollinator service is unsatisfactory and seed set pollen limited, anemophily is favoured because the wind
affords more reliable pollen transport. Indeed, Darwin (1876) proposed that wind pollination might evolve as an alternative to selfing when pollinators are unreliable:

‘If any entomophilous species ceased altogether to be visited by insects, it would probably perish unless it were rendered anemophilous, or acquired a full capacity for self-fertilisation’

Darwin 1876, p. 410.

And yet despite these early comments by Haldane and Darwin, the specific ecological mechanisms causing transitions from animal to wind pollination are poorly understood, especially in comparison with other reproductive transitions (e.g. the evolution of selfing from outcrossing and dioecy from hermaphroditism). Currently, remarkably little is known about the microevolutionary forces responsible for the origins of wind pollination. This is surprising because this transition represents one of the most significant transformations of character evolution in the evolutionary history of the flowering plants.

The evolution of wind pollination is likely to be favoured in species that possess morphological traits that fulfill the aerodynamic requirements of pollen dispersal and capture. A suite of morphological traits is commonly associated with wind pollination and these constitute the ‘anemophilous syndrome’ (Faegri & van der Pijl 1979). Many of the traits can be explained by functional arguments and the aerodynamic requirements for wind pollination. In general, wind-pollinated plants have small flowers, with highly reduced or no perianth parts. Nectaries are usually absent or non-functional (Faegri & van der Pijl 1979). Furthermore, regions of higher latitude, arid temperate environments, open vegetation, and wind-swept island floras have the highest representation of wind-pollinated plants (Whitehead 1968; Regal 1982). The physical and aerodynamic requirements for successful wind pollination may explain these ecological and geographical correlates. Several reviews have speculated on the environmental circumstances favouring the evolution of wind
pollination (Regal 1982; Whitehead 1983; Cox 1991; Culley et al. 2002), but the pathways and mechanisms involved in its origin are largely unexplored. In this thesis I examine the problem of the evolution of wind pollination from animal pollination by investigating morphological and ecological correlates of anemophily using comparative approaches.

**Pollination and mating in wind-pollinated plants**

The rich history that characterizes the fields of plant pollination and mating biology has almost exclusively focused on animal-pollinated plants. Moreover, the vast majority of theoretical investigations in plant reproductive biology have implicitly assumed animal pollination. However, the established paradigms for understanding the ecology and evolution of plant reproduction may not be appropriate for most wind-pollinated plants and a major goal of my thesis is to explore this possibility. There are a few key features of wind pollination that are in direct contrast to the situation found in most animal-pollinated species. First, in wind-pollinated species the capture of each pollen grain is an independent event, unlike animal-pollinated plants where pollen grains are often transported and delivered in clumps. Second, the wind cannot be saturated with pollen and thus male fitness should increase linearly with investment into male function. Finally, unlike animal-pollinated plants where the success of both female and male function relies on visits by pollinators, in wind-pollinated plants female and male function are largely independent of one another and have different aerodynamic requirements. These key differences have consequences for pollen dispersal, pollen discounting and geitonogamy, the function of sexual strategies including dichogamy and dicliny, and the patterns of sex allocation in wind-pollinated plants.

Prodigious pollen production is a characteristic feature of the anemophilous syndrome and pollen-to-ovule (P/O) ratios are generally much higher than in animal-pollinated taxa (wind – median 22150:1; animal – median 3450:1: Cruden 1977, 2000). Despite this striking
difference in P/O ratios there has been little consideration of differences in ovule number between animal- versus wind-pollinated plants. Low ovule number, including many taxa with uniovulate flowers, is a characteristic feature of anemophilous species (Linder 1998). The most common explanation for this association is the small chance of multiple pollen grains landing on individual stigmas of wind-pollinated plants (Pohl 1929; Dowding 1987). While this perspective incorporates the mechanics of pollen transfer, it does not account for the striking differences in relative costs of animal versus wind-pollinated flowers, or the consequences for pollen capture of distributing flowers and ovules throughout the inflorescence. I explore theoretically some of the factors that may account for the association between anemophily and uniovulate flowers in this thesis.

Wind can be a relatively imprecise vector of pollen and this might make it difficult for hermaphroditic species to avoid capturing their own pollen. Diverse anti-selfing mechanisms have been recognized in flowering plants including monoecy and dichogamy; however, to what extent they function to reduce selfing rates in wind-pollinated plants is not well understood. A widely recognized feature of wind pollination is the higher frequency of unisexual flowers (Bawa 1980; Charlesworth 1993, Renner & Ricklefs 1995; Vamosi et al. 2003). There is no comprehensive mechanistic explanation for this association although some conceptual arguments have been made (Charlesworth 1993). Several hypotheses have been proposed, including a reduction in shared fixed costs of floral display and pollinator rewards between female and male flowers (Lloyd 1982), a more linear male gain curve (Charnov et al. 1976), and the prevention of intra-floral self-fertilization (Lloyd & Webb 1986; Charlesworth 1993). In this thesis I evaluate the efficacy of anti-selfing mechanisms in anemophilous plants using experimental approaches.
Wind-dispersed pollen has a leptokurtic distribution from point sources (Bateman 1947; Gleaves 1973; Levin & Kerster 1974) so that most mating is quite local and neighbourhood composition can have profound consequences on mating. Although large population sizes and high densities often characterize populations of wind-pollinated species, several studies have suggested that seed set of recipients should decrease rapidly with distance from the pollen donor. Also, plants in high-density patches might be more highly outcrossed if they capture more pollen, in comparison with plants in low densities where stigmatic pollen loads might contain more self-pollen (Eppley & Pannell 2007). Thus, the demographic context of anemophilous populations is likely to strongly influence patterns of pollen dispersal and mating. I address several of these issues by examining the ecological and demographic context of pollination and mating in several taxa of wind-pollinated plants.

Sex allocation in anemophilous plants should reflect the fact that wind cannot be saturated with pollen, and that pollen grains travel independently. Thus, male fitness is usually assumed to be an approximately linear function of resource investment into pollen production (Charlesworth & Charlesworth 1981; Charnov 1982; Klinkhamer & de Jong 1997), and indeed there is some limited empirical support for this pattern (Schoen & Stewart 1986). Also, larger plants are more effective at dispersing pollen, because they tend to be taller and release pollen at higher points. It has been proposed that these conditions should select for size-dependent sex allocation (Burd & Allen 1998). Male-biased sex allocation commonly occurs in wind-pollinated species and is particularly associated with large plant size (McKone 1987; Solomon 1989; Aizen & Kenigsten 1990; Fox 1993; Dajoz & Sandmeier 1997; Pannell 1997; McKone et al. 1998). Plants growing at low density might also be more effective at dispersing pollen compared to plants at high density, which should be better at capturing pollen from neighbours. Thus, size-dependent sex allocation and plastic
response to different environments in wind-pollinated plants may be adaptive. Using experimental approaches I examine the extent to which sex allocation is plastic in a wind-pollinated species by growing plants in contrasting environmental conditions.

**Research objectives**

The objective of my thesis is to provide a comprehensive investigation of the evolution and ecology of wind pollination. To achieve this goal I have used a combination of approaches commonly employed in evolutionary biology research. These include comparative biology, theoretical modeling, population surveys, quantitative genetics, and field and glasshouse experiments involving floral manipulations and genetic markers. Below, I briefly outline the specific goals for each of the seven chapters in my thesis containing original research. These have been written as self-contained research papers for submission to journals. Consequently, there is inevitable repetition in the introductions and discussions of some chapters. The details for chapters that have been published are provided in the summaries below.

Chapter Two – *Wind of change: new insights on the ecology and evolution of pollination and mating in wind-pollinated plants*. This chapter is a comprehensive review of current knowledge on pollination and mating in wind-pollinated plants, and supplements areas of particular deficiency with new empirical data. I begin by outlining a basic theoretical framework for modeling the selection of wind pollination, and then discuss how wind pollination may commonly evolve to provide reproductive assurance. I present the most extensive empirical data evaluating if wind-pollinated plants are commonly pollen limited. My findings indicate that pollen limitation may not be especially common in anemophilous species. An important component of this chapter is the presentation of data on the proportion

Chapter Three – A phylogenetic analysis of the evolution of wind pollination in the angiosperms. In this chapter, I examine the correlated evolution of wind pollination and a suite of ecological, morphological, and sexual traits. I use a large-scale phylogeny of the angiosperms and maximum-likelihood methods to infer historical patterns of evolution. This approach enabled me to detect correlated evolution and the order of trait acquisition between pollination mode and each of nine characters. I provide functional explanations on why wind pollination may be associated with the traits I examine. One of the main findings of this study is that the evolution of wind pollination occurs more often in lineages with unisexual flowers. This finding is consistent with the hypothesis that wind pollination is selected in these lineages to provide reproductive assurance when pollinator service is unreliable. This chapter is published in a special issue of the International Journal of Plants Sciences, on Major Evolutionary Transitions in Flowering Plant Reproduction edited by S. C. H. Barrett. (Friedman, J., and S. C. H. Barrett. 2008. A phylogenetic analysis of the evolution of wind pollination in the angiosperms. International Journal of Plant Sciences 169: 49-58).

Chapter Four – The evolution of ovule number in wind-pollinated plants. The goal of
this chapter is to provide a mechanistic explanation for the association between wind pollination and uniovulate flowers. I challenge the common assumption that the association reflects an inability of wind-pollinated plants to capture large pollen loads and provide empirical data to support this assertion. In this chapter I develop a phenotypic selection model which demonstrates that when flowers are inexpensive it is beneficial to distribute ovules among numerous flowers on a plant. Aerodynamic considerations suggest that pollen capture saturates with increased allocation to flowers, so that making numerous flowers that sample more of the airstream maximizes fitness. This chapter will be submitted for publication in the near future.

Chapter Five – The consequences of monoecy and protogyny for mating in wind-pollinated Carex. Flowers of wind-pollinated species are frequently unisexual, or if hermaphroditic separate their sex functions temporally through well-developed dichogamy (Charlesworth 1993; Renner & Ricklefs 1995). It has often been proposed that the common association between monoecy and protogyny in anemophilous species is because these traits largely function to reduce the incidence of self-fertilization (Lloyd & Webb 1986; Bertin & Newman 1993; Sargent & Otto 2004). Here I test the effectiveness of monoecy and protogyny in limiting selfing in seven woodland Carex species from southern Ontario. I employ diverse experimental approaches including inflorescence enclosures, flower emasculations, supplemental pollinations and genetic markers. I assess whether plants are self-compatible, if they are pollen-limited, and whether monoecy and protogyny limit the capture of self-pollen thus reducing selfing rates. I found that in all of the Carex species that I investigated species are highly to moderately selfing. Therefore at least in these species monoecy and protogyny appear to be largely ineffective as anti-selfing mechanisms. This

Chapter Six – *Environmental influence on primary sex ratio in a dioecious plant*. The proximity of mates can influence mating opportunities and offspring quality, particularly in dioecious wind-pollinated species. Here, I explore the consequences of plant density for pollination, mating and sex ratios in a dioecious wind-pollinated species (*Rumex nivalis*) with heteromorphic sex chromosomes. Using field experiments and sex-specific markers I examine whether the density of neighbouring male plants influences pollination intensity and progeny sex ratios in six populations growing in the Swiss Alps. I found evidence in five of the six populations that female plants growing in neighbourhoods with more males captured more pollen and produce more female-biased sex ratios than females in neighbourhoods with fewer males. This result is consistent with the hypothesis that gametophytic competition between female- and male-determining pollen tubes (certation) plays a role in causing female-biased progeny sex ratios in *Rumex* species. This chapter was part of a collaborative project with Dr. Ivana Stehlik (University of Toronto) and is published in Proceedings of the National Academy of Sciences (Stehlik, I., J. Friedman, and S.C.H. Barrett. 2008. Environmental influence on primary sex ratio in a dioecious plant. Proceedings of the National Academy of Sciences 105: 10852-10857).

Chapter Seven – *High outcrossing in the annual colonizing species Ambrosia artemisifolia*. Variation in the local density of plants in wind-pollinated species is likely to have consequences for pollination and mating. For example, plants in high-density patches
are likely to capture more pollen and be more highly outcrossed, whereas plants at low density may be more highly selfed because stigmatic pollen loads contain less outcross pollen but more self-pollen (Eppley & Pannell 2007). I tested this idea in *Ambrosia artemisiifolia*, a weedy annual wind-pollinated species reported in the literature as self-compatible. Over two years I set up array experiments at three densities and measured pollen loads and outcrossing rates using genetic markers. Although I detected a relation between plant density and pollen load size, I found no significant differences among treatments in outcrossing rate. This result motivated a study of pollen-tube growth following controlled pollinations in which I demonstrated for the first time that *A. artemisiifolia* is self-incompatible not self-compatible. The maintenance of high outcrossing rates in this colonizing species is likely facilitated by the prodigious production of pollen, high seed production and extended seed dormancy. This chapter is published in Annals of Botany (Friedman, J., and S. C. H. Barrett. 2008. High outcrossing in the annual colonizing species *Ambrosia artemisiifolia*. Annals of Botany 101: 1303-1309).

Chapter Eight – Genetic and environmental components of sex allocation in *Ambrosia artemisiifolia*. In this chapter, I evaluated models predicting variation in patterns of sex allocation in response to environmental conditions, or in response to increasing age or size (Charnov 1092; Lloyd & Bawa 1984; de Jong & Klinkhamer 1989; Zhang 2006). I grew maternal half-sib families of the monoecious, annual, wind-pollinated plant *Ambrosia artemisiifolia* in the sun and shade in a glasshouse experiment to investigate genetic and environmental components of sex allocation over time. The results indicated a plastic response in sex allocation elicited by the different environmental treatments. The patterns observed were those predicted by theory with more male investment in the sun treatment and
a size-dependent adjustment with larger plants producing proportionally more male flowers. The timing of flower production differed between the treatments, with significant genetic variation for dichogamy within environments and plasticity between environments. I also demonstrated significant genetic variation and a maternal family genotype-by-environment interaction in sex allocation. This suggests that there is the potential for individuals with different genetically-determined patterns of sex allocation to respond adaptively to contrasting environmental conditions. I will submit this chapter for publication in the near future.
CHAPTER TWO

WIND OF CHANGE: NEW INSIGHTS ON THE ECOLOGY AND EVOLUTION OF POLLINATION AND MATING IN WIND-POLLINATED PLANTS

This chapter resulted from collaborations with Spencer C. H. Barrett. Spencer Barrett contributed to the ideas and writing of the manuscript published in Annals of Botany, 2009, 103: 1515-1529.

Summary

The rich literature that characterizes the field of pollination biology has focused largely on animal-pollinated plants. At least 10% of angiosperms are wind pollinated and this mode of pollination has evolved on multiple occasions among unrelated lineages and hence this discrepancy in research interest is surprising. Here, I discuss the evolution and functional ecology of pollination and mating in wind-pollinated plants, outline a theoretical framework for modeling the selection of wind pollination, and experimentally investigate pollen capture and the occurrence of pollen limitation in diverse wind-pollinated herbs. Wind pollination may commonly evolve to provide reproductive assurance when pollinators are scarce. I present evidence that pollen limitation in wind-pollinated plants may not be as common as it is in animal-pollinated species. Studies of pollen capture in wind-pollinated herbs demonstrate that pollen transfer efficiency is not substantially lower than in animal-pollinated plants as is often assumed. These findings challenge the explanation that the evolution of few ovules in wind-pollinated flowers is associated with low pollen loads. Flower and inflorescence architecture is crucial to pollination and mating because of the aerodynamics of wind pollination. I provide evidence for the importance of plant height, floral position, and stamen and stigma characteristics in promoting effective pollen dispersal.
and capture. Finally, I propose that geitonogamous selfing may alleviate pollen limitation in many wind-pollinated plants with unisexual flowers.

**Introduction**

‘As a large quantity of pollen is wasted by anemophilous plants, it is surprising that so many vigorous species of this kind abounding with individuals should still exist in any part of the world; for if they had been rendered entomophilous, their pollen would have been transported by the aid of the senses and appetites of insects with incomparably greater safety than by the wind.……. It seems at first sight a still more surprising fact that plants, after having been once rendered entomophilous, should ever again have become anemophilous’

Darwin 1876, p. 409.

Wind pollination (anemophily) is a derived condition in flowering plants and has arisen independently in numerous families. Charles Darwin was perplexed by the evolution of this seemingly inefficient pollination mechanism from animal pollination and yet this transition is commonplace among angiosperm families. Wind pollination has evolved at least 65 times from animal-pollinated ancestors and ~10% of angiosperm species rely on wind pollination (Linder 1998; Ackerman 2000). Phylogenetic analyses indicate that wind pollination evolves more often in certain clades, most likely because they have morphological features conducive to wind pollination. For example, traits such as small, unisexual flowers and dry pollen may facilitate transitions to wind pollination (Linder 1998; Chapter Three). However, in comparison with other angiosperm reproductive transitions (e.g. the evolution of selfing from outcrossing and dioecy from hermaphroditism), remarkably little is known about the microevolutionary forces responsible for the evolution of wind pollination and the paucity of plant groups showing intra-specific variation in pollination
mode, including animal and wind pollination, has impeded experimental investigations. Our knowledge of the reproductive ecology of most wind-pollinated plants is quite rudimentary and the functional relations between pollination and mating are poorly understood.

Previous reviews on wind pollination largely focused on morphological traits that facilitate pollen release and capture (Whitehead 1969; Regal 1982; Niklas 1985; Ackerman 2000), characters that facilitate the evolution of wind pollination (Linder 1998) and the prevalence of mixed wind and animal pollination - ambophily (Culley et al. 2002). The same suite of reproductive traits is commonly associated with anemophily in many unrelated families and comprises the wind-pollination syndrome (Faegri & van der Pijl 1979). The utility of pollination syndromes in animal-pollinated plants has been questioned (Waser et al. 1996), although their reality in many groups is surely beyond doubt (Fenster et al. 2004). The traits typically associated with wind pollination (Table 2.1) are well established and tend to be less variable and perhaps for this reason the notion of a ‘wind pollination syndrome’ has not attracted much skepticism.

My review provides an overview of current understanding of key processes in the pollination and mating of anemophilous plants, and challenges some traditionally held assumptions, which may have biased understanding of the functional biology of wind pollination. I draw contrasts with knowledge of similar processes in animal-pollinated plants and highlight important areas that lack empirical data or theory. I begin by discussing the conditions that may favour the evolution of wind pollination focusing on the role of pollinator loss, with wind pollination providing a reliable alternative. I then consider some of the demographic and genetic consequences of wind pollination including the effects of wind pollination on effective population size and gene flow. Having established a framework for understanding the evolution of wind pollination and its ecological consequences, I then turn
Table 2.1. Some characters typically associated with wind versus animal pollination. There are numerous exceptions to these associations but many of the traits listed for wind pollination collectively describe the wind pollination syndrome.

<table>
<thead>
<tr>
<th>Wind pollination</th>
<th>Animal pollination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Many flowers</td>
<td>Few flowers</td>
</tr>
<tr>
<td>Small or absent petals</td>
<td>Large petals</td>
</tr>
<tr>
<td>Floral colour greenish or whitish</td>
<td>Floral colours contrasting, bicoloured</td>
</tr>
<tr>
<td>Nectaries absent</td>
<td>Nectaries present</td>
</tr>
<tr>
<td>Flowers unscented</td>
<td>Flowers scented</td>
</tr>
<tr>
<td>Styles feathery</td>
<td>Styles solid</td>
</tr>
<tr>
<td>Ovules per flower few or one</td>
<td>Ovules per flower many</td>
</tr>
<tr>
<td>Pollen size less variable</td>
<td>Pollen size highly variable</td>
</tr>
<tr>
<td>Pollen grains many</td>
<td>Pollen grains few</td>
</tr>
<tr>
<td>Pollen ornamentation absent</td>
<td>Pollen ornamentation present</td>
</tr>
<tr>
<td>Protogyny</td>
<td>Adichogamous or protandrous</td>
</tr>
<tr>
<td>Unisexual flowers</td>
<td>Hermaphrodite flowers</td>
</tr>
<tr>
<td>Synchronous flowering</td>
<td>Less synchronous flowering</td>
</tr>
<tr>
<td>Open habitats</td>
<td>Closed habitats</td>
</tr>
<tr>
<td>Temperate distribution</td>
<td>Tropical or temperate distribution</td>
</tr>
<tr>
<td>High conspecific density</td>
<td>Low conspecific density</td>
</tr>
<tr>
<td>Higher gene flow</td>
<td>Lower gene flow</td>
</tr>
<tr>
<td>Lower net speciation rates</td>
<td>Higher net speciation rates</td>
</tr>
</tbody>
</table>
my attention to particular features of wind-pollinated species that are fundamental to
pollination and mating. I review current theory and empirical investigations on sex allocation
in wind-pollinated species, and provide novel perspectives on the role of floral morphology
and inflorescence architecture in pollen dispersal. Finally, I conclude by discussing how
strategies that promote outcrossing in animal-pollinated species are implemented in wind-
pollinated plants.

The evolution of wind pollination and its ecological and demographic consequences

Two principal questions concerning the evolution of wind pollination from animal-
pollinated ancestors that have defied simple answers are: 1) what are the selective
mechanisms driving the evolution of wind pollination? 2) which evolutionary pathways are
involved in the transition? We have remarkably little concrete information about either of
these issues, although it may be easier to speculate on the selective conditions under which
wind pollination evolves. Several review papers have speculated on environmental
circumstances favouring the evolution of wind pollination (Regal 1982; Whitehead 1983;
Cox 1991, Culley et al. 2002), but there has been no rigorous treatment of the issue.

Theoretical considerations

There is a rich theoretical literature on reproductive transitions in the angiosperms,
such as the evolution of selfing (e.g. Uyenoyama et al. 1993) and the evolution of gender
strategies (e.g. Charlesworth 1999). However, remarkably there has been no theory
developed for the evolution of wind pollination. Theoretical approaches for understanding
the evolution of wind pollination might include ESS models based on sex allocation and gain
curves, classical population genetic approaches, or simulation models that consider the
timing and effectiveness of pollen dispersal for animal versus wind pollination under various demographic scenarios.

A large body of theory investigates evolutionary strategies for dividing resources among female and male function in plants for various life history, reproductive and ecological situations (e.g. Charlesworth & Charlesworth 1981; Charnov 1982; Lloyd 1984). The transition from animal to wind pollination could be explored similarly with models that seek the ESS allocation to female and male function and pollinator attraction, which is not needed for wind pollination. Contrasting allocation scenarios might affect female and male fertilities differently, depending on gain curves and the effectiveness of animal versus wind pollination. Here, I briefly outline a modeling framework that could be elaborated further.

Consider a hermaphroditic plant species that is primarily animal pollinated, but wind coincidentally disperses some fraction of its pollen. The fixed pool of resources that individual plants invest in flowering is divided proportionally among primary female ($F$) function, primary male ($M$) function and pollinator attraction ($A$), with the constraint that $F+M+A=1$. The quantity of pollen delivered and removed by animals varies positively with investment in attraction, whereas the success of wind pollination depends only on investment in primary sex function and the effectiveness of wind as a vector ($\gamma$). The final model would consider two dispersal phenotypes, a common type and a rare invader, and then find the allocation pattern that cannot be invaded by any alternate pattern. Each phenotype would have its own version of equations 2.1 and 2.2 below for female and male fitness components, respectively. Here, I present equations for the common type. The female component of fitness is proportional to resources invested in ovules ($F$) multiplied by the fraction of ovules that are fertilized, which depends on the amount of pollen delivered by both insects and wind:
\[ w_f \approx F \left( \pi_a \left\{ A^{k_1} \right\} + \pi_w \left\{ \gamma^{k_2} \right\} \right) \]  
(2.1)

where \( A = 1 - F - M \), and \( \pi_a \) and \( \pi_w \) are the amounts of pollen delivered by insects and wind respectively, and are functions of \( A \) and \( \gamma \). Equation 2.1 holds when seed production is pollen limited, otherwise \( w_f \approx F \). The male component of fitness is proportional to the resources invested in pollen (\( M \)), the pollen removed by insects (\( p \)), which depends on a plant’s attractiveness, and the pollen removed by wind:

\[ w_m \approx M \left( p \{ A \}^{k_1} + \left[ 1 - p \{ A \} \right]^{k_2} \right) \gamma \]  
(2.2)

where \( A = 1 - F - M \). Using this approach one could identify the pollination conditions specified by exponents \( k_1, k_2, k_3 \) and \( k_4 \) that select for variants with reduced allocation to attraction, which instead rely more on wind pollination. The model above is for illustrative purposes only, and one would need to insert specific functions for \( \pi_a \{ A \}^{k_1} \), \( \pi_w \{ \gamma \}^{k_2} \), and \( p \{ A \} \), and account for competition of pollen from different dispersal phenotypes for ovule fertilization.

In addition to identifying conditions that might select for wind pollination, this approach could provide insight on the stability of ambophily.

**Ecological context for the evolution of wind pollination**

The evolution of wind pollination in animal-pollinated lineages is thought to occur when physical and biological conditions render biotic pollination less reliable (Whitehead 1969; Regal 1982; Cox 1991). Under such conditions it might be advantageous for individuals to decrease the proportion of pollen dispersed by animals, and increase the proportion dispersed by wind. Wind pollination might evolve when pollinators are scarce, absent or deliver poor quality pollen (Weller et al. 1998; Goodwillie 1999), such as when plants colonize areas with low insect abundance (Berry & Calvo 1989; Gomez & Zamora...
1996). In these situations, plants may use a combination of animal and wind pollination, either sequentially or simultaneously (reviewed in Culley et al. 2002). However, it is unclear whether ambophily is an evolutionary stable strategy selected for its flexibility, or is simply an intermediate condition during the transition to complete wind pollination. More work is needed on ambophily to establish its frequency and evolutionary relationships with animal and wind pollination.

The evolution of wind pollination will be favoured in taxa that possess morphological traits that fulfill the aerodynamic requirements of pollen dispersal and capture. Plants with small flowers, exerted stamens and short or no corollas may be more likely to evolve wind pollination, because pollen dispersal and capture are not impeded by large and/or complex perianth structures. Plants with these features are usually unshowy and pollinated by generalist pollinators, which may often deliver poor quality pollen because of their local foraging behaviour (Charlesworth 1993; Bawa 1994; but see Renner & Feil 1993). Genera with both animal- and wind-pollinated species, such as Schiedea and Thalictrum, are instructive in this regard because the animal-pollinated species have small relatively inconspicuous flowers, which are visited by generalist pollinators. The open, exposed morphology of flowers in these groups probably facilitated the evolution of wind pollination (Kaplan & Mulcahy 1971, Weller et al. 2006).

The most common explanation for why wind pollination evolves from animal pollination is that it is favoured by selection when pollinators are unreliable. This implies that populations receiving unsatisfactory pollinator service become pollen limited and wind pollination evolves because it provides reproductive assurance. However, selection for reproductive assurance is thought to be one of the major factors in the evolution of self-fertilization in animal-pollinated hermaphrodite plants (Eckert et al. 2006). Indeed, Darwin
believed that reproductive assurance was the chief reason for the evolution of selfing in plants (Darwin 1876). Interestingly, the concept of reproductive assurance has seldom been extended to consider wind-pollinated plants.

I conducted a large-scale comparative analysis and found that wind pollination evolves more often in animal-pollinated lineages with unisexual flowers (Chapter Three). In lineages with unisexual flowers the evolution of selfing through autonomous self-pollination would be unlikely and wind pollination may replace selfing as a mechanism providing reproductive assurance. According to this hypothesis, insufficient pollinator service resulting in pollen limitation could elicit two quite different evolutionary transitions, depending on the floral condition of ancestral populations. In populations with hermaphroditic flowers, autonomous self-pollination would relieve pollen limitation resulting in the evolution of selfing. In contrast, in populations with unisexual flowers, wind pollination may serve the same role by increasing cross-pollination. A similar scenario may have occurred in Schiedea in which diclinous species suffer from pollinator limitation and the origin of wind pollination is evident (Weller et al. 1998). Whether transitions from animal to wind pollination are commonly driven by pollinator limitation is not known. Unfortunately, the lack of species with both animal and wind-pollinated populations has impeded empirical investigation.

Pollen limitation and pollen dispersal

The idea that wind pollination evolves as a means of reproductive assurance implies that pollen limitation may be infrequent for wind-pollinated plants. Unfortunately, despite a burgeoning literature for animal-pollinated species (Burd 1994; Larson & Barrett 2000; Ashman et al. 2004) information on the prevalence of pollen-limited reproduction in wind-pollinated groups is sparse. Recently, there have been several studies showing pollen limitation in wind-pollinated trees in fragmented habitats (Fox 1992; Knapp et al. 2001;
To investigate this issue I studied ten herbaceous species, comprising eight monoecious *Carex* species and two dioecious species (*Rumex acetosella* and *Thalictrum dioicum*) growing at the Koffler Scientific Reserve, ON, Canada. I tested whether female fertility in populations was pollen limited by comparing seed set in open-pollinated plants to those that received supplemental pollen. My experiments revealed that only one of the ten species showed significantly greater seed set when flowers received supplemental pollen (Figure 2.1). The rarity of pollen limitation in these species suggests that pollen loads are sufficient to ensure full seed set. Further work is needed on a diversity of wind-pollinated taxa to assess the frequency of pollen limitation in comparison with animal-pollinated plants where it has proven to be surprisingly common occurring in 62% (Burd 1994) and 73% (Ashman et al. 2004) of the studies investigated. If wind pollination does evolve primarily as a mechanism of reproductive assurance I predict that pollen limitation will be less common than it appears to be in animal-pollinated plants.

Fundamental to any consideration of pollen limitation is the density of conspecific plants and pollen transport distances. Wind-dispersed pollen has a leptokurtic distribution from point sources (Bateman 1947; Gleaves 1973; Levin & Kerster 1974), so that the seed set of recipients should decrease rapidly with distance from the pollen donor. However, few studies have examined the role of density in governing pollen limitation in wind-pollinated plants. Although large population sizes and high densities often characterize populations of wind-pollinated species, several studies have demonstrated that mating is quite local and neighbourhood composition can have profound consequences. For example, pollination...
Figure 2.1. Pollen limitation is infrequent in wind-pollinated plants. Seed set (SE) for ten species that were open-pollinated or received supplemental pollen by hand pollination. All species were studied at the Koffler Scientific Reserve, (44° 03'N, 79° 29'W) ON, Canada in their natural habitats during summer 2004 and 2005. Values presented are the mean of 20 individuals for each treatment. Seed set was measured as the proportion seed set/spike for the Carex species, and seed set/plant for R. acetosella and T. dioicum. All species are uniovulate making classification of successful seed production straightforward. Carex scabrata is the only species for which there was a significant difference between the pollination treatments (intact mean=0.82, SE=0.02; supplemental mean=0.89, SE=0.04; $F_{1,33} = 11.33$, $P<0.001$).
success in *Taxus* was negatively correlated with plant spacing (Allison 1990), and in *Spartina alterniflora*, an invasive grass, the low-density leading edge of a local invasion suffered Allee effects (Davis et al. 2004b). In two dioecious *Thalictrum* species occurring at low density, increased distance to male plants reduced seed set (Steven & Waller 2007); and in *Festuca pratensis* most pollen was deposited within 75m of donors (Rognli et al. 2000). Similarly, in dioecious *Rumex nivalis* increasing distance between males resulted in reduced pollen loads and decreased seed set (Chapter Six). Trees in fragmented populations in anthropogenically-disturbed habitats can have average pollen dispersal distances as low as 65m (Sork et al. 2002; Knapp et al. 2001). Thus, there is evidence that relatively small-scale variation in plant density can have consequences for pollination but how commonly this translates into pollen limitation of seed set is unclear.

Both pollen dispersal and plant density can have consequences for the genetic structure of plant populations. Hamrick et al. (1979) reported that wind pollination was associated with higher levels of genetic diversity, larger effective neighborhood sizes, reduced inbreeding, and less genetic differentiation between populations than occurs in animal-pollinated plants. Similarly, Loveless & Hamrick (1984) proposed that because long-distance pollen dispersal and background pollen levels prevent genetic differentiation over large geographic areas, wind-pollinated species should have larger effective population sizes ($N_e$) and reduced subdivision within populations. However, the allozyme data used in these comparative surveys was comprised of only two taxonomic groups – conifers and grasses – so generalization is problematic. The very short duration of pollen viability in many wind-pollinated species (Dafni & Firmage 2000) may limit long-distance gene flow. As discussed above, the dispersal distance of most wind-borne pollen is surprisingly short and the overall significance of long-distance gene flow in wind-pollinated plants remains unclear.
Highly synchronous flowering and a short duration of anthesis can be viewed as strategies that limit the quantity of pollen that fails to reach stigmas in many wind-pollinated species. For example, wind-pollinated prairie species have narrower phenological curves than those that are insect-pollinated, due either to shorter flowering times or greater flowering synchrony (Rabinowitz et al. 1981). While synchronous, predictable phenology favours intraspecific pollen transfer, it may also reduce the amounts of heterospecific pollen transfer among sympatric species. Grass species flower at distinctive times of the day for brief periods of 15 to 20 minutes (Gregory 1973) and gene flow between two rice species, *Oryza sativa* and *O. glaberrima*, was partially reduced by diurnal differences in flowering (Sano 1989). Although there is no evidence of seasonal partitioning of mating among species in anemophilous communities (Bolmgren et al. 2003), wind-pollinated species may be able to discriminate between conspecific and heterospecific pollen prior to deposition on stigmas through different pollen behaviours in the aerodynamic environments generated around stigmas (Niklas & Buchmann 1987; Linder & Midgley 1996). If wind-pollinated species commonly limit their exposure to inter-specific pollen by flowering at predictable times, or by preferentially capturing conspecific pollen, this would challenge the traditional view that pre-zygotic barriers to inter-specific pollination are unlikely in wind-pollinated species (Grant 1949).

**Sex allocation and pollen dispersal**

*Male-biased sex allocation*

Male-biased sex allocation commonly occurs in wind-pollinated species and is particularly associated with large plant size (McKone 1987; Burd & Allen 1988; Solomon 1989; Aizen & Kenigsten 1990; Ackerly & Jasienski 1990; Fox, 1993; Dajoz & Sandmeier...
1997; Pannell 1997; McKone et al. 1998). Male fitness is usually assumed to be an approximately linear function of resource investment into pollen production, because wind is unlikely to saturate with pollen (Charlesworth & Charlesworth 1981; Charnov 1982; Klinkhamer & de Jong 1997) and indeed there is limited support for this (Schoen & Stewart, 1986). This pattern contrasts with the saturating gain curves generally assumed for animal-pollinated plants. However, male fitness may depend non-linearly on pollen dispersal, rather than linearly on pollen production (Burd & Allen 1998). Larger plants are more effective at dispersing pollen, because they tend to be taller and release pollen at higher points. Pollen dispersal distances are greater when pollen is released at elevated heights, because wind speeds are higher, there is often less intervening vegetation, and pollen takes longer to settle out of the airstream (Levin & Kerster 1974; Okubo & Levin 1989). Thus, unlike animal-pollinated plants, size-dependent sex allocation should select for increasing maleness with height (Bickel & Freeman 1993; de Jong & Klinkhamer 2005).

Theoretical research on sex allocation in wind-pollinated plants has considered the conditions under which male-biased sex allocation is maintained. Sakai & Sakai (2003) found that the relative size of the pollen dispersal to seed dispersal area in large versus small plants determines whether they should exhibit male-biased ESS sex allocation versus female-biased sex allocation, respectively. Pollen-transfer efficiency (the proportion of pollen captured) and competitive sharing among male flowers affects sex allocation, and more reproductive resources should select for increasing maleness (Masaka & Takada 2006). Thus, adaptive patterns of sex allocation should favour greater male function and particularly increased maleness in larger plants.

Fitness returns in wind-pollinated plants may depend more on relative plant height than absolute height. Smaller plants may perform better at capturing pollen while larger
plants are more effective at dispersing pollen to their neighbours. Size-dependent sex allocation occurs in monoecious *Ambrosia artemisiifolia*, where taller plants have relatively more male allocation than smaller plants (McKone & Tonkyn 1986; Traveset, 1992; Ackerly & Jasienski 1990; Lundholm & Aarssen 1994). By using shading treatments to simulate the effect of neighbours, Paquin & Aarssen (2004) demonstrated that when size is constrained because of shading a decrease in plant size is associated with a decrease in maleness and an increase in femaleness.

I investigated the effect of shading on plastic sex allocation and its influence on the expression of dichogamy in *A. artemisiifolia*. My results demonstrate that there is a trade-off in female and male function (Figure 2.2). Plants in shade treatments were protogynous and produced more female flowers, whereas plants in sun treatments were protandrous and produced more male flowers. Under field conditions small plants often occur in shaded environments and relatively tall plants are more likely to grow in full sun. Using light intensity to perceive neighbourhood composition may enable plants to maximize their success as either pollen donors or recipients. For wind-pollinated plants, plastic sex allocation may give individuals the flexibility to alter gender expression to match neighbourhood conditions thus optimizing fitness as maternal and paternal parents.

*Pollen-ovule ratios and pollen transfer efficiency*

Prodigious pollen production is a characteristic feature of the anemophilous syndrome and pollen-to-ovule ratios are generally much higher than animal-pollinated taxa (wind: median 22150:1; animal: median 3450:1: Cruden 1977, 2000). Cruden’s explanation for this pattern focused on the ‘efficiency’ of the pollination system gauged as the likelihood of pollen arriving on stigmas. However, as pointed out by Charnov (1982), this explanation assumes that pollen is produced in just the right quantities to ensure ovule fertilization and
Figure 2.2. Number of a) female and b) male flowers produced by *Ambrosia artemisiifolia* in sun versus shade treatments for the first three weeks of flowering. Plants were grown in 4” diameter pots under uniform glasshouse conditions during July-August 2006 at the University of Toronto, Ontario from open-pollinated seed collected at the Koffler Scientific Reserve. The values presented are the means (±SE) for N=77 sun plants and N=74 shade plants. The sun treatment involved ambient full sunlight. Plants in the shade treatment were placed under frames covered with black neutral-density shade cloth that reduced light levels by ~80%.
equates plant reproductive success with seed production thus ignoring male function. This view also assumes that much more pollen is wasted in anemophilous systems than with animal pollination. Indeed, the idea that wind pollination is an inefficient and wasteful system has pervaded the literature since Darwin (see quote at the beginning of this chapter; Proctor et al. 1996; Ackerman 2000). However, despite this widespread view there have been no studies comparing pollen capture in animal versus wind-pollinated taxa.

To investigate pollen transfer efficiency in wind-pollinated plants I collected data on the amount of pollen produced and the quantity of pollen captured by stigmas for 19 wind-pollinated herbaceous species. The species were located in their natural habitats at either the Koffler Scientific Reserve (44° 03'N, 79° 29'W, ON, Canada) or the Kananaskis Field Station (51° 02'N, 115° 03'W, AB, Canada). For each species I used 20 individuals and 3 flowers per plant. The distribution of pollen loads for each species are shown in Appendix 1. Mean pollen capture was 0.32% (range 0.01–1.19%) of the pollen produced (Figure 2.3). Harder (2000) reported that for 24 animal-pollinated species with granular pollen the proportion of pollen captured ranged from 0.03–1.9%. Although my values are lower they are within the same orders of magnitude and do not support the overall contention that pollen dispersal in wind-pollinated plants is considerably more wasteful than in animal-pollinated plants.

The timing and rate of arrival of pollen grains may affect opportunities for microgametophytic competition in wind-pollinated plants. If pollen arrives gradually, the first grains will likely be successful at fertilization, regardless of their competitive ability. However, anthesis in wind-pollinated plants tends to be highly synchronous within populations (Dowding 1987), and in grasses flowering can be over in less than 5 hours (Beddows 1931). Thus, pollen grains may arrive synchronously and compete to fertilize ovules. Also, anemophilous pollen grains arrive independently and so may be drawn more
Figure 2.3. Proportion of pollen produced that is captured by stigmas for 19 wind-pollinated species at Koffler Scientific Reserve, ON (summer 2004 and 2005) and Kananaskis Field Station, AB (summer 2001 and 2002). Species included: *Ambrosia artemisiifolia*, *Anthoxanthum nitens*, *Bromus inermis*, *Carex communis*, *C. hirtifolia*, *C. pedunculata*, *C. plantagineae*, *C. stipata*, *Chenopodium album*, *Elymus repens*, *Festuca campestris*, *Leymus innovatus*, *Phleum pratense*, *Plantago lanceolata*, *Rumex acetosella*, *R. crispus*, *Schizachne purpureascens*, *Scirpus microcarpus*, and *Thalictrum dioicum*. I counted the number of pollen grains in undehisced anthers using an Elzone 5380 particle counter (Micromeritics Inc. Atlanta, GA), assessed stigmatic pollen loads by staining stigmas with 1% basic fuschin, and counted pollen grains that had been deposited under a compound microscope (20x). Each species is categorized based on its sexual system (hermaphroditic flowers; monoecious, dioecious), and plotted values represent the mean of 20 individuals and 3 flowers each. The lines depict the percentage of pollen produced that was captured by stigmas and provide a reference guide.
evenly from potential sires than with animal pollination. The above considerations point to
the possibility of intense post-pollination competition in wind-pollinated plants leading to
gametophytic selection and this has the potential to result in greater progeny vigour
(Mulcahy & Mulcahy 1987). Thus, if wind-pollinated species do not generally exhibit
substantially lower pollen-transfer efficiencies in comparison with animal-pollinated species
(Figure 2.3), the copious pollen production of wind-pollinated taxa may instead reflect the
intensity of post-pollination competition, rather than any general inferiority of the wind as a
pollen vector.

Causes of low ovule number in wind-pollinated plants

There has been surprisingly little consideration of differences in ovule number
between animal- versus wind-pollinated plants despite their striking differences in P/O ratios.
Low ovule number, including many taxa with uniovulate flowers (e.g. Cyperaceae,
Juglandaceae, Poaceae), is a characteristic feature of wind-pollinated plants. Phylogenetic
evidence indicates that a reduction in ovule number usually occurs after the evolution of
anemophily (Linder 1998; Chapter Three). The most common explanation for this
association is the small chance of stigmas capturing multiple pollen grains (Pohl 1929;
Dowding 1987). Unlike animal-pollinated plants, where pollen grains commonly arrive in
clumps, pollen of wind-dispersed species is usually transported as single units, so the chance
of capturing each pollen grain is an independent event. However, stigmas of wind-pollinated
plants often capture amounts of pollen that far exceeds ovule number. For example in my
study of 19 species (Figure 2.3), mean stigmatic pollen load was 34.1 (SE = 3.8) grains.
Elsewhere pollen loads on open-pollinated stigmas of wind-pollinated taxa in Poaceae,
Proteaceae, Ranunculaceae, Restionaceae, and Rosaceae (mean pollen grains per ovule range
from 3 to 100: Honig et al. 1992; Linder & Midgley 1996; Davis 2004; Friedman & Harder
2004) are inconsistent with the proposal that low pollen loads lead to decreased ovule number in wind-pollinated species.

Several morphological and aerodynamic features of wind-pollinated plants may favor few ovules or a single ovule per flower. The low cost of producing flowers in wind-pollinated plants may favor a packaging strategy with more flowers per plant each containing few ovules. Burd (1995) provides indirect support for this idea by showing theoretically that low floral costs generally favor fewer ovules per flower, although his model only considered animal-pollinated plants. Also, by producing more flowers with fewer ovules, the spatial dispersion of flowers on a plant may enlarge the volume of air sampled and increase the probability of capturing more pollen grains. A more mechanistic understanding of the functional association between wind pollination and low ovule number would be informative.

**Floral and inflorescence architecture**

The floral morphology and inflorescence architecture of wind-pollinated plants largely reflect the aerodynamic requirements for successful pollen liberation from anthers and capture by stigmas. Fluid dynamic theory predicts that pollen removal is enhanced by traits that help move initially stationary pollen grains out of the boundary layer and into the airstream (Niklas 1992). Because the speed of flow rises with increasing distance from an object, anthers experience higher wind speeds when they are suspended beyond the boundary layer of still air created by other floral organs. This physical constraint has led to the evolution of highly diverse floral and inflorescence morphologies that distinguish wind- from animal-pollinated plants (Figure 2.4). The anthers of wind-pollinated species are typically extended on long flexible filaments enabling them to move in the slightest breeze. For example, *Pennisetum clandestinum* (Figure 2.4d) possesses extremely long filaments that project anthers away from the plant whereas stigmas are deployed near to the ground. Also,
Figure 2.4. Diverse inflorescence and floral morphology in four wind-pollinated species: a) *Carex pedunculata* (Cyperaceae) in Ontario, Canada. Plants are monoecious and protogynous, with male flowers clustered at the top of the inflorescence and female flowers below. b) Gynomonoecious *Gunnera peltata* (Gunneraceae), Isla Más Afuera, Juan Fernandez Islands. Inflorescences are very large and upright, perhaps to compensate for growing under extensive leaf canopies. *Gunnera* is frequently described as wind pollinated (e.g. Bernardello et al. 2001) but this has not been confirmed through experimental studies. c) Female (left side) and male (right side) individuals of *Leucadendron rubrum* (Proteaceae) in Cape Province, South Africa. The species is dioecious and has extreme sexual dimorphism between the sexes. d) *Pennisetum clandestinum* (Poaceae) in Cape Province, South Africa illustrating striking differences in the functional deployment of stigmas and anthers. The panel on the left shows anthers extended on very long filaments, with the arrow pointing to the stigmas. The panel on the right is a close-up of the feathery stigmas presented at ground level. All images by S.C.H. Barrett, except d) which is courtesy of L.D. Harder.
wind speeds increase with distance from the ground, so pollen dispersal is more effective when male flowers are presented in upper portions of the plant, which also reduces interference by vegetative structures. This leads to the prediction that in monoecious species male flowers should be presented above female flowers (for example, as in *Carex pedunculata* Figure 2.4a). Although the data is sparse, there appears to be some support for these positional relations (Freeman et al. 1981; Bickel & Freeman 1993; Fox 1993).

To examine whether the position of a flower within an inflorescence affects pollen capture, I collected data on four herbaceous wind-pollinated species from four families (Figure 2.5). Each species illustrates a different aspect of how architecture may influence pollen capture. In monoecious *Carex pedunculata*, female flowers in upper positions of the inflorescence captured significantly more pollen than flowers at lower positions. Because male flowers are presented at the top of the inflorescence above female flowers this pattern likely reflects increased self-pollen capture in upper female flowers since the species exhibits high selfing rates (Chapter Five). Like other grasses with diffuse panicles, florets in upper positions in *Festuca campestris* capture more pollen (Figure 2.5b) because they sweep through a larger volume of air during oscillation (Friedman & Harder 2004). *Plantago lanceolata* is protogynous, flowers acropetally, and the scape elongates during flowering, so that female and male functions are separated in height. Thus, stigmas at the top of the inflorescence have the potential to capture self-pollen from anthers below (Bos et al. 1985; Young & Schmitt 1995). Although in this species there was a trend for upper flowers to capture more pollen than lower flowers the difference was not significant (Figure 2.5c). Finally, there was no evidence of position effects in *Rumex acetosella* (Figure 2.5d) probably because it is dioecious and female and male plants are approximately the same height. If pollen is homogenously distributed in the airstream, then we expect flowers at different
Figure 2.5. Relation between the position of flowers within an inflorescence and stigmatic pollen loads for four wind-pollinated herbaceous species at the Koffler Scientific Reserve (summer 2004 and 2005), Ontario and Kananaskis Field Station (summer 2001), Alberta. In both *Carex pedunculata* (Cyperaceae) and *Festuca campestris* (Poaceae) flowers in upper positions of the inflorescence captured significantly more pollen than those in lower positions (*C. pedunculata*: $t_{2675}=3.43$, $P=0.01$; *F. campestris*: $t_{2863}=3.96$, $P=0.002$). In *Plantago lanceolata* (Plantaginaceae) and *Rumex acetosella* (Polygonaceae) there was no significant difference in pollen capture between flowers in upper and lower positions (*P. lanceolata*: $t_{2731}=1.08$, $P>0.5$; *R. acetosella*: $t_{2878}=0.11$, $P>0.5$). All $P$-values are adjusted for multiple contrasts.
positions to capture equivalent amounts of pollen. These examples illustrate how architecture, sexual system and aerodynamics may interact to affect pollen capture in wind-pollinated plants.

Pollen capture in wind-pollinated plants is also dependent on the morphology of flowers. The feathery stigmas of many anemophilous species have higher collection efficiencies than solid stigmas, because they have smaller radii, and because they produce proportionally thinner boundary layers (Niklas 1985). Furthermore, the reproductive morphology of wind-pollinated species creates a unique aerodynamic environment in the immediate vicinity of reproductive organs. Using wind-tunnel experiments, Niklas (1985) demonstrated that the morphology of ovulate organs influences the airflow through which pollen travels. Thus, pollen morphology and ovulate organs may act synergistically to generate aerodynamic environments that increase a species’ efficiency of pollen capture (Niklas & Buchmann 1987; Paw U & Hotton 1989; Friedman & Harder 2005). Linder & Midgley (1996) provided evidence for pollen discrimination by anemophilous species in a field study of four sympatric co-flowering species. They found high proportions of conspecific pollen on stigmas (range 40–80%) in comparison to pollen frequencies in the air. Whether this pollen filtering is a general phenomenon for sympatric wind-pollinated species remains to be determined; however, it does suggest that pollen capture may be a more refined process than a simple random sampling of the airstream and that prezygotic barriers to interspecific pollination may serve to reduce gamete wastage.

**Pollen-size evolution**

Unlike animal-pollinated taxa (see Harder 1998), the evolution of pollen size in wind-pollinated species depends strongly on pollen-transport conditions. Pollen size in anemophilous plants probably reflects stabilizing selection that balances two conflicting
demands (Whitehead 1969; Niklas 1985). Small pollen grains have low inertia, facilitating removal from anthers and low settling velocity, which allows pollen to travel farther before falling out of the airstream. On the other hand, large pollen grains have greater momentum, which increases the chance of breaking away from deflected streamlines to collide with stigmas. As a result of these influences, wind-pollinated species exhibit a smaller range of pollen sizes (17–58 µm) than animal-pollinated species (5–200 µm), although average pollen size does not differ greatly among these groups (Wodehouse 1935), contrary to popular misconception. Aerodynamic constraints on pollen size are evident in Plantago, where species with chasmogamous (open) flowers have much less variable pollen sizes than species with cleistogamous (closed) flowers (Primack 1978). Variation in pollen size among wind-pollinated species can be predicted by the size of the structure (either flower or inflorescence) that interacts with the airstream (Paw U & Hotton 1989; Friedman & Harder 2005).

**Inflorescence architecture**

The packaging of flowers within inflorescences affects how female and male reproductive parts interact with airflow, thereby influencing pollination. For example in the grasses, where a striking diversity of inflorescence architectures occur, wind-tunnel experiments demonstrate a dichotomy in the mechanics of pollen receipt depending on whether species posses compact or diffuse panicles (Niklas 1987). Compact panicles act as a bluff-bodied obstruction to airflow, so that much entrained pollen is deflected from windward stigmas and is instead captured by sedimentation from eddies on the leeward side of the inflorescence. In contrast, diffuse panicles disrupt airflow less, so that most pollen collides with windward-facing stigmas. Manipulative field experiments on grasses with compact and diffuse panicles demonstrate that the oscillation of grass inflorescences have contrasting effects on pollen removal and receipt (Friedman & Harder 2004). Specifically,
immobilization of culms by tethering in three species with compact panicles reduced pollen removal, but did not affect pollen receipt. In contrast, immobilization did not affect pollen removal from two species with diffuse panicles, but reduced pollen receipt. Together, these studies demonstrate that compact and diffuse inflorescences function differently to effect pollination. Studies comparing inflorescence architecture of plants growing in high-wind (e.g. open habitats) versus low-wind (e.g. forests) conditions would be informative in this regard.

There is growing evidence that inflorescence architecture in wind-pollinated plants has been shaped by selection for efficient pollen dispersal and capture; however, many unresolved questions remain. It is likely that there is also selection on inflorescence architectural traits for characteristics related to seed dispersal. For example, the position of flowers within inflorescences may affect seed provisioning by the maternal plant. If seeds are wind-dispersed, selection may act on traits that facilitate dispersal. Teasing apart the gender role and life history stage on which selection acts in hermaphroditic wind-pollinated species is likely to be tricky. However, comparing plant and inflorescence architectures between females and males in wind-pollinated dioecious species can circumvent this difficulty. In such cases the architecture of male plants should largely be selected for efficient pollen dispersal, whereas female plants will be influenced by selection pressures for pollen capture and seed dispersal. This seems should lead to greater levels of dimorphism in wind-pollinated dioecious species than in animal pollinated species where too much morphological divergence may influence pollinator behaviour and interfere with successful cross-pollination (Vamosi & Otto 2002). *Leucadendron rubrum* provides a spectacular example of the extreme sexual dimorphism that can arise in a wind-pollinated species because of these contrasting selection pressures (Figure 2.4c). To what extent selection acting on architectural traits
related to pollen dispersal, pollen capture and seed dispersal oppose one another is unknown, but could be evaluated in groups that possess both hermaphroditic and dioecious species.

**Strategies that promote outcrossing**

Plants are sessile and therefore require pollen vectors for cross-pollination. Natural selection shapes the interactions with pollen vectors so that pollen is dispersed effectively among conspecifics. In animal-pollinated species these interactions have given rise to the great structural diversity of flowers and inflorescences. Reproductive structures function to manipulate animal foraging in a predictable manner promoting pollen dispersal and reducing the incidence of self-pollination. I expect that the flowers and inflorescences of wind-pollinated plants are also under similar selective pressures, although precisely how floral and inflorescence traits influence pollination and mating has received scant attention in the literature.

*Prevalence of mixed mating*

One of the most recognized features of mating in wind-pollinated plants is the apparent low incidence of mixed mating leading to a bimodal distribution of outcrossing rates (Schemske & Lande 1985; Aide 1986; Barrett & Eckert 1990; Goodwillie et al. 2005), with species more frequently either predominantly selfing or outcrossing. However, the reported distribution of outcrossing rates for wind-pollinated plants should be viewed with some caution (and see Igic & Kohn 2006). In the most recent compilation (Goodwillie et al. 2005), all but 18 of the 76 wind-pollinated species belong to just two families (Poaceae and Pinaceae) and clearly further effort is required to broaden taxonomic representation. Nonetheless, if I assume that the apparent trend is real it is worth considering what factors
might contribute to a lower incidence of mixed mating in wind-pollinated plants compared with those that are animal-pollinated.

Paucity of mixed mating may reflect different selective pressures in wind-pollinated species including a less stochastic pollination environment (Aide 1986; Vogler & Kalisz 2001; Goodwillie et al. 2005). This implies that wind is a more predictable vector of pollen than animals and, provided population densities are sufficient, flowers should receive adequate outcross pollen. However, empirical data to support this ideas is scant and the occurrence in herbaceous wind-pollinated lineages (e.g. grasses, sedges) of many predominantly selfing species indicates that pollen dispersal is not always predictable.

The survey of pollen capture in wind-pollinated plants shows a tendency for higher pollen capture in hermaphrodite versus dioecious species, and between monoecious species versus those with perfect flowers (Figure 2.3). Although sample sizes are small, this order of increasing pollen load is consistent with the likelihood of self-pollen capture. I caution, however, that a large number of the hermaphrodite species in the survey were grasses (Poaceae) and most of the monoecious species were sedges (Carex). Although the lack of phylogenetic independence precludes statistical comparisons among sexual systems, the trend is interesting and should motivate further studies. It is possible that high levels of geitonogamous selfing are inevitable for many wind-pollinated plants, especially those of large size with many flowers in anthesis at the same time. Indeed, selection against this form of selfing may explain the common association between dioecy and wind pollination, and the frequent occurrence of self-incompatibility in wind-pollinated groups such as Poaceae.

Sexual strategies, pollen capture and mating

To investigate the effect of sexual system on pollen capture and its interaction with plant density, I investigated three wind-pollinated Rumex species. Two species, R. acetosella
and *R. nivalis*, are dioecious, and *R. crispus* is hermaphroditic. For each species I collected stigmas from at least 3 flowers on 20 plants and measured the distance to the nearest five pollen-producing neighbours. A significant relation between pollen load and mean distance to neighbours was evident in both dioecious species with more pollen captured on plants that had close neighbours (Figure 2.6a, 2.6c). There was no relation between plant density and pollen loads in hermaphroditic *R. crispus* (Figure 2.6b) and in this species self-pollination may buffer plants against the effects of low density. Although *R. crispus* is protandrous, there is considerable overlap in female and male function within an inflorescence and hence this form of dichogamy appears to be largely ineffective in promoting outcrossing (Bertin 1993; Routley et al. 2004).

There are strong associations between anemophily and protogyny (Bertin 1993; Sargent & Otto 2004) and transitions from biotic to abiotic pollination are more likely among protogynous species than among protandrous species (Sargent & Otto 2004). These patterns may occur because following a switch to wind pollination protogynous species are less likely to suffer from increased self-fertilization (Lloyd & Webb 1986; Bertin & Newman 1993; Sargent & Otto 2004). Protogyny is associated with self-compatibility, and protandry with self-incompatibility more often than expected under a random model (Bertin 1993; Routley et al. 2004). This finding is consistent with the inbreeding avoidance hypothesis for the evolution of protogyny (Lloyd & Webb 1986). The associations between protogyny and anemophily and protogyny and self-compatibility suggest that protogyny may be an effective outcrossing mechanism in many anemophilous species.

A widely recognized feature of wind pollination is the higher frequency of unisexual flowers in wind-pollinated versus animal-pollinated species (Bawa 1980; Charlesworth 1993, Renner & Ricklefs 1995; Vamosi et al. 2003). There is no comprehensive mechanistic
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Figure 2.6. Relation between mean distance to five nearest neighbours and stigmatic pollen load for three *Rumex* species. Both of the dioecious species, *R. acetosella* and *R. nivalis*, captured significantly more pollen when neighbours were close (*R. acetosella*: $t_{117}=2.66$, $P<0.01$, *R. nivalis*: $t_{53.4}=3.06$, $P<0.005$) while the relation is not significant for the hermaphrodite species, *R. crispus* ($t_{97.8}=1.46$, $P=0.15$). The equations for the relation between pollen receipt and distance to neighbours are: *R. acetosella* $y= 16.32x^{-1} + 0.78$; *R. crispus* $y= -14.06x^{-1} + 1.67$; *R. nivalis* $y= 27.45x^{-1} + 0.56$. *Rumex nivalis* was investigated during summer 2004 at Davos, Switzerland (46°41’N, 9°48’E) and *R. acetosella* and *R. crispus* during summer 2005 at Koffler Scientific Reserve, Canada (44°03’N, 79°29’W).
explanation for this association although some conceptual arguments are compelling (Charlesworth 1993). Several hypotheses have been proposed, including a reduction in shared fixed costs of floral display and pollinator rewards between female and male flowers (Lloyd 1982), a more linear male gain curve (Charnov et al. 1976), and the prevention of intra-floral self-fertilization (Lloyd & Webb 1986; Charlesworth 1993).

Although protogyny and unisexual flowers may limit self-fertilization in some wind-pollinated groups my own investigations indicate that this is not the case in some Carex species (Chapter Five). Emasculation experiments and estimates of mating patterns using genetic markers in seven monoecious, protogynous species demonstrated that geitonogamous self-fertilization predominated. Indeed, I proposed that unlike animal-pollinated species, where geitonogamy is rarely adaptive (Lloyd 1992), geitonogamy in wind-pollinated herbs with unisexual flowers may often provide reproductive assurance when ecological conditions limit outcross pollen dispersal. In the Carex I investigated low wind speeds in the woodland understory occupied by all seven species and the small stature of plants make this seem quite likely.

**Conclusions**

Many issues need to be addressed regarding evolution and adaptation in wind-pollinated plants. This review has largely focused on functional questions related to sex allocation, inflorescence design and mating strategies highlighting areas that need future work. In comparison with animal-pollinated plants, there is a paucity of basic information about the reproductive biology of wind-pollinated plants. Indeed, in some groups routinely described as wind pollinated (e.g. Gunnera, see Figure 4b) experimental evidence is needed to confirm the role of wind in pollen transport. Much more quantitative data on pollen dispersal, pollen limitation and mating patterns is required before generalizations can be
made concerning the efficacy of wind pollination in comparison with animal pollination. Three areas that are in particular need of future attention include: 1) theoretical investigations of the evolution of wind pollination; 2) measurement of fitness returns for male allocation strategies; and 3) studies of mating systems in wind-pollinated plants, particularly modes of selfing and patterns of paternity. Future mechanistic studies that extend the seminal work of Niklas (1985; 1987; Niklas & Buchmann 1987) linking reproductive morphology and plant architecture to the aerodynamics of pollen release and pollen capture, and efforts to link pollen dispersal and mating through the use of genetic markers, have the potential to reveal complex and fascinating new details on the reproductive biology of wind-pollinated plants.
CHAPTER THREE

A PHYLOGENETIC ANALYSIS OF THE EVOLUTION OF WIND POLLINATION IN THE ANGIOSPERMS

*This chapter resulted from collaborations with Spencer C. H. Barrett. Spencer Barrett contributed to the ideas and writing of the manuscript published in International Journal of Plant Sciences, 2008, 169: 49-58.*

**Summary**

Wind pollination is predominantly a derived condition in angiosperms and is thought to evolve in response to ecological conditions that render animal pollination less advantageous. However, the specific ecological and evolutionary mechanisms responsible for transitions from animal to wind pollination are poorly understood in comparison with other major reproductive transitions in angiosperms, including the evolution of selfing from outcrossing and dioecy from hermaphroditism. To investigate correlations between wind pollination and a range of characters including habitat type, sexual system, floral display size, floral showiness, and ovule number, I used a large-scale molecular phylogeny of the angiosperms and maximum likelihood methods to infer historical patterns of evolution. This approach enabled me to detect correlated evolution and the order of trait acquisition between pollination mode and each of nine characters. Log likelihood ratio tests supported a model of correlated evolution for wind pollination and habitat type, floral sexuality, sexual system, flower size, flower showiness, presence versus absence of nectar, and ovule number. In contrast, wind pollination and geographical distribution and number of flowers per inflorescence evolve independently. I found that in wind-pollinated taxa, nectar is lost more often and ovule number is reduced to one. I also found that wind pollination evolves more frequently in lineages already possessing unisexual flowers and/or unisexual plants. An
understanding of the ecological and life-history context in which wind pollination originates is fundamental to further investigation of the microevolutionary forces causing transitions from animal to wind pollination.

**Introduction**

Wind pollination (anemophily) has evolved at least 65 times in the angiosperms from biotically pollinated ancestors (Linder 1998). A recent survey estimates that abiotic pollination occurs in at least 18% of angiosperm families (Ackerman 2000) with wind pollination more commonly represented than water pollination. The evolution of wind pollination is thought to occur when environmental conditions render biotic pollination less advantageous (Regal 1982; Cox 1991). For example, a decline in pollinator abundance or changes in the abiotic environment limiting pollinator activity have been invoked to explain why wind pollination has evolved in particular taxa (Berry & Calvo 1989; Weller et al. 1998; Goodwillie 1999). However, the specific ecological mechanisms causing transitions from animal to wind pollination have not been investigated in detail and, in comparison with other reproductive transitions (e.g. the evolution of selfing from outcrossing and dioecy from hermaphroditism), little is known about the microevolutionary forces responsible for the evolution of wind pollination.

Comparative evidence clearly indicates that wind pollination evolves more frequently in certain clades (Ackerman 2000). As families and genera often share traits as a result of common ancestry, mapping traits on to phylogenetic trees is our best option for testing correlated evolution without bias from phylogenetic relationships (Felsenstein 1985; Donoghue 1989; Harvey & Pagel 1991). Taking phylogenetic relationships into account, I was interested in finding evidence for the existence of correlations between anemophily and specific morphological and ecological traits and determining the putative evolutionary
pathways leading to these associations. Linder (1998) first examined morphological traits and their associations with wind pollination in a comparative context. However, his study was limited to families of the lower rosids and commelinoid monocots and used the concentrated-changes test (Maddison 1990) to investigate correlations (see Schluter et al. 1997; Pagel 1999). The recent advent of maximum-likelihood phylogenetic comparative methods (Harvey & Pagel 1991; Pagel 1994; Freckleton et al. 2002) provides an opportunity to investigate correlations among life-history traits, ecology and biogeography, and to examine the evolutionary history of reproductive associations (e.g. dioecy – Vamosi et al. 2003; dichogamy and self-incompatibility – Routley et al. 2004; protogyny and pollination mode – Sargent & Otto 2004). I was interested in examining the relations between wind pollination and a range of characters that have been proposed to be associated with this condition. I now briefly review the traits chosen and the functional arguments that have been proposed to explain their correlations with anemophily.

Regions of higher latitude, arid temperate environments, open vegetation, and island floras have the highest representation of wind-pollinated plants (Whitehead 1968; Regal 1982). The physical and aerodynamic requirements for successful wind pollination may explain these ecological and geographical correlates. However, it is unclear whether these associations are robust to phylogenetic considerations, and if wind pollination is more likely to originate under these conditions, or is simply easier to maintain.

One of the more widely recognized features of wind pollination is the higher frequency of unisexual flowers in wind-pollinated versus animal-pollinated species (Bawa 1980; Renner & Ricklefs 1995). Several hypotheses have been proposed, including a reduction in shared fixed costs between female and male flowers (Lloyd 1982), a more linear male gain curve (Charnov et al. 1976), and limiting self-fertilization because of the
unavoidable geitonogamy that seems likely in wind-pollinated plants (Lloyd & Webb 1986; Charlesworth 1993). Some of these explanations are specific to dioecy, while others can be extended to dicliny (unisexual flowers) in general. These arguments imply that wind pollination precedes the evolution of unisexual flowers. This particular order of transition is supported in the Poaceae (Malcomber & Kellogg 2006) and *Fraxinus* (Wallander 2001); however, the opposite order appears to occur in *Leucadendron* (Midgley 1987; Hattingh & Giliomee 1989), *Thalictrum* (Kaplan & Mulcahy 1971), and possibly in *Schiedea* (Weller et al. 1998). Therefore, establishing the relative frequency of the two polarities that lead to a correlation between dicliny and anemophily is critical to understanding the functional basis of the correlation.

An association between wind pollination and low ovule number was reported by Linder (1998) in his comparative analysis of the evolution of anemophily. Wind-pollinated flowers have been presumed to produce only single ovules because of the small chance of multiple pollen grains landing on each stigma (Pohl 1929; Dowding 1987). Unlike animal-pollinated plants where pollen grains arrive in clumps, the pollen of wind-dispersed species is usually transported as single units, so that the chance of capturing each pollen grain is an independent event. These arguments imply that a reduction in ovule number occurs after the evolution of wind pollination, a sequence supported by the results of Linder (1998).

A suite of morphological traits is commonly associated with wind pollination and constitute the anemophilous syndrome (Faegri & van der Pijl 1979). Many of the traits can be explained by functional arguments and the aerodynamic requirements for wind pollination. However, it is unclear whether these traits facilitate the evolution of wind pollination or evolve after the origin of wind pollination in particular lineages. In general, wind-pollinated plants have small flowers, with highly reduced or no perianth parts. Nectaries are usually
absent or non-functional (Faegri & van der Pijl 1979). The presence of complex, large showy flowers may act as a constraint to the evolution of wind pollination, while it appears that nectaries are typically lost after the evolution of wind pollination (Linder 1998). Plants that already have small, simple flowers may be more suitable for transporting and capturing some portion of their pollen by wind, because the stigmas and anthers are likely to be exposed to air currents (Culley et al. 2002). Plants with a mixed pollination strategy involving both animal and wind pollination (ambophily) tend to have small, unshowy flowers (e.g. *Piper* spp. – de Figueiredo & Sazima 2000; *Salix* spp. – Tamura & Kudo 2000; Karrenberg et al. 2002). Similarly, plants that use pollen as a reward (rather than nectar) may produce more pollen and be at a selective advantage if conditions change to favour wind pollination. However, it is not always clear whether wind pollination evolves more frequently in lineages that have small inconspicuous flowers and no nectar, or if these traits are lost after the evolution of wind pollination because of energetic reasons associated with a loss of function.

Here, I use a recent molecular phylogeny of the angiosperms (Soltis et al. 2000) to investigate evidence for correlations between anemophily and specific morphological and ecological traits, and to evaluate the evolutionary pathways leading to these associations. Specifically, I investigated whether associations occur between wind pollination and 1) biogeographical and ecological traits including temperate distributions and open habitat types; 2) sexual traits including unisexual flowers (dichlory) and plants (dioecy) and low ovule numbers; and 3) a reduction in floral characteristics including flower size and showiness. In cases where I detected associations, I then investigated the order of transition between the traits using tests of contingent-evolution to assess the evolutionary pathways involved. This information enabled me to evaluate several potential hypotheses that have been proposed to explain the evolution of wind pollination from animal pollination.
Methods

Character coding and phylogenetic data

I use the “B series” tree and branch lengths of the molecular phylogeny of angiosperms by Soltis et al. (2000). This tree is based on 567 taxa and three gene sequences (18s rDNA, \textit{rbc}L, and \textit{atp}B). For each species included in the phylogeny, I assigned states for the following ten characters: pollination mode (animal or wind), floral sexuality (hermaphroditic or unisexual flowers), sexual system (cosexual or dioecious populations), ovules (one or more than one), flower size [small (<1cm) or medium to large (>1cm)], flower showiness [showy or plain (green, white, yellow-green)], number of flowers per inflorescence [few (<5) or many (>5)], nectar (present or absent), habitat type (open or closed) and geographical distribution (temperate or tropical).

I obtained information on these character states from Kubitzki (1993, 1998a, 1998b, 2003, 2004), Mabberley (1997), Hutchinson (1964), the database generously provided by Jana Vamosi (used in Vamosi et al. 2003), a variety of online floras and through extensive literature surveys (list of all sources available from the first author). All states were coded as binary characters. The Soltis et al. (2000) study depicts the relationships of genera; although the original phylogeny was estimated using representative species for each genus. I used these species for character coding. When information was unavailable for a particular species, I used the most common state (>50%) for the members of the genus. I obtained information for all 560 angiosperm species in the phylogeny, of which 68 are wind-pollinated.
Testing for correlated evolution and directionality

To test for correlated evolution between wind pollination and alternative character states, I used BayesTraits (Pagel & Meade 2006). I implemented the BayesDiscrete module, which investigates correlated evolution between pairs of discrete binary traits. The program fits continuous-time Markov models to the discrete character data and allows the trait to change states over infinitesimally small intervals of time. The model estimates transition rates and the likelihood associated with different states at each node of the tree and calculates transition probabilities across all possible character states at each node, eliminating the need to assign ancestral states. I used the branch scaling parameter, κ, suggested by Pagel (1994), which adjusted the weight of branch lengths in the model, and allowed it to take its maximum-likelihood value. In all cases κ < 1, which reduces the length of longer branches more than shorter ones.

BayesDiscrete tests for correlated evolution in two binary traits by comparing the fit (log-likelihood) of two of these continuous-time Markov models. The first is a model in which two traits (e.g. wind pollination and nectar) evolve independently on the tree. This creates two rate coefficients per trait. The other model allows the traits to evolve in a correlated fashion, such that the rate of change in one trait depends upon the background state of the other. The dependent model has four states, one for each combination of the two binary traits (0,0; 0,1; 1,0; 1,1; see Figure 3.1). To determine whether wind pollination is correlated with a trait, I compared the likelihood estimate of the independent model (L(I)) to the likelihood estimate of the dependent model (L(D)). Because likelihood ratios approximate a chi-square distribution, support for correlated evolution is indicated when L(D) is significantly greater than L(I), which can be tested by comparing -2[L(D)-L(I)] to a chi-square distribution with 4 degrees of freedom.
Figure 3.1. Rate parameters for the eight possible transitions between pollination mode and the binary characters under a model of dependent evolution. The code for pollination mode and states for the ten characters are provided above. The values of “0” and “1” do not necessarily indicate hypotheses about ancestral conditions.
I determined the statistical significance of each of the estimated parameters in the dependent model by restricting individual transition parameters to zero and recalculating the likelihood ratio of the model. I then compared the restricted seven-parameter model to the unrestricted dependent model with a 1 d.f. $X^2$ test. A significant likelihood ratio indicates that the transition rate is significantly different from zero. Finally, I tested specific hypotheses about contingent evolution by restricting two of the rates to be equal (e.g. unisexual flowers evolve equally in animal- and wind-pollinated lineages: $q_{12} = q_{34}$). This seven-parameter restricted model can be compared to the full dependent model using the likelihood ratio test, $-2[\text{L(D)} - \text{L}(q_{12} = q_{34})]$ with a 1 d.f. $X^2$ test. A significant likelihood ratio indicates that the parameters are significantly different from one another, demonstrating that the state of trait X influences the direction of evolution of trait Y (Pagel 1994). Because I performed multiple tests, I adjusted the $\alpha$-level using Bonferonni correction.

Results

Traits correlated with wind pollination

I first examined whether traits were associated with pollination mode regardless of phylogenetic considerations. I found that all of the floral characteristics I investigated were highly correlated with pollination mode, while none of the ecological traits were correlated with pollination mode (Table 3.1). When considering phylogenetic relationships, the results of the maximum likelihood analyses and the likelihood ratio tests indicated that wind pollination evolves in a correlated fashion with habitat type, floral sexuality, sexual system, ovule number, flower size, flower showiness, and nectar presence or absence (Table 3.2). Wind pollination and geographical distribution and number of flowers per inflorescence evolve independently (Table 3.2). To investigate these associations further, and to determine
Table 3.1. Distribution of species among the pollination modes and states of the nine characters used in this study. Chi-square tests determine whether there is an association between pollination mode and the distribution of species among the two states of each trait.

<table>
<thead>
<tr>
<th>Trait</th>
<th>State</th>
<th>Animal</th>
<th>Wind</th>
<th>$\chi^2$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geographical distribution</td>
<td>Tropical</td>
<td>293</td>
<td>33</td>
<td>2.98</td>
</tr>
<tr>
<td></td>
<td>Temperate</td>
<td>199</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Habitat type</td>
<td>Closed</td>
<td>238</td>
<td>26</td>
<td>2.47</td>
</tr>
<tr>
<td></td>
<td>Open</td>
<td>254</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Floral sexuality</td>
<td>Hermaphrodite</td>
<td>394</td>
<td>18</td>
<td>88.31***</td>
</tr>
<tr>
<td></td>
<td>Unisexual</td>
<td>98</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Sexual system</td>
<td>Cosexual</td>
<td>440</td>
<td>38</td>
<td>53.80***</td>
</tr>
<tr>
<td></td>
<td>Dioecious</td>
<td>52</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Number of ovules</td>
<td>More than one</td>
<td>385</td>
<td>33</td>
<td>27.89***</td>
</tr>
<tr>
<td></td>
<td>One</td>
<td>107</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Number of flowers</td>
<td>Few</td>
<td>143</td>
<td>11</td>
<td>4.98*</td>
</tr>
<tr>
<td></td>
<td>Several to many</td>
<td>349</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Flower size</td>
<td>Medium to large</td>
<td>177</td>
<td>4</td>
<td>24.73***</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>315</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Flower showiness</td>
<td>Showy</td>
<td>325</td>
<td>11</td>
<td>61.94***</td>
</tr>
<tr>
<td></td>
<td>Plain</td>
<td>167</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Nectar</td>
<td>Present</td>
<td>398</td>
<td>7</td>
<td>148.76***</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>94</td>
<td>61</td>
<td></td>
</tr>
</tbody>
</table>

*P<0.05; **P<0.01; ***P<0.0001
Table 3.2. Likelihood-ratio values for tests of correlated evolution between pollination modes and ecological and morphological traits for 560 species using the BayesTraits program and the phylogeny of Soltis et al. (2000). Likelihood-ratio values for tests of four hypotheses, which are not mutually exclusive, about contingent evolution.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Likelihood ratio of dependent versus independent model</th>
<th>Trait (state 1) evolves more often in wind-pollinated clades</th>
<th>Wind pollination evolves more often in clades with trait (state 1)</th>
<th>Wind pollination lost more often in clades with opposite trait (state 0)</th>
<th>Opposite trait (state 0) evolves more often in animal-pollinated clades</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geographical distribution</td>
<td>6.92</td>
<td>1.06</td>
<td>2.43</td>
<td>0.10</td>
<td>2.34</td>
</tr>
<tr>
<td>Habitat</td>
<td>14.85**</td>
<td>0.53</td>
<td>2.50</td>
<td>7.37*</td>
<td>0.06</td>
</tr>
<tr>
<td>Floral sexuality</td>
<td>58.32***</td>
<td>0.27</td>
<td>13.63***</td>
<td>0.78</td>
<td>6.15</td>
</tr>
<tr>
<td>Sexual system</td>
<td>53.35***</td>
<td>0.62</td>
<td>19.30***</td>
<td>7.92*</td>
<td>9.62**</td>
</tr>
<tr>
<td>Ovules</td>
<td>21.43***</td>
<td>6.79*</td>
<td>0.13</td>
<td>1.50</td>
<td>1.47</td>
</tr>
<tr>
<td>Number of flowers</td>
<td>8.20</td>
<td>2.32</td>
<td>0.00</td>
<td>2.34</td>
<td>2.22</td>
</tr>
<tr>
<td>Flower size</td>
<td>34.83***</td>
<td>0.82</td>
<td>1.04</td>
<td>5.55</td>
<td>2.03</td>
</tr>
<tr>
<td>Flower showiness</td>
<td>55.63***</td>
<td>0.34</td>
<td>0.42</td>
<td>5.69</td>
<td>2.93</td>
</tr>
<tr>
<td>Nectar</td>
<td>132.14***</td>
<td>15.44***</td>
<td>6.09</td>
<td>10.31**</td>
<td>1.26</td>
</tr>
</tbody>
</table>

*P<0.05; **P<0.01; ***P<0.0001
the direction and order of transitions underlying correlated evolution, I tested specific hypotheses about each association.

**Ecological traits**

Results indicated that pollination mode and habitat type (closed habitats or open habitats) do not evolve independently. A model of correlated evolution fit the data better and the transition rates predicted that wind pollination and open habitats are positively associated (Table 3.2). Furthermore, wind pollination was more often lost in closed habitats (Table 3.2). I was unable to reject the hypothesis that geographical distribution (tropical or temperate) and pollination mode evolved independently.

**Floral traits**

Pollination mode and flower size (small or large) do not evolve independently. The transition rates I obtained predict that wind pollination and small flowers are positively correlated (Table 3.2). Although none of the contingent-changes tests were significant, the transition rates indicate that wind pollination evolves more often in small-flowered lineages, and once established is seldom lost. Similarly, flower showiness (showy or plain) and pollination vector do not evolve independently. The model of correlated evolution fit the data significantly better than the model of independent evolution and transition rates predicted that plain flowers and wind pollination are positively associated. In contrast, I cannot reject the hypothesis that pollination mode and flower number (few or many) evolve independently. Finally, nectar (presence or absence) and pollination mode evolve in a dependent way. Transition rates predict that an absence of nectar and wind pollination are positively correlated. The correlation arises because nectar is lost significantly more often in wind-pollinated clades (Table 3.2).
Sexual traits

Pollination mode and both floral sexuality and sexual system do not evolve independently. In both cases, a model of correlated evolution fit the data better (Table 3.2). Individual transition rates predicted that wind pollination and unisexual flowers are positively correlated. Furthermore, the contingent-change tests reveal that wind pollination evolves more often in clades with unisexual flowers (usually in species with either monoecious or dioecious sexual systems; Table 3.2). To assess if the presence of dioecious species is driving this association, I removed all dioecious species from the dataset and re-ran the analysis. Again, I found that the model of correlated evolution fit the data better and the transition rates predicted that wind pollination and monoecy are associated (data not shown). None of the contingent-changes tests were significant, possibly due to a lack of power, although the trend suggested that wind pollination evolves more often in monoecious lineages (data not shown). With respect to the correlation between dioecy and pollination mode, individual transition rates predicted that wind pollination and dioecy are positively associated. Furthermore, the contingent-change tests indicated that wind pollination evolves more often in dioecious clades (Table 3.2).

The analysis indicated that ovular condition (single ovule versus multiple ovules) and pollination mode evolved in a dependent way. The transition rates revealed that single ovules and wind pollination are positively correlated (Table 3.2). Furthermore, the contingent-change tests indicated that single ovules evolve from multiple ovules more often in wind-pollinated clades (Table 3.2).

Discussion

The results of the phylogenetic analysis indicate that wind pollination evolves in a correlated way with open habitats, unisexual flowers, dioecy, the uniovulate condition, small
plain flowers, and a lack of nectar. In contrast, wind pollination and geographical distribution and the number of flowers per inflorescence evolve independently. Although several of these associations have been examined previously (e.g. Regal 1982; Linder 1998; Vamosi et al. 2003) this study is the first to investigate correlations across the angiosperm phylogeny. Furthermore, in several cases I was able to detect contingent evolution and identify the most common background on which the evolution of a trait occurs. I now discuss potential adaptive explanations for the associations that the comparative analyses have revealed and comment on some of the limitations of using large-scale phylogenies for this type of analysis. Finally, I discuss unresolved questions and propose several future avenues of research that might be profitably pursued.

*Ecological traits*

I found no evidence for correlated evolution between geographical distribution and wind pollination. This result may at first appear to challenge many observations of the higher frequency of wind-pollinated species in temperate regions (e.g. Regal 1982; but see Bawa et al. 1985; Bullock 1994). However, here I was specifically interested in the correlated evolution of wind pollination, not in the frequency of wind pollination in contrasting geographical regions. For example, the high abundance of anemophily in temperate regions found by Regal (1982) may largely reflect the predominance of Poales (grasses, sedges, rushes, etc) and Fagales (beeches, oaks, etc) in many ecosystems. In the dataset presented here, each of these groups was represented by a single evolutionary transition. Although there is a greater abundance of wind-pollinated versus animal-pollinated plants in many temperate ecosystems, the analysis provides no evidence that ecological conditions in temperate regions preferentially select for the evolution of wind pollination from animal
pollination, or that wind-pollinated plants have migrated from tropical to temperate regions because of more favourable environments.

The aerodynamic requirements for wind pollination occur in habitats with open vegetation that allow for moderate wind speeds (Whitehead 1983; Niklas 1985; Dowding 1987). Although the finding of independent evolution between pollination mode and geographical region (temperate or tropical) may seem to contradict this, I did not limit the coding of tropical regions to tropical forest but included species from other tropical ecosystems including savannahs and grasslands. However, I specifically tested the effect of vegetation structure by looking for correlated evolution with open versus closed habitats because plant size and density are likely to influence the efficacy of pollen dispersal in wind-pollinated plants. I found that open habitats and wind pollination evolve in a dependent way and are positively associated. In addition, wind pollination was lost more often when it occurred in closed habitats.

These results suggest that there may be constraints on the origin of wind pollination in closed habitats and that wind-pollinated plants are more likely to persist in open habitats. Support for this idea is indicated by the frequency with which plants in closed forests in tropical regions use a mixture of wind and insect pollination (e.g. Piperaceae – de Figueiredo & Sazima 2000; Arecaceae – Uhl & Moore 1977; Listabarth 1993; Berry & Gorchov 2004). Additionally, although I did not test for the effect of altitude (due to insufficient data), increasing altitudinal gradients are often coupled with a decrease in vegetative cover. Several studies have shown that altitudinal gradients, which impact both the pollinator community and habitat type, can select for wind pollination (Berry & Calvo 1989; Gomez & Zamora 1996). Findings suggest that most wind-pollinated species are likely to be limited in distribution by the structure of the surrounding vegetation including the seasonal phase of
canopy cover.

**Floral traits**

Not unexpectedly, I found strong evidence that nectar and pollination mode evolve in a correlated manner. Results indicate that nectar is lost more often in wind-pollinated clades as one might expect on energetic grounds. However, this result also suggests that the presence of nectar in animal-pollinated species does not act as a constraint to the evolution of wind pollination. It has been proposed that wind pollination evolves more readily in nectarless lineages in which pollen is used as reward for pollinators (e.g. *Thalictrum* – Kaplan & Mulcahy 1971). Although this may be true in particular cases I found no evidence that nectar generally limits possibilities for transitions to anemophily. In contrast, although the pattern was less clear, it appears that large showy flowers may constrain the evolution of wind pollination. I found that wind pollination evolves more often in taxa with small unshowy flowers. Linder (1998) proposed that wind pollination evolved more often in animal-pollinated groups with poorly developed or undifferentiated perianths, such as the magnoliids, caryophyllids and rosids, and rarely in groups with zygomorphic flowers. Finally, I found no evidence for correlated evolution between the number of flowers per inflorescence and wind pollination. Weller et al. (2006) reported that the number of flowers, *per se*, in *Schiedea* was less important for wind pollination in comparison with inflorescence condensation, a composite measure of the number of flowers and the length of the inflorescence. Hence, inflorescence architecture probably plays a more important role in the evolution of wind-pollinated plants than the number of flowers produced within an inflorescence.

A strong association between wind pollination and reduced floral morphology is apparent throughout the literature. However, it is unclear whether this association arises as an
adaptation to wind pollination, or because large complex flowers constrain the evolution of wind pollination. Results suggest that large flowers act as a constraint to the evolution of wind pollination, perhaps because they limit the exposure of anthers and stigmas to the airstream. However, there is no *a priori* expectation for why colorful flowers should also constrain the evolution of wind pollination. Therefore, it is possible that these associations arise because wind pollination evolves more often in species that are pollinated by generalist insects, including flies and small pollen-collecting bees, which are often associated with plants that have small white or pale-colored unshowy flowers. This is supported by the observation that most ambophilous taxa are pollinated by generalist pollinators (e.g. *Salix* – Tamura & Kudo 2000; Peeters & Totland 1999, *Piper* – de Figueiredo & Sazima 2000, *Linanthus* – Goodwillie 1999, *Thalictrum* – Kaplan & Mulcahy 1971 and *Schiedea* – Weller et al. 1998, 2006). These patterns suggest that the evolution of wind pollination occurs in lineages with reduced floral morphology. Subsequent selection against attractive structures would then intensify the correlation with wind pollination.

*Sexual traits*

The finding that wind pollination is strongly correlated with dicyliny and dioecy is in accord with several previous studies (e.g. Bawa 1980; Charlesworth 1993, Renner & Ricklefs 1995; Vamosi et al. 2003). There is no comprehensive mechanistic explanation for the association between wind pollination and unisexual flowers, although some conceptual arguments are compelling (see Charlesworth 1993). In hermaphrodite animal-pollinated plants, female and male function usually share the costs of floral display and pollinator rewards (Lloyd 1982). Pollinators perform two services in one visit – both delivering pollen to the stigma and picking up pollen from anthers. However, in wind-pollinated plants the removal and capture of pollen are independent events and different structural requirements
are necessary for optimal pollen dispersal and pollen capture (Niklas 1985; Friedman & Harder 2004). Indeed, spatial interference between female and male structures in a flower may be directly disadvantageous and explain the high incidence of dichogamy and herkogamy in wind-pollinated species (Lloyd & Webb 1986; Webb & Lloyd 1986). Nonetheless, geitonogamous selfing may be inevitable for wind-pollinated plants, resulting in strong selection for dioecy as a mechanism of inbreeding avoidance. Benefits of sexual segregation such as flexibility for altering female and male investment and differential positioning of flowers for optimal pollen capture versus dispersal are likely to be important in the evolution of unisexual flowers. Mechanistic studies examining the benefits of unisexual flowers in wind-pollinated species are necessary for understanding the selective factors responsible for this frequent association.

An important finding of this study concerns the order of transitions involving dicliny and wind pollination. I found that wind pollination evolves more often after the establishment of dicliny in unrelated lineages. This pattern was evident for species with unisexual flowers and also for those that were purely dioecious. This is the first time this pattern has been identified in a large-scale comparative analysis and is important because case studies of particular taxa provide conflicting scenarios. For example, it has been reported that dicliny precedes wind pollination in *Leucadendron* (Proteaceae – Midgley 1987) and *Thalictrum* (Ranunculaceae – Kaplan & Mulcahy 1971) whereas the opposite order apparently occurs in the Poaceae (e.g. *Buchloe, Distichlis, Scleropogon, Spinifex* – Connor 1979 and references therein) and in *Fraxinus* (Oleaceae – Wallander 2001), where wind pollination has originated at least three times, with dioecy evolving from androdioecy after wind pollination on at least three occasions. There are likely to be different selective factors favoring unisexual flowers
depending on the ecological conditions, but the finding that wind pollination evolves more often in diclinous lineages suggests a common functional basis for this association.

Here I outline an evolutionary scenario for why wind pollination evolves more often in diclinous lineages. Dioecy is correlated with small, unshowy flowers (Vamosi et al. 2003) that are usually pollinated by generalist pollinators (Charlesworth 1993; Bawa 1994). The floral morphology of these plants may make the evolution of wind pollination an especially feasible option because pollen dispersal and capture are not impeded by large or complex perianths. If pollinators become scarce or ineffective, reducing fertility as a result of pollen limitation, diclinous species may evolve wind pollination to ensure more effective pollen dispersal between plants (reviewed in Culley et al. 2002). These same ecological conditions commonly promote the evolution of selfing as a mechanism of reproductive assurance (reviewed in Eckert et al. 2006). However, the presence of unisexual flowers would in most lineages preclude the evolution of selfing through autonomous self-pollination (although see Ågren & Schemske 1993). According to this hypothesis, insufficient pollinator service resulting in pollen limitation could elicit two quite different evolutionary transitions in pollination systems, depending on the floral condition of ancestral populations. In populations with hermaphroditic flowers autonomous self-pollination would relieve pollen limitation resulting in the evolution of selfing. In contrast, in populations with unisexual flowers wind pollination may serve the same role by increasing the proficiency of cross-pollen dispersal. Thus, similar ecological conditions and selective agents could result in two very different evolutionary outcomes due to contrasting ancestral traits.

Several studies provide support for a scenario in which wind pollination evolves in dioecious lineages as a response to pollinator scarcity. Weller et al. (1998) report that some diclinous species of *Schiedea* (Caryophyllaceae) suffer from pollinator limitation, with the
evolution of wind pollination a common outcome. In *Leucadendron* the genus is exclusively
dioecious and the vast majority of species animal-pollinated. However, there are at least four
independent transitions from animal pollination to wind pollination (Midgley 1987; Hattingh
& Giliomee 1989; Barker et al. 2004). *Leucadendron* is endemic to the fynbos shrublands of
the Cape Floristic region of South Africa where competition for pollinators may be intense
and pollen limitation of seed set is commonplace (Steiner 1988; Johnson & Bond 1997).

Whether transitions from animal pollination to wind pollination in *Leucadendron* are driven
by pollen limitation and the requirements of more effective cross-pollination is not known.
The finding that wind pollination evolves more often in lineages with unisexual flowers
suggests that wind pollination evolves to relieve pollinator limitation (but see Cox 1991 for
alternative explanations), particularly because a reversion to perfect flowers and autonomous
self-pollination would be highly unlikely.

A similar argument might suggest that wind pollination should evolve more
frequently in self-incompatible lineages following pollinator loss. However, this transition
may be rare because self-incompatibility can break down quite readily in some taxa (see Igic
et al. 2008), and certainly occurs more easily than transitions from dioecy to
hermaphroditism (Bull & Charnov 1985). In self-incompatible *Linanthus parviflorus*, the
evolution of wind pollination offers reproductive assurance against unreliable pollinators
(Goodwillie 1999). In other species of *Linanthus*, the breakdown of self-incompatibility and
the evolution of self-fertilization provide reproductive assurance (Goodwillie 2001). It would
be interesting to determine the ecological and life-history contexts in which these contrasting
outcomes occur. The transition to wind pollination in self-incompatible lineages has also
been reported in the genus *Espeletia* (Asteraceae – Berry & Calvo 1989). In species where
the evolution of selfing is prevented due to strong incompatibility and limited genetic
variation for self-compatibility there may be selection for wind pollination when pollinators are scarce.

I found strong evidence for a reduction in ovule number to one after the evolution of wind pollination. This suggests that the uniovulate condition is an adaptation to wind pollination. Further evidence for this transition is evident in the Poales where there have been repeated reductions from multiple ovules per carpel to solitary ovules (Linder 1998; Linder & Rudall 2005). The most common explanation for this association is that wind-pollinated plants are unlikely to capture sufficient pollen grains to fertilize many ovules. However, experimental studies involving the measurement of pollen loads of naturally pollinated taxa of Poaceae, Restionaceae, Rosaceae and Proteaceae (Honig et al. 1992; Linder & Midgley 1996; Friedman & Harder 2004) found significant amounts of pollen on stigmas that far exceeded ovule number (mean pollen grains per ovule range from 3 to 100). It is therefore unlikely that pollen limitation alone is responsible for selection of decreased ovule number in wind-pollinated species.

Several morphological and aerodynamic features of wind-pollinated plants may favour an optimal strategy of few ovules per flower. The relatively low cost of producing flowers in wind-pollinated plants may favour a packaging strategy with few ovules per flower and more flowers per plant. The model developed by Burd (1995) provides support for this idea by showing that higher floral costs generally favour more ovules per flower, although his model only considered animal-pollinated plants. Also, because wind pollination is a stochastic process where plants may capture pollen from a variety of potential mates, uniovulate carpels may be a mechanism to increase pollen-tube competition. Finally, by producing more flowers with fewer ovules, the spatial separation of flowers may increase the volume of air sampled by a plant and the probability of capturing pollen grains. A more
mechanistic understanding of the functional relation between wind pollination and ovule number would be informative.

Caveats and future research

There are a suite of other traits that may be correlated with wind pollination that would be interesting to investigate. For some of these, I attempted to include them in the analysis but was unable to compile sufficient data for all taxa. Most noticeably are those traits associated with pollen. Copious pollen production and smooth, dry, small pollen grains are commonly cited as attributes of anemophilous pollen (Faegri & van der Pijl 1979; Whitehead 1983; Proctor et al. 1996). Linder (2000) reported a correlation between pollen aperture type and wind pollination across angiosperm families, and he also proposed that circular apertures may be linked to speciation. Other attributes that could yield informative results include altitude, life form, and characteristics of stigmas.

Some of the traits associated with wind pollination may not be independent and could complicate some of the correlations I report. Analytical methods to investigate correlated evolution for more than two traits simultaneously in a phylogenetic context are unavailable. The effects of a third trait could best be examined by looking at correlated evolution within higher-level phylogenies that are variable in only the trait of interest. However, there are few clades that have the repeated evolution of wind pollination and adequate variation in ecological and morphological traits (but see Wallander 2001). In an effort to understand the associations between traits, I examined all pairwise inter-correlations between the traits in this study that evolve in a correlated way with wind pollination (21 different pairwise associations; results not shown). I found that many of the morphological traits evolve in a correlated way with one another but that habitat type did not evolve in a correlated way with any trait except the presence of nectar. Unfortunately, where I detected significant inter-
correlations between traits (e.g. the positive association between unisexual flowers and single ovules, \( P < 0.001 \)) I cannot determine if this association is driving correlations with wind pollination, or if the association with wind pollination is causing the inter-correlation. Until multivariate methods become available, it is impossible to tease apart the relative contributions of different associations to the overall positive correlation of a trait with wind pollination.

A potential bias in the methodology using transition rates may occur when the character states at the tips of the phylogeny are unequal and are poor indicators of the stationary frequencies. This issue has been recently addressed in the context of ecological specialization of insects (Nosil & Mooers 2005) where it was shown that there can be false detection of higher transition rates to the more common state. Currently implemented methods of character state reconstruction assume that the rate of character change is the same over the entire phylogeny, which is unlikely to be the case, particularly if the trait itself can influence speciation and/or extinction. However, because I found about equal occurrences of traits evolving before or after wind pollination (Table 3.2) this problem is unlikely to be a major factor influencing results.

A serious concern for comparative studies of reproductive transitions is the frequent use in the literature of floral characters to infer pollination mode. Here, I was looking for correlations between morphological traits and wind pollination. For some of the taxa included in the study, it is likely that their assignment as wind-pollinated species in the literature was based purely on morphological characters associated with the ‘anemophilous syndrome’ (Faegri & van der Pijl 1979). Clearly, it is then circular to test for correlations with wind pollination. Definitively determining pollination mode in some groups can be time consuming (e.g. large tropical trees). Pollinator exclusion experiments using bags are
commonly employed, but this method is inherently flawed as it changes the aerodynamic environment around inflorescences. Studies have shown that the structure of the stigma and/or inflorescence can have significant consequences for pollen flow (Niklas 1987; Niklas & Buchmann 1987; Linder & Midgley 1996; Friedman & Harder 2005) and so bagging experiments may inadvertently limit pollen dispersal by wind. Unfortunately, without detailed field observations and experiments it is difficult to know with certainty whether a plant is insect- or wind-pollinated, or both.

The study included only a few species per family and only one species per genus. As a result, many transitions occurring at higher taxonomic levels will be undetected in this analysis. For example, in the Poaceae, which are entirely wind-pollinated, unisexuality has evolved multiple times and evidence also suggests frequent reversions to hermaphroditism (Connor 1981; Malcomber & Kellogg 2006). However, neither of the species included in this study are dioecious (Oryza sativa and Zea mays) and so these transitions are not accounted for. Similarly, Dodd et al. (1999) report a single origin of wind pollination in the Fagales, a result that I also observed in this study. However, a phylogeny of the Fagaceae indicates that there may be multiple origins of wind pollination (Manos et al. 2001). Applying comparative approaches similar to those used here to well-resolved phylogenies of particular angiosperm clades should further distinguish traits that facilitate the evolution of wind pollination from those that are direct adaptations to wind pollination.

There are many fundamental questions about wind pollination that remain unresolved. For example, the comparative study by Dodd et al. (1999) showed that transitions between biotic and abiotic pollination are strongly asymmetric, so that shifts from biotic to abiotic pollination happen much more frequently and are also correlated with a net decrease in speciation rate. However, there are important exceptions to this, including the
Poaceae, which is highly species-rich with over 10000 species (Doust & Kellogg 2002) and
the Fagales which is a species-rich group of wind-pollinated trees but may in fact have
relatively low rates of diversification (Magallón & Sanderson 2001). My study indicates that
wind pollination and geographical distribution do not evolve in a correlated way, although
there may be much greater abundance of wind-pollinated plants in temperate regions (Regal
1982). It is possible that wind-pollinated lineages in temperate regions are more likely to
persist and undergo speciation – a hypothesis I was unable to test with this dataset.

The transition from animal pollination to wind pollination remains a central problem
in plant evolutionary biology. Using phylogenetic evidence, I demonstrated correlated
evolution between wind pollination and a range of traits, including open habitats, unisexual
flowers, dioecy, uniovules, small plain flowers, and a lack of nectar. For five of these traits
(open habitat, unisexual flowers, dioecy, uniovules, and lack of nectar) I found evidence of
contingent evolution, allowing me to make predictions about the mechanisms responsible for
the associations. The study raises novel predictions about the causes of correlations between
wind pollination and unisexual flowers and between wind pollination and a reduction in
ovule number. These ideas would benefit from further exploration. Studies that target
specific groups and test mechanistic hypotheses are essential for understanding the functional
basis for the evolution and maintenance of wind pollination.
CHAPTER FOUR
THE EVOLUTION OF OVULE NUMBER IN WIND-POLLINATED PLANTS

Summary
In flowering plants, the expenditure of maternal resources on reproduction occurs in three stages: the production of flowers, the development of ovules within ovaries, and the maturation of seeds and fruit. Because of the modular construction of plants, ovules and seeds are ‘packaged’ within individual flowers and an optimal strategy should occur depending on pollination and resource conditions. In contrast to the wide variation in ovule packaging strategies in animal-pollinated species, uniovulate flowers are the most frequent condition in anemophilous plants. This pattern is most often explained as an adaptive response to low pollen receipt. Here I develop a phenotypic model for ovule number per flower that incorporates the aerodynamics of pollen capture and a fixed resource pool for provisioning of flowers, ovules and seed development. The results challenge the prevailing explanation for uniovulate flowers in wind-pollinated plants. I demonstrate that when flowers are inexpensive, as they are in anemophilous species, ovule number should be minimized and lower than the average number of pollen tubes per style, even under stochastic pollination and fertilization regimes. The model predicts that plants benefit from producing many inexpensive flowers, even though some capture too few pollen grains to fertilize all their ovules. Wind-pollinated plants with numerous flowers each with few or a single ovule distributed throughout the inflorescence are able to sample more of the airstream and this should maximize seed set.
Introduction

Flowering plants are characterized by diverse reproductive structures indicating that the deployment of finite resources to reproduction can be optimized in many different ways. The success of a plant as a seed parent depends on partitioning resources among flowers for ovule production, ensuring that flowers receive an adequate supply of pollen, and maintaining resources for seed maturation (Lloyd 1980; Primack 1987). The number of ovules that are produced within a flower can reflect selection arising from any number of these influences (Burd 1995; Burd et al. 2009). A striking feature of ‘ovule packaging’ (the deployment and partitioning of resources to ovules within and between flowers of a plant) in angiosperms is the frequency of very low ovule numbers, including many species with uniovulate flowers, in wind-pollinated (anemophilous) species. Comparative phylogenetic evidence indicates that a reduction in ovule number usually occurs after the evolution of anemophily, suggesting that it is an adaptation to wind pollination (Linder 1998; Chapter Three). The association between anemophily and low ovule number contrasts with the six-orders of magnitude variation in angiosperms in general (Stebbins 1974; Greenway & Harder 2007) and suggests that optimal ovule packaging in wind-pollinated species may be highly constrained.

Various hypotheses have been proposed for the evolution of ovule packaging in angiosperms. The incidence of shared paternal parentage within a flower or plant (correlated mating) might affect ovule number. Kress (1981) argued that ovule number, pollen unit size and pollen dispersal evolve together to regulate sibling competition among developing embryos within a fruit. In contrast, in a study of the relation between size-number trade-offs in ovule and seed size, Greenway & Harder (2007) suggested that ovule size and packaging might reflect maternal control in parent-offspring conflict. Microgametophyte competition
for ovules might also select on variation in ovule number (Mulcahy 1975; Lee 1984). Finally, two hypotheses have been proposed to explain the over-production of ovules in some species. Excess ovules might be adaptive if they enable plants to take advantage of pollinator stochasticity, particularly the random deposition of particularly high pollen loads (Burd 1995; Burd et al. 2009). Alternatively, the over-production of ovules might allow maternal plants to screen embryos for viable offspring without compromising seed production (‘reproductive compensation’: Harder et al. 2008). These hypotheses to explain optimal ovule packaging have almost exclusively considered animal-pollinated plants.

The most frequently invoked hypothesis for the association between wind pollination and uniovulate flowers posits that there is small chance of stigmas of anemophilous species capturing multiple pollen grains (Pohl 1929; Dowding 1987). According to this view ovules would be wasted if they were concentrated within a small number of anemophilous flowers and this has resulted in the production of many flowers each with a single ovule. Unlike animal-pollinated plants, where pollen grains are commonly transported in clumps (Harder & Johnson 2008), pollen of wind-dispersed species is usually transported in the air stream as individual pollen grains, so that the chance of capturing each grain is an independent event. This explanation for the association between uniovulate flowers and anemophily rests on the assumption that stigmas of wind-pollinated plants capture few pollen grains.

Wind is generally viewed as a random and relatively inefficient mode of pollen dispersal in comparison with animal pollination (Wessells & Hopson 1988 p.560; Campbell et al. 1999 p. 734; Maseuth 2009 p. 205; but see Niklas 1985). As a consequence, it is widely believed that anemophilous species must produce copious pollen to assure successful pollination (Faegri & van der Pijl 1979; Whitehead 1983), and survey data of pollen-to-ovule ratios has been used to support this view. For example, Faegri & van der Pijl (1979) reported
pollen-to-ovule ratios for wind-pollinated species exceeding $10^6:1$ and Cruden (2000) reported a median pollen-ovule ratio of 22150:1 for hermaphroditic, wind-pollinated plants. These ratios are orders of magnitude higher than those for outcrossing animal-pollinated species, which have a median ratio of 3450:1 (Cruden 1977, 2000). However, it is important to note that these interpretations of high pollen-to-ovule ratios in anemophilous taxa were not accompanied by studies that measured pollen loads in anemophilous species.

Recent investigations of pollen loads in anemophilous species challenge the notion of inefficient pollination and demonstrate that stigmas of wind-pollinated plants often capture amounts of pollen that far exceed ovule number. For example, in Chapter Two I reported mean stigmatic pollen load of 34.1 (SE = 3.8; median = 7) grains for 19 wind-pollinated herbaceous species from seven families. In addition, experimental studies involving the measurement of pollen loads of naturally pollinated taxa of Poaceae, Protaceae, Ranundulaceae, Restionaceae, and Rosaceae (Honig et al. 1992; Linder & Midgley 1996; Davis 2004; Friedman & Harder 2004) reported quantities of pollen on stigmas that far exceeded ovule number (mean pollen grains per ovule range from 3 to 100). Thus the current empirical data are not consistent with the proposal that low pollen loads lead to decreased ovule number in wind-pollinated species.

The aerodynamics of wind pollination can provide insights into the functional advantage of uniovulate flowers for anemophilous taxa. In wind-pollinated plants, pollen capture is a function of the size of the female receptive surface and there are significant aerodynamic constraints on stigma size (Niklas 1987). Furthermore, stigmas (and ovules) that are spatially separated throughout the inflorescence of wind-pollinated species will sample a greater portion of the airstream than stigmas and ovules that are concentrated in a few flowers. For animal-pollinated plants, the optimal resource allocation may be to group
ovules within a flower and allocate to large shared attractive structures. However, for wind-pollinated plants the resource allocation to individual flowers is considerably less, due to the lack of nectar or showy floral parts, and so producing many uniovulate flowers may be more adaptive. Burd (1995) provides some indirect support for this idea by showing theoretically that low floral costs generally favor fewer ovules per flower, although his model only considered animal-pollinated plants.

Here I develop a phenotypic model for the evolution of ovule number in wind-pollinated plants. I begin by considering the deterministic case where pollen receipt is a function of stigma size and outline the aerodynamic properties of pollen capture by stigmas. Next, I incorporate a stochastic component to pollination and fertilization and use empirical data on stigmatic pollen loads in wind-pollinated species to parameterize the model. These analyses help to provide an explanation for the association between reduced ovule number and anemophily. Following the presentation of my results, I discuss some of the evolutionary consequences of a reduction in ovule number on seed size. I also consider how several recent hypotheses for ovule number in animal-pollinated plants, including stochastic pollination, the occurrence of correlated mating (shared paternity within fruits), and reproductive compensation, might influence the evolution of ovule number in wind-pollinated plants.

**Ovule-number model**

Maternal fitness in plants is generally measured through seed set, which is dependent on the receipt of pollen and fertilization of ovules. Thus selection should favour a strategy in which the maximum number of ovules are fertilized and develop into seed, given resource constraints. Here I develop a phenotypic model that considers a situation of hierarchical allocation in which resources are subdivided first to flower production and then to ovules and seed provisioning (see Lloyd 1980; Venable 1996; Worley et al. 2003). Thus, available
resources and flower cost determine flower production, which is not directly influenced by ovule costs. I also include in the model the cost of maturing a fertilized embryo into a seed, as this places significant resource demands on maternal function and is therefore likely to constrain optimal ovule production. I incorporate several features that are specific to wind pollination; in particular the fact that pollen capture will be, in part, a function of stigma size (Niklas 1987). I also parameterize the model to reflect low allocation to individual flowers, consistent with observations from wind-pollinated species.

Pollen capture

In wind-pollinated plants, pollen capture will be a function of the size of the female receptive surface (Niklas 1987). A series of physical models developed by Paw U & Hotton (1989) indicate that the total pollen deposited on stigmas is a function of the area of the stigma (per unit downwind length), the concentration of pollen in the airstream and the efficiency of impaction. The efficiency of impaction is defined as the ratio of pollen grains captured to those which would have passed through the area if the stigma had not been present (Davies & Peetz 1956; Gregory 1973). Streamlines of airflow near a stigma will be deflected around it in proportion to the boundary layer produced by the stigma, and large stigmas produce proportionally larger boundary layers. Whether a pollen grain impacts a stigma will depend on the wind speed, the size of the stigma, and the size of the pollen grain. Heavy, large pollen grains will be captured more often, while small light grains will be carried by the airflow around the stigma. In this model, I make the simplifying assumption that all pollen grains are of uniform size, so that the efficiency of capture will depend only on stigma size.
The relationship between capture efficiency \( E \) and receptor size \( D \) is usually expressed in terms of the Stokes number (see Table 4.1 for a list of all parameters and variables):

\[
S = \frac{V_{\text{set}} U}{D g}
\]  

(4.1)

where \( V_{\text{set}} \) is the settling velocity of a pollen grain (m sec\(^{-1}\)), \( U \) is mean wind speed (m/s), \( D \) is the diameter of the receptor (m), and \( g \) is gravitational acceleration (9.8 m sec\(^{-2}\)). The explicit relationship between \( E \) and \( S \) is derived empirically (Gregory 1973) or from numerical integration (Davies & Peetz 1956), and typically takes the form of an S-shaped curve. Paw U & Hotton’s (1989) equation for the total number of pollen grains deposited,

\[
P = ECD
\]  

(4.2)

where \( C \) is the concentration of pollen in the airstream, and \( D \) is the effective female receptive area (a function of stigma size), can be shown to have a saturating relation through the range of stigma sizes likely to occur, with pollen capture actually decreasing at very large stigma sizes due to their larger boundary layers.

To describe the number of pollen grains captured as a function of stigma size I use a hyperbolic function that approximates the shape described above. In particular I define pollen capture as:

\[
P = k \frac{c_f}{\beta + c_f}
\]  

(4.3)

where \( k \) is a scaling parameter that reflects the concentration of pollen in the airstream, \( \beta \) is a parameter that affects the degree of saturation, and \( c_f \) is the size of a stigma measured as its cost. I do not consider the decreasing part of the curve, as it is unrealistic that stigmas
Table 4.1. Definitions of parameters and variables

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B$</td>
<td>number of flowers produced by a plant</td>
</tr>
<tr>
<td>$\beta$</td>
<td>half-saturation constant for pollen capture function</td>
</tr>
<tr>
<td>$C$</td>
<td>concentration of pollen in the airstream</td>
</tr>
<tr>
<td>$c_f$</td>
<td>resource cost of producing one flower (excluding ovules)</td>
</tr>
<tr>
<td>$c_o$</td>
<td>resource cost of producing one ovule</td>
</tr>
<tr>
<td>$c_p$</td>
<td>resource cost of developing one fertilized ovule into a seed</td>
</tr>
<tr>
<td>$D$</td>
<td>diameter of the stigma</td>
</tr>
<tr>
<td>$E$</td>
<td>capture efficiency</td>
</tr>
<tr>
<td>$F$</td>
<td>proportion of ovules that are fertilized</td>
</tr>
<tr>
<td>$g$</td>
<td>gravitational acceleration</td>
</tr>
<tr>
<td>$g(\alpha, \sigma)$</td>
<td>gamma distribution with mean $\alpha \sigma = P$</td>
</tr>
<tr>
<td>$G(P,n)$</td>
<td>cumulative probability function of $g(\alpha, \sigma)$ evaluated at $n$ ovules</td>
</tr>
<tr>
<td>$k$</td>
<td>asymptote reflecting pollen concentration</td>
</tr>
<tr>
<td>$1/\lambda$</td>
<td>proportion of resources spent on flowers</td>
</tr>
<tr>
<td>$n$</td>
<td>number of ovules per flower</td>
</tr>
<tr>
<td>$n^*$</td>
<td>number of ovules per flower at the transition between pollen and resource limitation</td>
</tr>
<tr>
<td>$R$</td>
<td>total resources available for flower, ovule and seed production</td>
</tr>
<tr>
<td>$P$</td>
<td>number of pollen grains received by a stigma</td>
</tr>
<tr>
<td>$\pi$</td>
<td>seed production when resources are not limiting</td>
</tr>
<tr>
<td>$\rho$</td>
<td>seed production when resources are limiting</td>
</tr>
<tr>
<td>$S$</td>
<td>Stokes number</td>
</tr>
<tr>
<td>$W$</td>
<td>expected (mean) maternal fitness</td>
</tr>
<tr>
<td>$V_{set}$</td>
<td>settling velocity of a pollen grain</td>
</tr>
</tbody>
</table>
would evolve to be so large, as the plant would only incur additional costs without receiving further benefits.

**Female fitness through seed set**

A plant’s fitness through seed set will depend on the total number of flowers produced, the number of ovules in each flower and their probability of fertilization.

\[ W = BnF \]  

(4.4)

The number of flowers \((B)\) depends on resource availability and the cost of flowers, and is defined as \(B = R / \lambda c_f\), so that plants never spend more than a proportion, \(1/\lambda\), of their resources on producing flowers. Thus, optimizing ovule production does not simultaneously determine flower production. Instead, a plant produces flowers in proportion to their cost, and then ovule number is optimized given the relative costs of flower production, ovule production and seed provisioning. Because pollen capture is a saturating function of flower size (see equation 4.3) there will be a trade-off in which producing larger stigmas that capture more pollen results in the production of fewer flowers. I expect that at a threshold stigma size it will be optimal for a plant to allocate resources to additional flowers, rather than investing in larger flowers.

**Resource constraints**

Plants must allocate fixed amounts of resources to flowers, ovules and fruits in order to develop seeds (Primack 1987). To reflect these resource constraints on seed set, I use an approach that incorporates elements first outlined in a graphical model by Haig & Westoby (1988), and those by Harder et al. (2008). If a plant experiences high pollen capture and fertilization, and has too few resources to mature all of its fertilized ovules, then final seed production will be depressed relative to the number of fertilized ovules (‘resource
limitation’). Alternatively, if a plant receives less pollen than the number of ovules produced, then all fertilized ovules are matured but there are resources remaining (‘pollen limitation’). A third form of limitation, ‘ovule limitation’ (Harder et al. 2008), occurs when the number of pollen tubes exceeds the number of ovules, and is mathematically equivalent to pollen limitation in this model, because neither condition uses all the resources available for reproduction.

The analysis of ovule and pollen limited seed set is straightforward. In this situation all fertilized ovules develop into seed so that seed set is:

$$\pi = BnF. \quad (4.5)$$

With resource limited seed set, the number of fertilized ovules that develop into seed will be determined by how resources have been partitioned and the availability of resources to provision seeds, so that seed set is:

$$\rho = \frac{R - B(c_f + nc_o)}{c_p}, \quad (4.6)$$

where $R$ is the total amount of resources available, $c_o$ is the cost of an ovule, and $c_p$ is the additional cost of maturing a fertilized ovule. As outlined above, pollen limitation occurs when $\pi > \rho$; and resource limitation occurs when $\pi < \rho$. Both pollen and resource limitation represent sub-optimal alternatives to perfectly matching the requirements of floral production, ovule production and seed provisioning to the resources available. Thus optimal ovule production occurs at the transition between resource and pollen limitation, where $\pi = \rho$, which can be shown to occur when:

$$n^* = \frac{(\lambda - 1)c_f}{c_o + c_pF} \quad (4.7)$$
This solution cannot be solved explicitly for an optimal ovule number, because $F$, the probability of fertilization, is itself a function of $n$, the number of ovules. However, I explored the consequences of different parameter values for optimal ovule production through numerical integration.

The cost of producing an ovule ($c_o$) is a very small fraction of the cost of maturing an embryo, so that the denominator of equation 4.8 is largely driven by $c_p$. Thus, irrespective of the pollination environment and the probability of fertilization, if the cost of maturing a seed were reduced, it would be better to make more ovules. Because this is a relatively uninteresting result, I chose to fix the cost of seed provisioning and the cost of ovules and instead explore the consequences of changing flower cost on ovule packaging strategies. This is because flower cost affects the quantity of pollen captured and also the number of flowers produced. Throughout the subsequent analyses, I set the following parameters $\beta = 0.3; k = 20; c_o = 0.01; c_p = 0.1; R = 20; \lambda = 2$ because they represent biologically relevant values.

Pollen capture data to parameterize the model

From May-August 2004 and 2005, I sampled 13 species at the Koffler Scientific Reserve at Jokers Hill, Ontario (44° 03’N, 79° 29’W) to assess stigmatic pollen loads. I collected data from: Ambrosia artemisiifolia (Asteraceae), Chenopodium album (Chenopodiaceae), Carex arctata, C. hirtifolia, C. pedunculata, C. pensylvanica, C. plantagineae, C. laxiflora, C. scabrata (Cyperaceae), Scirpus microcarpus (Cyperaceae), Plantago lanceolata (Plantaginaceae), Rumex acetosella, R. crispus (Polygonaceae), and Thalictrum dioicum (Ranunculaceae). During June-August 2001 and 2002 I collected data from 5 species near the Barrier Lake Field Station of the Kananaskis Field Stations, Alberta (51° 02’N, 115° 03’W), including: Anthoxanthum nitens, Bromus inermis, Elymus repens,
Festuca campestris, Leymus innovatus and Phleum pratense (all Poaceae). All 19 species in the sample were herbs and each possessed uniovulate flowers.

For each species, I selected 20-30 plants from populations and collected stigmas from at least 6 flowers per plant. Stigmas were stored in separate micro-centrifuge tubes containing 1.5 mL of 70% ethanol. To assess pollen capture, I stained stigmas with 1% basic fuschin and counted pollen loads under a compound microscope (20x).

Results

Ovule number under deterministic pollination and fertilization

I begin with the simplest scenario, in which the probability of fertilization is the ratio of the number of pollen grains to the number of ovules, \( F = \frac{P}{n} \). It is probably unrealistic to assume that every pollen grain that is captured fertilizes an ovule, so I will later add stochasticity to the fertilization function. When pollination and fertilization are deterministic, as in equation 4.3, then the optimal ovule production occurs at the transition from pollen to resource limitation (equation 4.7). Fitness increases linearly with ovule production until the transition from pollen to resource limitation, then it decreases linearly, resulting in a sharp peak at the resource-limitation boundary. Figure 4.1a illustrates the consequences of different flower costs on optimal ovule number under resource and pollen limitation. When flowers are relatively cheap, optimal ovule production per flower should be very low (\( c_f=0.1; n=0.9 \)), and as flowers become more expensive, ovule number per ovary should be higher (\( c_f=0.5; n=4.5; c_f=1; n=9.1 \)). The reason for this is twofold. First, plants capture more pollen grains when they have larger stigmas, so that there is the potential for more ovules to be fertilized. And second, the relative cost of an ovule is considerably less than the cost of a flower, so plants pay little penalty for producing more ovules within each flower. Under this
Figure 4.1. Relation of female fitness to ovule number for three floral costs. Solid lines represent $c_f=0.1$, long-dash represents $c_f=0.5$, and short-dash represents $c_f=1$. Panel a) indicates deterministic pollination based on eq. 3 and 7. Panel b) depicts stochastic pollination according to a gamma distribution with the mean determined by eq. 3. Panel c) shows stochastic fertilization according to a distribution based on empirical values (see Figure 4.2). Fitness for stochastic pollination is calculated according to eq. 8.
deterministic situation, total seed production (fitness) for individuals of the three flower costs are the same.

*Ovule number under stochastic pollination and deterministic fertilization*

I now consider a situation where pollen receipt is stochastic with values drawn from a gamma distribution with the mean equal to equation 4.3. This is a skewed distribution with high variance. Fitness is calculated similarly as above, either equaling $\pi$ or $\rho$; however, the probability of fertilization is now:

$$F = (1 - G(P,n))$$

(4.8)

where $G(P,n)$ is the cumulative probability function of the gamma distribution evaluated at $n$ ovules, so that $F$ represents the probability of capturing sufficient pollen to fertilize $n$ ovules. Figure 4.1b illustrates the results of this model and indicates that stochasticity in pollen receipt is associated with selection for greater ovule number per flower, particularly with higher flower costs ($c_f=0.1: n=1.5; c_f=0.5: n=7.3; c_f=1: n=13.5$). This occurs because plants are pollen-limited (rather than resource-limited) over a greater range of ovule numbers per flower, so that they pay little penalty for producing extra ovules. Unlike the deterministic case above, the fitness of individuals with the three flower costs are not identical, because the fewer flowers produced by plants with costly flowers is not necessarily matched by increased pollen receipt.

*Ovule number using empirical pollen loads and deterministic fertilization*

The observed distribution of stigmatic pollen loads for the 19 anemophilous species is illustrated in Figure 4.2. The distribution is truncated at a maximum of 50 grains per stigma. The grand mean of the distribution was 34.1 (SE=3.8, median=7) pollen grains per stigma and for the truncated distribution 11.3 (SE=0.2, median=6).
Rather than pollen capture being related to flower costs according to aerodynamic expectations, I now consider the case where the probability of a given pollen load is determined by the observed distribution of stigmatic pollen loads (Figure 4.2). I use a similar fertilization function as equation 4.8 above, but rather than drawing from a gamma distribution, I draw from the distribution illustrated in Figure 4.2. The results of this model are shown in Figure 4.1c ($c_f=0.1: n=1; c_f=0.5: n=10; c_f=1: n=10$). Fitness is substantially reduced for plants with greater flower costs, because the number of flowers decreases without the benefit of increased stigmatic pollen load. This result demonstrates the benefits of producing numerous flowers that each sample the airstream with equivalent probabilities of pollen capture. In this situation, optimum ovule number is the same for plants with $c_f=0.5$ and $c_f=1$ because pollen capture is independent of $c_f$ and neither are resource limited.

**Ovule number under stochastic fertilization**

I now consider the case in which not every pollen grain successfully fertilizes an ovule. Instead I define the probability of fertilization according to a Poisson process, which accounts for the observation that plants typically require more pollen grains than ovules to achieve full fertilization, and also incorporates an assumption that pollen tubes distribute randomly among ovules. Similar to Aizen & Harder (2007), if $P$ is the total number of pollen grains captured by a stigma, then

$$F = 1 - e^{(-P/n)}$$

(4.9)

is the probability of an ovule being fertilized. I first consider the case where pollen receipt is deterministic (equation 4.3). Compared to the case of deterministic pollination and fertilization (Figure 4.1a), optimum ovule number is shifted upwards for all flower costs.
Figure 4.2. Distribution of stigmatic pollen loads for 19 wind-pollinated herbaceous species with uniovulate flowers at Koffler Scientific Reserve, ON and Kananaskis Field Station, AB sampled in May-August 2004, 2005 and June-July 2001, 2002. See text for a list of individual species. See Appendix 1 for each species distribution.
(Figure 4.3a). This is because there is always a finite possibility that an ovule will remain unfertilized. The degree to which this shift occurs depends on flower cost, because the probability of fertilization now increases curvilinearly with pollen receipt (and thus flower cost) according to equation 4.9. In all cases optimum ovule number is lower than the number of pollen grains captured ($c_f=0.1: n=0.95$, pollen receipt=5 grains; $c_f=0.5: n=4.9$, pollen receipt=12.5 grains; $c_f=1: n=12.3$, pollen receipt=15 grains). Finally, I combine the cases of stochastic pollination and stochastic fertilization (Figure 4.3b). When both processes are stochastic, fitness is reduced, as plants are more often pollen and fertilization limited. Fitness is highest for plants with cheap flowers because they offset stochasticity in pollen receipt by producing more flowers. Optimum ovule number shifts upwards, but for the two lower flower costs it remains lower than the average number of pollen grains captured by a flower ($c_f=0.1: n=2; c_f=0.5: n=11.3; c_f=1: n=16$).

**Discussion**

The model developed here demonstrates that a reduction in ovule number per flower is advantageous for plants that invest limited resources in flowers, as occurs in wind-pollinated species. When pollen receipt is a function of flower size the benefits of producing larger flowers that capture more pollen are matched by the costs of producing fewer flowers (Figure 4.1a). However, when pollination and/or fertilization are stochastic, these benefits are diminished and fitness is reduced in comparison with plants that produce many inexpensive flowers (Figure 4.1b, 4.3a,b). Both stochastic pollination and fertilization select for greater ovule number compared to the deterministic case. This is because plants pay less of a penalty in terms of resource limitation if they produce more ovules. The model indicates that optimal ovule number is lower than average pollen receipt, except for the largest flowers under both
Figure 4.3. Relation of female fitness to ovule number for three floral costs when fertilization is a stochastic process. Solid lines represent $c_f=0.1$, long-dash represents $c_f=0.5$, and short-dash represents $c_f=1$. Panel a) indicates deterministic pollination and stochastic fertilization based on eq. 3, 7 and 9. Panel b) shows stochastic pollination and stochastic fertilization, where pollen receipt varies according to a gamma distribution with the mean determined by eq. 3.
stochastic pollination and stochastic fertilization.

These results have several general implications for the allocation strategies of wind-pollinated plants. First, they suggest that plants that produce many, inexpensive flowers might accrue greater benefits than those with costly flowers, even if they capture less total pollen. Second, when pollination and/or fertilization are stochastic, plants that make many inexpensive flowers, with few ovules in each, suffer reduced fitness consequences. Finally, stochasticity selects non-linearly for greater ovule number so that plants with more costly flowers realize greater benefits from increased ovule provisioning. I now discuss the implications of these results for several features of the allocation strategies of wind-pollinated species, including optimal ovule number per flower, seed provisioning and reproductive compensation. I also consider how several other features of wind-pollinated plants, including the incidence of correlated mating and pollen limitation, might influence the evolution of ovule number.

*Ovule size, number, and seed provisioning*

I have focused on how the pollination process in wind-pollinated plants may influence the evolution of ovule number per flower. However, it is also possible that ovule number variation could be driven by selection on seed number per flower. It has been well established that there is a size-number trade-off between seed number per flower and seed size, so that species with many seeds commonly produce smaller seeds and vice versa (Harper et al. 1970; Willson 1983; Westoby et al. 1992). In species with many ovules per flower, the potential to abort developing embryos during seed development may mean that final seed number is only partially determined by ovule number. However, in the uniovulate flowers that characterize many wind-pollinated plants, seed set per flower will always be either one or zero. Thus, it is possible that selection for relatively larger seeds is driving the
evolution of ovule number. However, I can think of no adaptive explanation why wind-pollinated species, given their enormous range of life histories and the diversity of environments that they occupy, should collectively require larger seeds than plants with other pollination systems. Indeed, none of the reviews on seed size have suggested that pollination mode may be an attribute that is correlated with substantial variation in seed size (e.g. Harper et al. 1970; Baker 1972; Westoby et al. 1996; Leishman et al. 2000), other than indirectly through its effect on flower size (Primack 1987). Thus I am inclined to favour an explanation that the evolution of ovule number in wind-pollinated plants is largely associated with factors related to pollination and mating rather than because of indirect selection on seed size.

The direct costs of seed production are paid through investments in ovule number and seed development. Although considerable information is available on seed development, very limited data exists on the size and costs of ovule production. Given the uncertainty that an ovule will be fertilized, investment in ovules might be limited so that most investment is expended after fertilization when embryos develop into seeds (Lloyd 1980; Westoby & Rice 1982). Some support for this hypothesis is demonstrated by the 93-fold conversion ratio of ovule volume into seed volume that occurs after fertilization (Greenway & Harder 2007). However, plants do not appear to produce the smallest possible ovules because at the interspecific level there is a positive association of ovule volume with flower mass and a negative association of ovule size and number. Also within species there is a threefold variation in ovule volume among individuals (Greenway & Harder 2007). Thus, despite the expectation that selection should minimize resource investment into ovules there is evidence for substantial variation within and among species in ovule size.

Maternal parents may allocate more resources to the sporophytic tissue of ovules allowing greater control over resource investment in developing seeds (Westoby & Rice
1982). In contrast to the general size-number trade-off hypothesis, Greenway & Harder (2007) found that species with uniovulate flowers have the smallest ovules, although the species in their dataset did not include any that were wind-pollinated. If resource allocation among ovules occurs primarily within rather than between flowers, then species with one ovule per ovary have the least opportunity for parent-offspring conflict because optimal resource allocation would be similar for both the maternal plant and the developing seed. Thus uniovulate ovaries may eliminate the requirement of producing large ovules to allow maternal dominance in parent-offspring conflict (Greenway & Harder 2007). Wind-pollinated plants that have inexpensive flowers each with a single ovule might accrue additional gains by making cheaper ovules.

*Low incidence of correlated mating*

Wind-pollinated plants may have a low incidence of shared paternal parentage (correlated mating, Ritland 1988) at the plant level (when individuals are uniovulate) and the fruit level (when individuals have more than one ovule). Unlike animal-pollinated flowers where pollinators often deliver clumps of related grains (e.g. Harder & Johnson 2008), pollen grains in wind-pollinated species are sampled randomly from the airstream and arrive independently of one another. As a result the occurrence of multiple paternal parentage in anemophilous species should be relatively high assuming that maternal parents occur in neighbourhoods with many potential paternal parents. It has been proposed that high levels of competition among unrelated embryos for maternal resources could have selected for the uniovulate condition in wind-pollinated plants (Kress 1981). The model developed here does not include the potential effects of paternal parentage and competition for maternal resources on seed provisioning or seed viability; however, it is worth considering how these might influence the evolution of ovule number.
Opportunities for microgametophytic competition within a flower may be enhanced as a consequence of pollen receipt involving unrelated pollen grains. In general, when pollen arrival schedules are gradual rather than synchronous the first pollen grains to be deposited on a stigma are more likely be successful at fertilization (pollen precedence, Epperson & Clegg 1987), regardless of their competitive ability (Thomson 1989). However, anthesis in wind-pollinated plants tends to be highly synchronous within populations (Dowding 1987); for example, in grasses pollen dispersal and stigma receptivity can be completed in less than five hours (Beddows 1931). In this case, pollen grains may arrive during a brief time period and competition to fertilize ovules may be quite severe. Intense post-pollination competition can lead to gametophytic selection and this has the potential to result in greater progeny vigour (Mulcahy & Mulcahy 1987). Studies that have attempted to demonstrate a relation between pollination intensity (pollen load size) and progeny vigour have been equivocal (e.g. Snow 1990; Quesada et al. 1993; Davis 2004; Marshall et al. 2007). Nonetheless, this influence may be of particular importance for wind-pollinated plants if they receive pollen from many potential paternal parents of varying quality.

The rapid germination of pollen grains upon arrival on the stigma would be advantageous if they are competing intensely for fertilization in uniovulate flowers. Several large wind-pollinated families have trinucleate pollen including Poaceae and Restionaceae, as does the uniovulate family Asteraceae (Brewbaker 1967). Trinucleate pollen, containing twin sperm cells and a vegetative cell, is a derived condition in the angiosperms (Brewbaker 1967). The pollen grains contain fully developed mitochondria at the time of dehiscence allowing for rapid germination on stigmas, but very brief viability (Hoekstra 1979). A more detailed examination of the distribution of binucleate and trinucleate pollen with respect to ovule number and pollination mode may reveal interesting patterns and could provide novel
insights on the consequences of the uniovulate condition on pollination and mating in wind-pollinated plants.

Pollen quantity and quality and the evolution of ovule number

Model results demonstrate that stochastic pollination favours greater ovule production, especially in plants with high flower costs (e.g. compare Figure 4.1a to 4.1b). This result has two implications for anemophilous species. First, the high incidence of uniovulate flowers in wind-pollinated plants suggests that stochastic pollination may play only a minor role on selection for optimal ovule number. Second, if flower costs are low then plants should benefit from producing numerous inexpensive flowers, even if some capture too few pollen grains to fertilize their ovules (Figure 4.2a, b). Indeed, not surprisingly wind-pollinated plants tend to have much smaller and inexpensive flowers than animal-pollinated plants (Chapter Three), presumably due to the aerodynamic constraints on pollen dispersal associated with larger flowers. However, it is not clear whether the pollination process is more or less variable for wind-pollinated plants in comparison with those that are animal pollinated.

It has been proposed that stochastic variation in pollen receipt selects for increased ovule number thus allowing plants to take advantage of unpredictably good pollination (Burd 1995, 2008; Burd et al. 2009). In a survey of 40 animal-pollinated species, Burd et al. (2009) found that ovule number was positively associated with the statistical dispersion of pollen receipt (reflecting stochastic variation). Significantly, a comparison of the coefficient of variation reported in their study ($CV = 441$; calculated from Table 1 in Burd et al. 2009) with that from pollen loads for 19 wind-pollinated species ($CV = 103$) indicates a striking difference suggesting that pollen receipt may be less variable for wind-pollinated plants. Pollination in anemophilous species may be more predictable because of the more
synchronous anthesis period and the possibility that all stigmas on a plant will have approximately the same probability of pollen capture. On the other hand, wind pollination is at the mercy of uncertain atmospheric conditions and these could increase the stochasticity of pollen dispersal.

Pollen limitation appears to be a common feature of animal-pollinated populations (Burd 1994; Larson & Barrett 2000; Ashman et al. 2004; Knight et al. 2005) and occurs when there are insufficient pollinators or a scarcity of mates. Of course only the latter cause of pollen limitation is relevant for wind-pollinated plants, which might suggest that they are less often pollen limited, particularly when population densities are adequate. Several studies have demonstrated pollen limitation in wind-pollinated trees in fragmented habitats (Fox 1992; Knapp et al. 2001; Sork et al. 2002; Koenig & Ashley 2003). However, many wind-pollinated plants occur in dense populations and studies in relatively undisturbed habitats that have compared seed set in open-pollinated versus supplemental pollinated flowers of anemophilous species have failed to demonstrate pollen limitation (Davis et al. 2004a; Chapter Two).

Studies of pollen limitation have typically considered only the effects of pollen quantity, but poor-quality pollen (e.g. self pollen) can also reduce seed production (Aizen & Harder 2007). The production of surplus ovules can diminish costs associated with poor pollen quality. Theoretical studies suggest that plants may benefit from producing extra ovules within flowers to compensate for embryo losses due to genetic culling as a result of fertilization by poor quality pollen (Harder & Routley 2006; Harder et al. 2008). However, the strong association between uniovulate flowers and anemophily suggests that this form of reproductive compensation may not be an important process in wind-pollinated species. Alternatively, it raises the possibility that wind-pollinated plants may be using whole flowers
as the unit to overcompensate. But the potential for selfed and outcrossed embryos to
compete for maternal resources is likely to be diminished when they occur in different
flowers, because architectural effects will influence the availability of resources to flowers in
different parts of the inflorescence (Diggle 2003). The absence of reproductive compensation
within anemophilous flowers and the reduced ability of selfed and outcrossed zygotes in
different flowers to compete for resources may contribute to the apparently low incidence of
mixed mating in wind-pollinated species in comparison with animal-pollinated species
(Goodwillie et al. 2005).

In conclusion, this investigation suggests that the uniovulate condition that
characterizes most wind-pollinated species most probably reflects the very small costs of
individual flowers rather than low pollen capture, as most workers had previously assumed.
The model I developed here demonstrates that making numerous, inexpensive flowers
favours a strategy of fewer ovules in each, even when flower production does not trade-off
directly with ovule number. This suggests that the observed reduction of ovule number once
wind-pollination has established in angiosperm lineages (Chapter Three) results from
selection on resource partitioning when flower cost is minimized for aerodynamic reasons.
When pollination is stochastic, plants with fewer costly flowers suffer greater fitness
consequences than plants with many inexpensive flowers, even though each small flower
may capture too few pollen grains to fertilize all of its ovules. Plants that distribute flowers
and ovules throughout the inflorescence sample more of the airstream and this ovule
packaging strategy maximizes fitness.
CHAPTER FIVE

THE CONSEQUENCES OF MONOECY AND PROTOGYNY FOR MATING IN WIND-POLLINATED CAREX

This chapter resulted from collaborations with Spencer C. H. Barrett. Spencer Barrett contributed to the ideas and writing of the manuscript published in New Phytologist, 2009, 181: 489-497.

Summary

Monoecy and protogyny are widespread in wind-pollinated plants and have been interpreted as outcrossing mechanisms, though few studies have investigated their function. Carex, a large genus of anemophilous herbs, is predominantly monoecious and many species are protogynous. I investigated whether monoecy and protogyny limit self-pollination in seven Carex species. I conducted field experiments comparing stigmatic pollen loads and seed set between intact and emasculated stems. I tested for self-compatibility and evaluated pollen limitation of seed set by supplemental pollination. Finally, I measured outcrossing rates in open-pollinated and emasculated stems using allozyme markers. Emasculated stems captured significantly less pollen than open-pollinated stems and set less seed. Pollen deposition during the female-only phase for intact stems was only 12% of the total captured. Outcrossing rates for three species indicated high selfing (range $t=0.03$–0.39). Allozyme loci in the remaining species were monomorphic also suggesting high selfing. Results demonstrate that neither monoecy nor protogyny is particularly effective at limiting self-fertilization. Selection for the avoidance of selfing is unlikely to maintain monoecy in many Carex species although protogyny may provide limited opportunities for outcrossing. I propose that geitonogamy in self-compatible wind-pollinated species with unisexual flowers may be widespread and provides reproductive assurance.
CHAPTER FIVE: MONOECY AND PROTOGYNY IN CAREX

Introduction

Flowers of wind-pollinated plants are frequently unisexual (dichliny), or if hermaphroditic separate their sex functions temporally, through well-developed dichogamy (Charlesworth 1993; Renner & Ricklefs 1995; Ackerman 2000). Many monoecious species are wind pollinated and in general abiotic pollination is strongly associated with this sexual system (Webb & Lloyd 1986; Renner & Ricklefs 1995; Chapter Three). In some cases monoecy may have evolved as a mechanism to promote outcrossing (e.g. Lloyd 1972; Charlesworth & Charlesworth 1978). However, a survey of 588 angiosperms by Bertin (1993) found that the presence of self-compatibility versus self-incompatibility was independent of whether species were monoecious or possessed hermaphroditic flowers. This result is inconsistent with a major role for the avoidance of selfing in the evolution of monoecy. Because wind direction is often unpredictable, the spatial segregation of sex functions through monoecy may be ineffective at preventing geitonogamy in wind-pollinated plants (Harder et al. 2000).

The common association between monoecy and protogyny in wind-pollinated plants (Lloyd & Webb 1986; Bertin & Newman 1993; Sargent & Otto 2004) may largely function to reduce the incidence of self-fertilization. Abiotically-pollinated species are more frequently protogynous than protandrous, whereas biotically-pollinated species exhibit the reverse pattern (Sargent & Otto 2004). Moreover, Bertin (1993) and Routley et al. (2004) demonstrated that protogyny was associated with self-compatibility, and protandry with self-incompatibility, more often than expected under a random model. This finding is consistent with the inbreeding avoidance hypothesis for the evolution of protogyny (Lloyd & Webb 1986). The associations between protogyny and anemophily and protogyny and self-
compatibility suggest that protogyny may be an effective outcrossing mechanism in many anemophilous species.

In animal-pollinated plants, geitonogamy involves mating costs as it reduces outcross siring success through pollen (Harder & Barrett 1995; Harder & Wilson 1998; Lau et al. 2008) and seed discounting (Lloyd 1992), and can also result in inbreeding depression (Eckert & Barrett 1994a; Eckert 2000). However, neither the frequency nor the reproductive consequences of geitonogamy in wind-pollinated species have been investigated (but see Pannell 1997; de Jong et al. 1999). Wind-pollinated plants have a bimodal distribution of outcrossing rates (Schemske & Lande 1985; Aide 1986; Barrett & Eckert 1990; Goodwillie et al. 2005), with species more frequently either predominantly selfing or outcrossing. It has been argued that the apparent low incidence of mixed mating, compared with animal-pollinated plants, might reflect different selective pressures in wind-pollinated species, including infrequent geitonogamy (Aide 1986; Vogler & Kalisz 2001; Goodwillie et al. 2005).

The occurrence of separate female and male flowers in monoecious species requires that any selfing that occurs through geitonogamy must involve the same pollination processes as would occur with outcrossing. Because in animal-pollinated species geitonogamy requires the action of a pollinator, it offers no reproductive assurance in the absence of pollinators (Lloyd 1992). Reproductive assurance in animal-pollinated plants is achieved through autonomous selfing, and is particularly beneficial when pollinators are scarce or mates are lacking (Herlihy & Eckert 2002; Kalisz et al. 2004). Wind-pollinated plants do not suffer from unpredictable pollinators, so it is unclear whether the capacity for self-fertilization provides reproductive assurance. Eckert et al. (2006) surveyed the literature for experimental tests of reproductive assurance and found results for 29 species, all animal-pollinated (but see
Jacquemyn & Brys 2008 for an ambophilous example). Reproductive assurance can be tested by comparing seed set of intact control flowers to those of emasculated flowers, where anthers are removed before they dehisce (Cruden & Lyon 1989; Lloyd & Schoen 1992; Eckert et al. 2006). In wind-pollinated plants the capacity for pollen to fall from anthers onto stigmas below may provide a simple mechanism for self-pollination (Lloyd & Webb 1986). However, the degree to which monoecy and protogyny may limit geitonogamy is currently unknown and therefore forms the basis of this study.

Carex L. (Cyperaceae) is near exclusively wind-pollinated and is one of the largest genera of angiosperms with over 2000 species (Kükenthal 1909; Reznicek 1990). Flowers are exclusively unisexual and species are predominantly monoecious and rarely dioecious (Standley 1985a; Ball & Rezenicek 2002). Sedge flowers are arranged on a condensed spike, which may contain flowers of only one sex or both sexes (Smith & Faulkner 1976). Most species exhibit interfloral protogyny where stigmas on a given stem are receptive before male flowers on the same stem release their pollen (Bertin & Newman 1993). Despite the ubiquitous occurrence of Carex species in diverse plant communities in Northern temperate regions (Ball 1990), there have been virtually no experimental field studies on the floral biology of populations (but see Handel 1976; Stenström 1999). Recently, Bertin (2007) investigated whether monoecy in six Carex species permitted flexibility in the allocation of resources to female and male function under changing environmental conditions. He found only modest effects of altered nutrients, water and light on sex allocation and suggested that the avoidance of self-pollination may be particularly important in the evolution and maintenance of monoecy in sedges.

Here, I investigate seven sympatric Carex species to evaluate the functional roles of monoecy and protogyny in pollination and mating. I addressed three main questions: 1)
Through the use of pollen enclosures and supplemental pollen, I determined whether plants are self-compatible and if they are pollen limited. 2) Using emasculation experiments and genetic markers, I asked whether monoecy inhibits the capture of self-pollen and therefore limits rates of self-fertilization. 3) By comparing pollen loads on stigmas of intact and emasculated stems throughout flowering, I determined whether protogyny influences pollen capture and promotes outcrossing. I use results from these experiments to evaluate the efficacy of monoecy and protogyny in limiting selfing, and discuss the implications of geitonogamy for wind-pollinated plants. I also comment briefly on the rarity of dioecy in Carex and speculate on the puzzle of such a species-rich but potentially highly selfing group.

**Methods**

*Species and location of study*


All Carex species in this study consist of many flowering stems (or culms) on a plant. Each stem is characterized by a terminal staminate spike comprised of numerous stamens,
with several pistillate spikes arranged throughout the inflorescence below. Each pistillate spike produces many single-ovule flowers. The mean number of stems per plant for each species was: *C. arctata* (4.62 SE=0.29); *C. hirtifolia* (5.75, SE=0.36); *C. laxiflora* (6.25, SE=0.37); *C. pedunculata* (16.10, SE=0.93); *C. pensylvanica* (29.49, SE=2.11); *C. plantaginea* (6.58, SE=0.55); and *C. scabrata* (30.44, SE=3.06). The differences among species in stem number reflect, in part, variation in overall plant size.

**Pollination and seed set**

For each species I used 30 pairs of plants separated by at least 5m to ensure they were distinct clones. I applied treatments to two labeled stems on each experimental plant and these were either left intact or emasculated prior to flowering. Emasculation involved removing the staminate spike from the stems. One stem was used for stigma collection and the second for measuring seed set. For each day during flowering I recorded the sexual phase of stems. All species are protogynous and, within a stem, stigmas mature from the top of the inflorescence to the bottom. After several days of stigma exposure, anthers emerge at the top of the stem, while female flowers continue to open below. Thus, initially only female flowers are functional, followed by a phase where both female and male flowers are in anthesis. For intact and emasculated stems I collected stigmas every other day during the flowering period and stored them in 70% ethanol. To assess pollen capture, I stained stigmas with basic fuschin and counted all pollen on the three stigmas of a flower using a compound microscope (20x).

I tagged an additional 40 plants per species – 20 stems were bagged and received only self pollen to test for self-compatibility, and 20 received supplemental cross pollen using hand pollination to test for pollen limitation by comparing them to open-pollinated flowers. To determine whether plants are self-compatible, I secured pollen enclosures around the
stems of plants before stigmas had emerged to exclude wind-borne pollen, and then I applied self pollen to stigmas. The enclosure consisted of three layers of pollen-proof spun-nylon material secured to the ground with thin metal spikes. When anthers were dehiscent, I opened the enclosure around the bagged stem and brushed the stigmas with anthers from the same plant. The bag was then re-secured. For each bagged stem, I applied pollen on at least two separate days. Supplemental pollen was applied to stigmas by brushing them with anthers from three donors, located at least 25m away. Again, pollen was applied on at least two separate days.

Once seeds were fully mature (4-6 weeks) I harvested all treatment infructescences from intact, emasculated, bagged and supplementally-pollinated stems, and counted the number of mature seeds and the number of aborted or unfertilized flowers to assess proportion seed set. I then used seeds from intact and emasculated infructescences to investigate mating patterns.

I analysed pollen receipt with repeated-measures, general linear models (SAS PROC MIXED; 9.1; SAS Inst. 2002; Neter et al. 1996), with the dependent variable log-transformed to assure normally distributed residuals. I analyzed seed set using generalized linear models (SAS PROC GENMOD; 9.1; SAS Inst. 2002; Allison 1999) with logit transformations to accommodate the binomial distribution of data. Likelihood-ratio tests were used to determine the significance of each effect in the model. Analyses considered species, treatment, and sex phase (where appropriate) as categorical independent variables, and their interactions. I excluded terms from the model by backward elimination ($\alpha=0.05$) if they did not explain a significant proportion of the variation in the dependent variable. The interaction between species and treatment was always retained in the model to reflect the experimental design. Because I sampled multiple flowers per stem, I employed restricted maximum likelihood
(Jennrich & Schluchter 1986) to characterize the covariance between responses by individual plants. I calculated denominator degrees of freedom for F-tests of the general linear models by Kenward & Roger’s (1997) approximation, which can result in fractional degrees of freedom. I adjusted all plotted values to account for the other components in the model.

**Mating patterns**

I used allozyme markers and starch gel electrophoresis to determine the outcrossing rate of a single population of each species. Seed families of a minimum of 10 seeds from each maternal family (all harvested intact and emasculated stems) were ground in three drops of 0.1M Tris-HCl extraction buffer (Soltis et al. 1983) and the extract absorbed onto 3-mm chromatography paper wicks and placed directly onto 11–12% starch gels. I used a histidine-citrate (pH 6.5) and lithium-borate buffer system (pH 8.3), gels were stained for enzyme activity following recipes in Wendel & Weeden (1991). Four species (C. hirtifolia, C. arctata, C. pedunculata and C. pensylvanica) exhibited no allozyme polymorphism among a minimum of 11 loci. For the other three species, I resolved two variable loci each [C. scabrata: triosephosphate isomerase (Tpi: 2 alleles), phosphoglucomutase (Pgm: 2 alleles); C. laxiflora: alcohol dehydrogenase (Adh: 2 alleles), triosephosphate isomerase (Tpi: 2 alleles); C. plantaginea: phosphoglucomutase (Pgm: 3 alleles), phosphoglucose isomerase (Pgi: 2 alleles)].

Genotypes were inferred based on segregation patterns characteristic of either dimeric or monomeric codominant enzymes. For each species I estimated the outcrossing rate (t) using the program MLTR (vers. 3.0, Ritland 1990). This program uses maximum-likelihood procedures to infer the genotypes of maternal parents, allele frequencies in the pollen pool and the proportion of progeny that are the result of outcrossing. I derived the standard errors of the outcrossing rate estimates from the standard deviation of 1000 bootstrap values, using
the seed family as the unit of resampling. To determine whether estimates of outcrossing rates were significantly different between emasculated and intact stems, I examined the pairwise comparisons of 1000 bootstrap estimates following methods outlined in Eckert & Barrett (1994b). Using this method, treatments were considered to differ significantly if 100[1-(\(\alpha_{PC}/2\)] percentage of the differences between randomly paired bootstrap values were all greater than zero or all less than zero (where \(\alpha_{PC}\) represents the Type 1 error rate per contrast).

Results

Pollen receipt

Overall, stigmas of emasculated stems captured significantly less pollen than those of open-pollinated (intact) stems (Table 5.2, Figure 5.1a; mean decrease in pollen capture: 33.83%, range: 0–83%). Average stigmatic pollen loads of intact stems ranged from 4.13–13.19 among the seven species (Table 5.1). There was no significant species by treatment interaction indicating that the Carex species do not generally differ in their response to emasculation. However, emasculated stems of C. plantaginea received higher pollen loads than intact stems (emasculated adjusted mean=3.11, SE=0.36; intact adjusted mean=2.67, SE=0.22), although this difference was not significant (\(t_{922} = 1.07, P>0.25\)).

In each Carex species, stigmas received significantly more pollen during the combined sex phase than during the female-only phase (Table 5.2, Figure 5.1b; percentage of pollen capture during female-only phase: 12.44%, range: 6.46–30.87%). There was a significant treatment by gender phase interaction (Table 5.2). This interaction arises because
Table 5.1. Summary of average stigmatic pollen load and average proportion seed set for seven open-pollinated Carex species at Koffler Scientific Reserve, Ontario, Canada.

<table>
<thead>
<tr>
<th>Species</th>
<th>Stigmatic pollen load (SE)</th>
<th>Proportion seed set (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. arctata</td>
<td>8.87 (2.67)</td>
<td>0.81 (0.01)</td>
</tr>
<tr>
<td>C. hirtifolia</td>
<td>5.49 (0.53)</td>
<td>0.80 (0.05)</td>
</tr>
<tr>
<td>C. laxiflora</td>
<td>4.33 (0.83)</td>
<td>0.73 (0.03)</td>
</tr>
<tr>
<td>C. pedunculata</td>
<td>13.19 (2.63)</td>
<td>0.90 (0.01)</td>
</tr>
<tr>
<td>C. pensylvanica</td>
<td>10.41 (3.61)</td>
<td>0.65 (0.03)</td>
</tr>
<tr>
<td>C. plantaginea</td>
<td>4.13 (0.58)</td>
<td>0.82 (0.03)</td>
</tr>
<tr>
<td>C. scabrata</td>
<td>4.73 (0.70)</td>
<td>0.82 (0.02)</td>
</tr>
</tbody>
</table>
Table 5.2. Summary of analyses on pollen receipt and seed set during emasculation experiments on seven *Carex* species at Koffler Scientific Reserve, Ontario, Canada.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Stigmatic pollen load</th>
<th>Proportion seed set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>$F_{6,896}=12.34$ ***</td>
<td>$F_{6,336}=6.70$ ***</td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{1,1017}=5.51$ *</td>
<td>$F_{1,366}=19.41$ ***</td>
</tr>
<tr>
<td>Gender</td>
<td>$F_{1,1069}=182.38$ ***</td>
<td>$NA$</td>
</tr>
<tr>
<td>Treatment x species</td>
<td>$F_{6,749}=2.01$</td>
<td>$F_{6,366}=1.50$</td>
</tr>
<tr>
<td>Gender x species</td>
<td>$F_{6,887}=2.98$ **</td>
<td>$NA$</td>
</tr>
<tr>
<td>Gender x treatment</td>
<td>$F_{1,678}=25.01$ ***</td>
<td>$NA$</td>
</tr>
</tbody>
</table>

*P<0.05, ** P<0.01, *** P<0.0001
Figure 5.1. Results from emasculation experiments on seven monoecious Carex species at Koffler Scientific Reserve, Ontario, Canada. a) Influence of emasculation treatment on mean (SE) stigmatic pollen load; b) Interaction between emasculation treatment and gender phase during flowering on mean (SE) stigmatic pollen load; and c) Influence of emasculation treatment on mean (SE) proportion seed set. Letters indicate significant differences. See Table 5.2 for statistical details.
stigmas of intact stems received significantly more pollen than those of emasculated stems during the combined sex phase (intact mean=11.33, range=6.82–21.91; emasculated mean=5.15, range=1.60–11.30; \( t_{681} = 6.88, P<0.0001 \); Figure 5.1b). However, there was no significant difference between intact and emasculated stems during the female phase when self-pollen was unavailable (intact mean=1.41, range=0.69–2.17; emasculated mean=1.65, range=0.89–2.51; \( t_{915} = 1.38, P>0.15 \); Figure 5.1b).

Patterns of seed set

Open-pollinated stems set significantly more seed than emasculated stems (Table 5.2, Figure 5.1c; mean decrease in seed set: 24.01%, range: 10–47%). The absence of a significant species by treatment interaction (Table 5.2) indicates that all species responded similarly to emasculation. Maximum seed set ranged from 65–88% among intact stems of the seven species (Table 5.1).

In six of the seven species investigated, stems that received supplemental pollen set equivalent amounts of seed as open-pollinated stems (Figure 5.2; statistics not shown). Carex scabratata was the only species that set significantly more seed with the addition of supplemental pollen (intact mean=0.82, SE=0.02; supplemental mean=0.89, SE=0.04; \( F_{1,33} = 11.33, P<0.001 \); Figure 5.2) indicating some pollen limitation of seed set.

Bagged stems that had self-pollen applied to stigmas set the same amount of seed as intact stems in six of the seven species investigated (Figure 5.2; statistics not shown). This demonstrates that the species investigated are fully self-compatible. Stems of Carex pensylvanica that were bagged and received self-pollen set significantly less seed than open-pollinated stems (intact mean=0.65, SE=0.03; bagged mean=0.55, SE=0.05; \( F_{1,57} = 22.34, P<0.0001 \); Figure 5.2).
Figure 5.2. Comparison of three treatments (open-pollinated, supplemental pollen, bagged plus self pollen) on mean (SE) proportion seed set for seven *Carex* species. Asterisks indicate treatments that differed significantly from the open-pollinated treatment, see text for statistical details.
Outcrossing rates

Open-pollinated stems of Carex species with polymorphism at allozyme loci exhibited high levels of self-fertilization with two species predominantly selfing (Intact: C. laxiflora $t=0.09$, SE=0.03; C. scabrata $t=0.03$, SE=0.02) and the remaining species showing mixed mating (Intact: C. plantaginea $t=0.39$, SE=0.06). Comparisons of outcrossing rate between intact and emasculated stems in each species failed to detect significant differences. However, in all three species emasculated stems had slightly higher outcrossing rates than intact stems (Emasculated: C. laxiflora $t=0.16$, SE=0.07; C. plantaginea $t=0.45$, SE=0.08; and C. scabrata $t=0.09$, SE=0.03) consistent with the occurrence of within-stem geitonogamy. The high degree of selfing in emasculated stems also indicates substantial between-stem geitonogamy.

Discussion

Pollination and mating in monoecious wind-pollinated species has seldom been investigated experimentally and we lack a functional explanation for the widespread association between monoecy and wind pollination. Here I used Carex, a species-rich and predominantly monoecious genus, to assess whether monoecy limits geitonogamous selfing. Field experiments with seven species demonstrated that monoecy is not effective at limiting this mode of self-fertilization. Self-pollen contributes significantly to stigmatic pollen loads and seed set. Emasculated stems captured significantly less pollen than intact stems (Figure 5.1a) and set significantly fewer seeds (Figure 5.1c). During the stage when most plants were in both female and male phases, intact stems received more pollen than emasculated stems (Figure 5.1b), indicating that they are probably capturing a substantial portion of self pollen from the same inflorescence. However, the co-occurrence of monoecy and protogyny in
*Carex* provides a brief window of opportunity for outcross pollen to be captured, although I found only 12% of the total pollen is captured during the female phase (Figure 5.1b). Results demonstrate that geitonogamy is a pervasive feature of the floral biology of *Carex* species and that it occurs both within and between stems.

In the *Carex* species I investigated the combination of self-compatibility, modest clonal growth and multiple flowering stems per plant provides opportunities for between-stem geitonogamy. Protogyny provides some protection against within-stem geitonogamy, but asynchrony in sex phases among stems of a plant permits pollen transfer between stems. Although it was not practical to quantify the between-stem component of geitonogamy by whole plant emasculation, the high selfing rates in emasculated stems indicate that pollen dispersal between flowering stems on a plant likely contributes significantly to geitonogamous self-fertilization.

**Monoecy and protogyny fail to limit self-pollination**

Monoecious wind-pollinated plants may be unable to avoid self-pollination when female and male parts are presented simultaneously, which has been interpreted as an explanation for the high incidence of dichogamy in wind-pollinated species (Lloyd & Webb 1986; Webb & Lloyd 1986). Sargent & Otto (2004) found that transitions from biotic to abiotic pollination were more likely among protogynous species than among protandrous species. They suggested that this result occurs because protogynous species are less likely than protandrous species to suffer from increased self-fertilization upon a switch to wind pollination. An initial period of stigma presentation provides an opportunity for cross-pollination before selfing is possible. However, results demonstrate that although the protogynous phase allows some pollen to be captured the amounts involved are significantly less than during the combined sex phase (Figure 5.1b). Although theory suggests that
protogyny provides better protection against selfing than protandry (Lloyd & Webb 1986),
the data indicate that protogyny is not particularly effective at preventing self-pollination, at
least in the species I investigated. Authors of other studies of Carex (Vonk 1979; Whitkus
1992) have explained high selfing rates by suggesting that an overlap in anthesis of female
and male flowers among stems could result in geitonogamy, although this was not
demonstrated experimentally.

Despite wind pollination and unisexual flowers there is considerable evidence that
many Carex species are highly selfing. Self-compatibility is widespread in Carex and in the
Cyperaceae more generally (Faulkner 1973; Handel 1978; Vonk 1979; Schmid 1984;
Standley 1985b; Whitkus 1988; Snyder & Richards 2005). Although the possibility of self-
incompatibility in a few species has been raised (Ford et al. 1991; Stenström et al. 2001) there is no convincing experimental evidence for its occurrence in Carex. Studies examining
the partitioning of genetic diversity within and among populations using genetic markers
have revealed low levels of intra-population variation, but high inter-population genetic
differentiation; a pattern consistent with high selfing (Bruederle & Fairbrothers 1986;
Reinhammar 1999; Tyler et al. 2002; Kull & Oia 2007). Other studies have provided direct
evidence that some Carex species are predominantly selfing (Handel 1978; Schmid 1984;
Whitkus 1988). Handel (1985) and Ford et al. (1991) suggested that both inflorescence
morphology and growth form facilitate selfing in many Carex species. Like most of the taxa
discussed here, selfing species are characterized by a cespitose growth habit and by having
multiple pistillate and staminate spikes on each stem or spikes with both sexes. These traits
increase opportunities for geitonogamous selfing, both within and between stems. In contrast,
rhizomatous species with relatively few, widely-spaced, unisexual stems have a different
population genetic structure with genetic diversity partitioned within rather than among populations (Ford et al. 1991; McClintock & Waterway 1993; Jonsson et al. 1996; Stenström et al. 2001), a pattern consistent with higher levels of outcrossing. This suggests that clonal architecture plays an important role in governing mating patterns and population genetic structure in Carex.

In six of the seven species I investigated there was no evidence that female fertility was pollen limited. The addition of supplemental pollen did not significantly increase seed set (Figure 5.2). However, intact plants set significantly more seed than emasculated plants (Figure 5.1c) demonstrating that self pollen enables plants to achieve maximum seed set. The overall prevalence of pollen limitation in wind-pollinated species is unclear, although it is often assumed that pollen availability does not limit reproduction. Recent empirical work suggests that pollen capture and seed set may decrease rapidly with increasing distance from pollen donors, and that pollen limitation in wind-pollinated plants may often occur in sparse populations (Handel 1976; Knapp et al. 2001; Koenig & Ashley 2003; Davis et al. 2004b; Eppley & Pannell 2007; Chapter Six). The Carex species in this study all grow in the understory of forests, a relatively unusual habitat for most wind-pollinated species. Forests are typically characterized by slow wind speeds and disrupted wind profiles. Although the seven species flower in early to late spring before the forest canopy has closed, wind speeds may still be low and unpredictable. Furthermore, the relatively small stature of the species I investigated means that they may experience short pollen dispersal distances in comparison with wind-pollinated plants of open habitats. These factors, combined with the clonal growth patterns and presence of multiple spikes on a plant probably explain the low incidence of outcross pollination.
Geitonogamy and reproductive assurance in wind-pollinated plants

Virtually all work on geitonogamy deals with animal-pollinated plants where it is unlikely to be adaptive (Lloyd 1992). This is, in part, because geitonogamy often results from pollinators successively visiting flowers of one plant (Lloyd 1992). Thus, geitonogamous selfing limits opportunities for outcrossing because it removes pollen directly from the pollen pool that pollinators transport between flowers (Harder & Barrett 1995; Harder et al. 2008). However, in wind-pollinated species self-pollination between flowers should not directly impact opportunities for cross-pollination. Although a small portion of the total pollen produced will be used for selfing it is unlikely to diminish the success of the plant as a pollen donor. This arises because wind cannot be saturated with pollen and pollen removal is not limited by pollinator visitation. Thus geitonogamy can be increased without additional metabolic cost for the plant and without decreasing cross-pollination. de Jong et al. (1999) modeled a similar scenario involving the effects of geitonogamy on sex allocation in hermaphrodite plant populations. They found that the absence of pollen discounting resulted in increased relative male allocation and, in the absence of inbreeding depression, individuals benefited from the automatic advantage of selfing. Therefore if pollen discounting is negligible the consequences of geitonogamy in wind-pollinated species may be quite different from animal-pollinated species, and may be beneficial.

In animal-pollinated plants, selection for geitonogamy as a mechanism of reproductive assurance is unlikely because it requires the aid of a pollinator and so offers no reproductive assurance in the absence of pollinators. However, recent work by Pannell (2006) illustrates that in a metapopulation when mates are limiting, geitonogamy can be selected as a mechanism of reproductive assurance. Empirical work on *Mercurialis annua*, a wind-pollinated androdioecious annual, has shown that geitonogamous selfing gives
hermaphrodites at low density a reproductive advantage over males during colonization (Pannell 1997, 2001; Eppley & Pannell 2007). The data suggest that even in stable populations, geitonogamous selfing can be beneficial for wind-pollinated plants by alleviating pollen limitation in environments not conducive to extensive pollen dispersal.

Alternative functions for monoecy

There are several reasons why monoecy may be beneficial in wind-pollinated plants that are independent of any influences on selfing. Unisexual flowers in wind-pollinated plants may facilitate specialization for efficient pollen removal versus receipt. Unlike hermaphrodite animal-pollinated plants, where pollinators deliver pollen to stigmas and pick-up pollen from anthers in a single visit, in wind-pollinated plants the removal and capture of pollen are largely independent events. Interference between female and male structures within a flower may be directly disadvantageous in hermaphroditic wind-pollinated plants because the optimal conditions for pollen dispersal and pollen capture are usually different (Lloyd 1982; Niklas 1985; Young & Schmitt 1995; Friedman & Harder 2004). In accordance with aerodynamic predictions, male flowers are most successful when presented at elevated heights, while female flowers capture more pollen when they are below the source of pollen release.

The Carex species studied here are characterized by several pistillate spikes throughout the inflorescence arranged adjacent to or below a staminate spike comprised of numerous stamens. This morphology suggests very different optima for female and male function in encouraging cross-pollination. However, this arrangement may also facilitate self-pollination within a stem as pollen can easily fall from anthers onto the stigmas below. Carex plantaginea differed from the other six species investigated in being the only one in which intact and emasculated plants captured equivalent amounts of pollen. Furthermore, it
exhibited higher levels of outcrossing than the other species ($t=0.39$ SE=0.06). *Carex plantaginea* was shown by Handel (1976) to have less restricted pollen flow than the other *Carex* species in his study. He suggested that this difference was due to the elevated height of the staminate spikes. Spatial segregation of female and male function may enhance outcrossing by allowing differential positioning and morphology of flowers for optimal pollen dispersal versus capture, but in the species I investigated it clearly did not preclude opportunities for selfing.

*Rarity of dioecy and the puzzle of diversification in Carex*

There is a strong association between anemophily and dioecy in flowering plants (Renner & Ricklefs 1995; Linder 1998; Vamosi et al. 2003). A common evolutionary pathway to dioecy is from monoecy and the two sexual systems are strongly associated within genera (Bawa 1980; Renner & Ricklefs 1995). In *Carex* most species are monoecious and dioecy is relatively rare in the genus (ca. 10 species) apparently having originated several times (Standley 1985a). Given the very large size of the genus this raises the fascinating question as to why the evolutionary transition from monoecy to dioecy is so infrequent and why dioecious lineages are not more successful, particularly since monoecy appears to be ineffective as an outbreeding mechanism.

Stebbins (1957) first proposed that self-fertilization may be an evolutionary dead-end, since selfing lineages may have limited potential for adaptive diversification and are more likely than outcrossing lineages to go extinct (Takebayashi & Morrell 2001; Igic et al. 2008). Thus, the possibility that species-rich *Carex* may contain many selfing species is puzzling. Other features of the genetic system may be responsible for promoting diversification independent of mating patterns. For example, species richness in *Carex* may be a consequence of unusual and rapid chromosomal evolution (Hipp 2007). Sedges have
holocentric chromosomes, which evolve rapidly by fission and fusion, resulting in exceptional agmatoploid chromosome series ranging from $n = 6$ to $n = 66$ (Tanaka 1949). Although unusual chromosomal evolution may, in part, account for the great diversity of sedges, the factors responsible for their remarkable radiation remain largely unresolved. Determining whether high selfing is widespread among Carex species is needed before this problem can be addressed in more detail. Nevertheless, at least in the Carex species I investigated, monoecy and protogyny are largely ineffective at limiting high rates of self-fertilization.
CHAPTER SIX

ENVIRONMENTAL INFLUENCE ON PRIMARY SEX RATIO IN A DIOECIOUS PLANT

This chapter resulted from collaborations with Ivana Stehlik and Spencer C. H. Barrett. Ivana Stehlik contributed to the ideas, data collection, analysis, and writing of the manuscript; Spencer Barrett contributed to the ideas and writing of the manuscript published in Proceedings of the National Academy of Sciences, 2008, 105: 10852-10857.

Summary

The proximity of mates can influence mating opportunities and the quantity and quality of offspring, especially in dioecious plant species. Progeny sex ratios modulated by environmental conditions is one of the most radical ways in which offspring quality may be influenced; yet it has rarely been reported in plants. A mechanism proposed to influence progeny sex ratios in dioecious plants involves competition between female- and male-determining microgametophytes (certation) as a result of variation in pollination intensity. However, the role of selective fertilization in dioecious plants is controversial and has not been demonstrated under field conditions. Here, I investigate whether natural variation in the spatial arrangement of females and males influences pollination intensity and progeny sex ratios in the wind-pollinated herb Rumex nivalis. Based on previous experimental manipulation of pollination intensity in this species, I predicted maternal parents in close proximity to males would produce more strongly female-biased progeny sex ratios. I tested this prediction in six alpine populations in Switzerland by measuring the distance between focal females and neighboring males and assessing pollen loads and seed sex ratios of maternal parents. In four of the six populations, females positioned in close proximity to males captured more pollen and exhibited more female-biased sex ratios. The results
CHAPTER SIX: ENVIRONMENTAL INFLUENCE ON SEX RATIO

demonstrate, for the first time in plants, that demographic aspects of the maternal mating environment can influence progeny sex ratios. The most probable explanation for biased primary sex ratios in *Rumex* is selective fertilization resulting from pollen tube competition.

**Introduction**

The spatial context in which reproduction occurs is of critical importance for plants because of their sessile habit. Most plants mate and disperse offspring locally, so that mating success is context dependent and influenced by plant density and the phenotypic composition of neighborhoods (Antonovics & Levin 1981; Davis et al. 2004b; Stehlik et al. 2006). Dioecious species are especially sensitive to spatial structure and composition because of the restricted number of mating groups within populations. Female reproductive success can be influenced by male flowering density, depending on the extent of pollen dispersal (Barrett & Thomson 1982; Heilbuth et al. 2001; Wilson & Harder 2003). Patch density and the local sex ratio also have the potential to influence parental fitness through their influence on pollination intensity. The amount of pollen captured by stigmas could potentially affect both the quality of offspring and progeny sex ratios through gametophytic competition (certation) and selective fertilization (Correns 1928; Jones 1928; Mulcahy 1975). However, the relative roles of genetic and environmental factors in governing primary sex ratios in dioecious populations are still poorly understood and evidence for environmentally induced variation in primary sex ratios is limited, despite considerable heterogeneity in seed sex ratios among species, populations and families (de Jong & Klinkhamer 2005).

Sex determination induced by the environment is one of the most direct ways in which progeny sex ratios can vary. Environmental sex determination is expected to be adaptive if the environment experienced during development is variable and exerts a sex-dependent influence on fitness (Charnov & Bull 1977; Bull 1983). There are numerous
examples in the animal kingdom in which environmental sex determination results in biased progeny sex ratios, with environmental triggers such as temperature or parental condition commonly involved (e.g. Clutton-Brock et al. 1981; Lagomarsino & Conover 1993; Janzen & Phillips 2006). However, because plant species tend to be more phenotypically plastic than animals in gender expression, they typically show environmentally influenced sex-allocation plasticity, or sex inconstancy during flowering (Lloyd & Bawa 1984; Korpelainen 1998; Delph & Wolfe 2005). Evidence for environmental sex determination early in development is relatively rare in seed plants (e.g. Spinacia oleracea: Freeman et al. 1994), and how frequent environmental influences interact with genetic sex determination mechanisms to influence progeny sex ratios in dioecious plants is unclear.

In the dioecious herb Rumex nivalis (Polygonaceae), sex determination is governed by heteromorphic sex chromosomes with females homogametic XX and males heterogametic XY₁Y₂ (Żuk 1963, Wagenitz 1981). Similar to several other dioecious species of Rumex with sex chromosomes (Correns 1922, 1928; Smith 1963; Rychlewski & Zarzycki 1975; Conn & Blum 1981), there is experimental evidence that progeny sex ratios are also influenced by non-genetic factors, specifically the amount of pollen deposited on stigmas. By experimentally manipulating the distance between male pollen donors and female recipients of R. nivalis in a common garden, Stehlik & Barrett (2006) demonstrated that seed sex ratios were dependent on the specific maternal pollination environment. Females at closer distances to males had higher stigmatic pollen loads and produced more strongly female-biased seed sex ratios compared with more distant females. However, attempts to demonstrate relations between male proximity and progeny sex ratios have been inconclusive in other Rumex species (Rychlewski & Zarzycki 1975, Conn & Blum 1981), and the role of maternal
pollination environment in affecting progeny sex ratios in natural populations of dioecious species has not been investigated.

Here, I investigate whether the composition of the local mating neighborhood in natural populations of dioecious *R. nivalis* influences progeny sex ratios. If so, this would represent a hitherto unique interaction of environment and offspring sex ratios in a dioecious plant species. Under thecertation hypothesis, progeny sex ratios of maternal parents of *R. nivalis* located in close proximity to males should be more female biased as a result of higher pollen loads, leading to increased competition between female- versus male-determining pollen tubes. I tested elements of this prediction in six natural populations of *R. nivalis* in the Swiss Alps. I mapped the location of plants in each population and measured pollen loads of focal females. Sex-specific molecular markers and censuses of flowering sex ratios of maternal families were then used to determine the sex of offspring. The results provide the first evidence for an environmental influence on primary sex ratios in a dioecious plant.

Methods

Study species

*Rumex nivalis* (Polygonaceae) is a wind-pollinated perennial herb restricted to the European Alps and mountains of Bosnia-Herzegovina (Wagenitz 1981). Populations occur almost exclusively in snowbeds interspersed among alpine meadows above tree line. Individual plants have a basal rosette and one to several inflorescences. Clonal expansion via basal branching is limited and genets easily identified. Females have uniovulate flowers and mature adults produce up to 200 seeds per season. As in other *Rumex* species of section *Acetosa*, females of *R. nivalis* possess one pair of X chromosomes (homogametic sex; 2n =
14), whereas males are characterized by one X and two Y chromosomes (heterogametic sex; 
$2n = 15$; Wagenitz 1981).

Selection of populations, measurements and sex determination

In summer 2004, I selected six natural populations of *R. nivalis* in Switzerland that 
varied in sex ratios and densities of flowering males and females (Table 6.1). To assess local 
sex ratios, I subdivided each population into 4 m × 4 m grids and counted all flowering 
females and males per quadrat. Within each population, I haphazardly chose 30 focal females 
from neighborhoods with a range of male densities. To assess the pollination environment of 
each focal female, I measured the distance to the 50 nearest male inflorescences. If an 
individual male produced more than one inflorescence, I treated the inflorescences as 
independent. For each focal female, I measured stigmatic pollen loads, seed set and seed sex 
ratios.

At peak flowering (end of June to beginning of August 2004), I collected 16 flowers 
per focal female throughout the inflorescence (top to bottom) and preserved them 
individually in 70% ethanol in microcentrifuge tubes for measurements of stigmatic pollen 
loads. Stigmas varied in their duration of exposure to pollen. I stained stigmas in 1% fuchsin 
and counted pollen grains on stigmas under a compound microscope with a 100× 
magnification. In September 2004, I collected entire inflorescences of focal females to assess 
seed set and sex ratios. I counted the number of seeds produced and calculated the total seed 
set of each female by dividing the number of fully developed seeds by the total number of 
flowers. Due to grazing by animals I only recovered 88% of focal females.

Seed sex ratios were calculated for each of the focal females in all six populations as 
the number of female offspring divided by the total number of offspring assessed. I
Table 6.1. Summary of population parameters for six natural populations of dioecious *Rumex nivalis* in Switzerland used in the study of progeny sex ratio bias.

<table>
<thead>
<tr>
<th>Population</th>
<th>Geographic coordinates</th>
<th>Altitude (m a.s.l.)</th>
<th>Population size</th>
<th>Male density (#/m²)</th>
<th>Sex ratio</th>
<th>No. of focal females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arosa1</td>
<td>9°37'7.2&quot;/46°46'6.1&quot;</td>
<td>2500</td>
<td>1333</td>
<td>0.98</td>
<td>0.61</td>
<td>25</td>
</tr>
<tr>
<td>Arosa2</td>
<td>9°37'7.3&quot;/46°46'9.3&quot;</td>
<td>2250</td>
<td>614</td>
<td>0.12</td>
<td>0.85</td>
<td>21</td>
</tr>
<tr>
<td>Davos1</td>
<td>9°49'20.0&quot;/46°41'32.3&quot;</td>
<td>2500</td>
<td>816</td>
<td>0.40</td>
<td>0.72</td>
<td>30</td>
</tr>
<tr>
<td>Davos2</td>
<td>9°48'33.7&quot;/46°41'49.4&quot;</td>
<td>2330</td>
<td>1542</td>
<td>1.26</td>
<td>0.77</td>
<td>29</td>
</tr>
<tr>
<td>Flims</td>
<td>9°16'21.8&quot;/46°52'40.5&quot;</td>
<td>2500</td>
<td>981</td>
<td>0.53</td>
<td>0.78</td>
<td>26</td>
</tr>
<tr>
<td>Saentis</td>
<td>9°21'65.9&quot;/47°14'30.1&quot;</td>
<td>2120</td>
<td>1589</td>
<td>0.54</td>
<td>0.78</td>
<td>28</td>
</tr>
</tbody>
</table>
determined the sex ratio of progenies following Stehlik & Barrett (2005, 2006) by either (i) growing maternal halfsib progenies to flowering in the glasshouse, or (ii) by determining gender using a male-specific SCAR marker (Stehlik & Blattner 2004; see below). An analysis using a generalized linear model to assess whether the method of sex determination (glasshouse vs. SCAR marker), treated as a fixed factor, affected progeny sex ratios yielded no significant difference between the two techniques (SAS PROC GENMOD; 9.1; SAS Inst. Inc. 2002; $\chi^2_1 = 0.91, P = 0.34$). Due to the loss of some maternal plants in the field, I sexed offspring from a total of 159 females in the six populations (mean number of females per population = 26.5, SE = 1.34, range = 21–30; Table 6.1) for a total of 4903 offspring.

Using method i, I grew all available seeds per maternal parent to flowering for the populations Davos1, Davos2 and Saentis. Seeds were fully ripe at the time of harvest in these populations and the mean number of offspring per female was 38.31 seeds (SE = 2.13). Germination and growth conditions closely resembled those used in Stehlik & Barrett (2005, 2006) and, as in these studies, germination rates were high (>90%). At the time of the final census for sex determination, 98.5% of plants had flowered yielding a mean sample size of 32.9 (SE = 2.11) offspring per female, for a total of 2862 individuals. Previous work on *R. nivalis* (Stehlik & Barrett 2005) established that the sex ratio of germinated vs. ungerminated seeds was not significantly different. I am therefore confident that flowering sex ratios are equivalent to seed sex ratios. Indeed, this was reflected in the non-significant influence of the mode of sex determination in the ANOVA described above.

I assessed the seed sex ratios in populations Arosa1, Arosa2 and Flims using method ii, involving the SCAR marker. DNA extractions, PCR conditions, and sex scoring were identical to those used in Stehlik & Barrett (2005). Although seeds were fully developed at the time of seed harvest in these populations, they were still green which can reduce
germination levels. I determined the sex of 30 seeds per female, unless they produced fewer seed and I then used all available offspring. This resulted in a mean of 28.35 (SE = 0.50) seeds per female and a total of 2041 seeds assessed.

**Statistical analyses**

To investigate the relationships among stigmatic pollen load, seed set and seed sex ratio with each focal female’s specific pollination environment, I investigated the fit of the three response variables to measures of male proximity. As occurs in many wind-pollinated plants, pollen dispersal in *R. nivalis* follows a leptokurtic distribution with females directly adjacent to males receiving the highest pollen loads and a steep decrease occurring over relatively short distances (Stehlik & Barrett 2006). Pollen loads should therefore be dependent on the proximity to males in the local neighborhood. I initially ran statistical analyses with the distance to the nearest male per focal female as the independent variable. I then re-ran the analyses using the distance to the second-nearest male, the third-nearest male etc. up to the inclusion of all 50 males to determine the model with the best fit to the data, as judged by the model with the lowest log-likelihood. For pollen load and sex ratio, the most relevant response variables for the certation hypothesis, the model with the lowest log-likelihood was the distance to the fourth-nearest male as the independent variable. For seed set, the goodness-of-fit increased slightly with increasing distance past the fourth male but then dropped. I also investigated the relationships between male density and the three response variables. I assessed the number of males within 0.25m incremental distance classes from focal females (i.e. 0m–0.25m, 0m–0.5m, 0m–0.75m etc.). I used the same technique as above and found that the inclusion of males at a distance of up to 1.75m yielded the best fit; however, the fit using the distance to the fourth-nearest male still provided a better fit. Because the difference between these two measures of maternal pollination environment
resulted in only small qualitative differences, I only present results based on analyses using the distance to the fourth-nearest male as the independent variable.

I investigated stigmatic pollen load with a generalized linear model (SAS PROC GENMOD; 9.1; SAS Inst. 2002; Neter et al. 1996) with a negative binomial distribution and log-link function. I used this distribution to accommodate the zero-inflated data structure and heterogeneous variance. Seed set and seed sex ratios were analyzed with generalized linear models (SAS PROC GENMOD; 9.1; SAS Inst. 2002; Allison 1999) with logit transformations to accommodate the binomial distribution of data. In all of the analyses, I treated population as a block and female pollination environment (distance to the fourth-nearest male) as a fixed factor. Likelihood-ratio tests were used to determine the significance of each effect in the model (Allison 1999). I also examined the relation between sex ratio and stigmatic pollen load irrespective of female-specific pollination environment using generalized linear models (SAS PROC GENMOD). For all analyses, I initially included all factors and all their interactions. I excluded non-significant terms by stepwise backward elimination ($\alpha = 0.05$) if they did not explain a significant proportion of the variation.

Results

Variation in population size, density and flowering sex ratios

The six populations varied in flowering population size (mean number of individuals $= 1145.8$, SE $= 163.1$, range $= 614–1589$) and flowering male density (mean males per $m^2 = 0.64$, SE $= 0.08$, range $= 0.12–1.26$; Table 6.1). All populations were strongly female-biased (mean sex ratio $= 0.75$, SE $= 0.03$, range $= 0.61–0.85$; Table 6.1), a feature typical of *R. nivalis* populations (Stehlik & Barrett 2006). As a result of variation in male density, there was a wide range of distances between focal females and surrounding males. In the case of
the distance between a focal female and its fourth-nearest male (the independent variable in statistical analyses), the mean distance was 1.25m (SE = 0.07, range = 0.24–6.7).

Pollen load, seed set and seed sex ratios

The distance between a focal female and the fourth-nearest male had a significant effect on pollen capture in populations of *R. nivalis* (Figure 6.1a, Table 6.2). Stigmatic pollen loads were largest in females with males in close proximity, whereas pollen loads decreased in females with male neighbors at further distances (Figure 6.1a, Table 6.2). There was significant variation in pollen capture among the six populations of *R. nivalis* (Table 6.2). This effect was partially due to significantly lower female pollen loads in Flims compared to other populations (partial regression coefficient, \( b = -0.269, \ SE = 0.208, X^2 = 6.76, P<0.05 \)), and Arosa2, in which pollen capture did not decrease with increasing distance to males (partial regression coefficient, \( b = 0.512, \ SE = 0.681, X^2 = 0.57, P=0.45 \)). I detected no pollen on 44% of all sampled stigmas. The mean number of pollen grains per stigma for all females in the six populations was 1.65 (SE = 0.5). If I exclude stigmas that captured no pollen, the mean pollen load was 2.95 (SE = 0.08).

The distance between a focal female and its fourth-nearest male had a significant influence on seed set in *R. nivalis* (Figure 6.1b, Table 6.2), despite high average seed set in all populations (mean = 0.83, SE = 0.01). Females in closer proximity to males set more seed than females further away from males (Figure 6.1b, Table 6.2). Additionally, there was a significant population effect and a significant interaction between population and distance to the fourth-nearest male (Table 6.2). This pattern was largely driven by Arosa1, in which seed set increased with increasing distance to males (partial regression coefficient, \( b = 0.906, \ SE = 0.254, X^2 = 13.63, P<0.001 \)).
Table 6.2. Summary of statistical analyses for the influences on stigmatic pollen load, seed set, and seed sex ratio for six natural populations of *Rumex nivalis*.

<table>
<thead>
<tr>
<th>Model</th>
<th>Sources of variation</th>
<th>Test results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stigmatic pollen load</td>
<td>Population</td>
<td>$\chi^2_5 = 13.10^*$</td>
</tr>
<tr>
<td></td>
<td>Distance to fourth-nearest male</td>
<td>$\chi^2_1 = 15.76^{***}$</td>
</tr>
<tr>
<td></td>
<td>Population × distance to fourth-nearest male</td>
<td>$\chi^2_5 = 11.38^*$</td>
</tr>
<tr>
<td>Seed set</td>
<td>Population</td>
<td>$\chi^2_5 = 110.78^{***}$</td>
</tr>
<tr>
<td></td>
<td>Distance to fourth-nearest male</td>
<td>$\chi^2_1 = 7.46^{**}$</td>
</tr>
<tr>
<td></td>
<td>Population × distance to fourth-nearest male</td>
<td>$\chi^2_5 = 117.12^{***}$</td>
</tr>
<tr>
<td>Sex ratio</td>
<td>Population</td>
<td>$\chi^2_5 = 12.72^*$</td>
</tr>
<tr>
<td></td>
<td>Distance to fourth-nearest male</td>
<td>$\chi^2_1 = 8.83^{**}$</td>
</tr>
<tr>
<td></td>
<td>Population × distance to fourth-nearest male</td>
<td>$\chi^2_5 = 12.57^*$</td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
Figure 6.1. Relation between a) stigmatic pollen load, b) seed set, and c) seed sex ratios of focal females and their distance to the fourth nearest male in six natural populations of *Rumex nivalis* in Switzerland. See text and Table 6.2 for statistical details. The predicted relations based on the generalized linear models are depicted. The equations are a): 

\[ y = e^{3.51 - 0.66x} \]

b): 

\[ y = \frac{e^{2.38 - 0.14x}}{1 + e^{2.38 - 0.14x}} \]

c): 

\[ y = \frac{e^{1.56 - 0.18x}}{1 + e^{1.56 - 0.18x}} \].
The distance between focal females and their male neighbors had a significant influence on the degree of female bias in the progeny sex ratios of seeds (Figure 6.1c, Table 6.2). Females with males in close proximity had the strongest female bias with decreasing bias with increasing distance between female and male plants (Figure 6.1c, Table 6.2). There was a significant population effect and a significant interaction between population and distance to the fourth-nearest male (Table 6.2). These effects were largely driven by the absence of an association between seed sex ratios and distance to males for Davos1 (partial regression coefficient, $b = 0.014$, SE = 0.122, $X^2 = 0.01$, $P = 0.91$) and Arosa2 (partial regression coefficient, $b = 0.121$, SE = 0.126, $X^2 = 0.92$, $P = 0.34$). Fifty-seven of the 159 females produced significantly female-biased seed sex ratios and no female produced a significant bias towards male offspring. Overall, the mean progeny sex ratio was significantly biased toward daughters with a ratio of 0.65 (SE = 0.01; $X^2 = 9.76$, $P<0.0001$).

There was a positive relation between seed sex ratio and pollen load ($X^2 = 3.88$, $P<0.05$; Figure 6.2), as well as a significant effect of population ($X^2 = 13.99$, $P<0.05$). Females with higher pollen loads produced more female-biased seed sex ratios than females with lower pollen loads, although there was considerable variation in this relation (partial regression coefficient, $b = 0.133$, SE = 0.068, $X^2 = 3.86$, $P<0.05$; Figure 6.2).

Discussion

This study is the first to demonstrate that the local pollination environment can influence progeny sex ratios in populations of a dioecious plant. Females of *R. nivalis* positioned in close proximity to males captured the most pollen (Figure 6.1a), produced the highest seed set (Figure 6.1b), and exhibited the most female-biased sex ratios (Figure 6.1c). The most probable explanation for the association between higher stigmatic pollen loads and
Figure 6.2. Relation between the seed sex ratio and stigmatic pollen load of females in six natural populations of *Rumex nivalis* in Switzerland. For statistical details refer to the text. The predicted relation based on the generalized linear model is depicted. The equation for the relation is: \[ y = \frac{e^{0.52 + 0.13x}}{1 + e^{0.52 + 0.13x}}. \]
female-biased primary sex ratios in *R. nivalis* (Figure 6.2) is selective fertilization resulting from differential pollen-tube growth of female- versus male-determining pollen (certation). However, the role of certation in affecting sex-ratio variation in dioecious species is controversial (Carroll & Mulcahy 1990; Purrington 1993; Taylor et al. 1999), and previous attempts to demonstrate relations between pollination intensity and female bias under field conditions have failed (Rychlewski & Zarzycki 1975; Conn & Blum 1981).

**Pollen dispersal and female bias in seed sex ratios**

I detected a significant bias towards female offspring in open-pollinated seed families of *R. nivalis* sampled from six populations in the Swiss Alps compared to the 1:1 ratio expected based on the sex determination system. No family exhibited an excess of males. The average frequency of females (0.65) in seed families was somewhat higher than those obtained in a survey of seed sex ratios in 18 Swiss populations (mean = 0.59; Stehlik & Barrett 2005), but within the range (0.74–0.63) recorded in a common garden experiment conducted at Toronto, Ontario involving females positioned at different distances from males (Stehlik & Barrett 2006). Female-biased seed sex ratios have also been reported in other *Rumex* species. By manipulating the amount of pollen applied to stigmas of *R. acetosa* and *R. hastatalus*, Rychlewski & Zarzycki (1975) and Conn & Blum (1981), respectively, increased female bias with heavier pollen loads. Collectively, these results indicate that variation in pollination intensity can influence the degree of female bias in progeny sex ratios and this appears to be a general feature of the reproductive system in several *Rumex* species.

Wind dispersal of pollen typically follows a leptokurtic distribution with most pollen deposited near the source and a long flat tail characterized by low deposition (Tonsor 1985; Honig et al. 1992). A previous experiment conducted at Toronto with small experimental arrays of *R. nivalis* indicated a steep decrease towards the flat end of the pollen dispersal
curve (Stehlik & Barrett 2006). The range of distances employed in that experiment (5cm–150cm) was shorter than I investigated in the field, where the mean distance between a female and her closest male neighbor was 92.7cm (SE = 7.6). Pollen dispersal under natural conditions did not show as steep a decrease, probably because of the much larger number of males available for pollen donation. Higher wind speeds in the mountainous habitats of the Swiss Alps, in comparison with the earlier artificial array experiment, may have also played a role. In both studies no pollen was detected on a large proportion of stigmas that were sampled (44% this study; 68%, Stehlik & Barrett 2006). However, this was not reflected in a concomitant decrease in seed set in natural populations, which was generally high (mean = 0.83). The stigma samples almost certainly underestimated the total pollen loads that were captured because many stigmas were harvested prior to the duration of maximum longevity and some pollen may have been washed off stigmas during preservation in ethanol and slide preservation (see Stehlik & Barrett 2006).

The signal of a leptokurtic decrease in pollen capture with distance and its effect on female bias was evident in population comparisons. Arosa1, the population with the highest density of males (Table 6.1), showed the strongest decrease in female bias with increasing distance to males (mean distance between focal females and their closest males = 52.6cm, SE = 1.3; partial regression coefficient in the generalized linear model, $b = -0.532$, SE = 0.216, $\chi^2 = 6.04$, $P<0.05$), whereas Arosa2 and Davos1 had the lowest male densities and showed the weakest responses (mean distances for Arosa2 and Davos 1 = 126.7cm, SE = 7.5; and 110.7cm, SE = 4.6, respectively; partial regression coefficients, $b = 0.121$, SE = 0.126, $\chi^2 = 0.92$, $P>0.05$; $b = 0.014$, SE = 0.122, $\chi^2 = 0.01$, $P>0.05$).

Effective wind-pollination relies on efficient pollen removal and capture. This is best achieved in habitats unobstructed by vegetation and abiotic barriers, thus allowing the wind
to move through the landscape in a laminar way (Niklas 1985; Dowding 1987). *Rumex nivalis* was the dominant vegetation with no physical obstructions in most populations I sampled, including Arosa1. In contrast, nearby Arosa2 had the highest level of intermixing of *R. nivalis* with other alpine meadow plants and the terrain was also interspersed with large boulders. These differences in composition likely contributed to populations varying in their response of seed sex ratio to male distance, illustrating the potential role of ecological context in affecting progeny sex ratios.

*Mechanisms governing biased primary sex ratios*

In species with genetic sex determination, Mendelian inheritance should produce primary sex ratios of 1:1. However, both genetic and environmental factors can potentially modify the sex ratio of seeds, although the specific mechanisms involved are still poorly understood (de Jong & Klinkhamer 2005). In theory, genes modifying primary sex ratios could alter the quantity or quality of female versus male-determining pollen in males, or females could selectively abort seeds based on their gender. As yet, there is little evidence to support either mechanism (although see Taylor et al. 1999) and in *Rumex* gametophytic selection involving competition between female- and male-determining pollen tubes remains the most viable hypothesis based on the available evidence (Correns 1922, 1928; Smith 1963; Rychlewski & Zarzycki 1975; Conn & Blum 1981; Stehlik & Barrett 2006). Evolutionary stable strategy models can predict either female- or male-biased primary sex ratios depending on the relative distance of seed and pollen dispersal (de Jong et al. 2002), but the limited empirical data that is available (see Table 10.2 in de Jong & Klinkhamer 2005) are not consistent with sex allocation theory.

Investigations of sequential life cycle stages in glasshouse and field populations of *R. nivalis* have revealed increasing amounts of female bias (Figure 6 in Stehlik et al. 2007). On
average, male plants produce a small (0.52) excess of female-determining pollen with family means ranging from 0.46–0.59. A similar pattern of female bias has also recently been reported in the pollen of *R. acetosa* (Blocka-Wandas et al. 2007). Although female bias in *R. nivalis* pollen appears to be general, the degree of bias is too small to account for the observed bias in seed sex ratios. Rather, the small bias appears to be amplified by post-pollination processes leading to the selective fertilization of uniovulate flowers. Abortion of developing seeds can largely be ruled out in *R. nivalis* since female bias occurs commonly in plants with near maximum seed set and the uniovulate flowers provide limited scope for ovular competition.

Several lines of evidence indicate that environmental factors influencing the progamic phase of reproduction cause female-biased primary sex ratios in *R. nivalis*. First, Stehlik & Barrett (2005) reported an association between the degree of female bias in seed sex ratios and the flowering sex ratio of natural populations. Females in populations with strongly female-biased flowering sex ratios (proportionally fewer males) produced less female-biased offspring, whereas populations with higher frequencies of flowering males produced more female-biased progeny. This association is consistent with the certation hypothesis since larger pollen loads would be expected in populations with higher male frequencies. Second, direct evidence for the relation between pollination intensity and female biased progeny sex ratios in *R. nivalis* was obtained by manipulating pollen loads experimentally (Stehlik & Barrett 2006). The results of the present study now corroborate this finding under field conditions. Finally, supporting experimental evidence from pollination experiments on two other *Rumex* species (Correns 1928; Smith 1963; Rychlewski & Zarzycki 1975; Conn & Blum 1981) points to the role of gametophytic selection as the principle mechanism governing female-biased sex ratios in *R. nivalis*. However, it is important to emphasize that I
have not provided definitive evidence for the certation hypothesis because pollen-tube competition was only inferred and not measured directly. Obtaining direct evidence for certation remains a daunting challenge because of the technical difficulties of distinguishing female- from male-determining pollen tubes in styles of dioecious species.

The sex ratios of flowering and non-flowering (vegetative) plants in natural populations of *R. nivalis* are strongly female biased (flowering mean = 0.87, range = 0.72–0.99; vegetative mean = 0.78, range = 0.36–0.94; N=18 populations; Stehlik & Barrett 2005). This indicates that gender-based mortality also plays a role in amplifying biases established during the gametophytic and progamic phases of the life cycle. Extending ideas initially suggested by Smith (1963) and Lloyd (1974), Stehlik & Barrett (2005) proposed that the poor performance of male microgametophytes and sporophytes in *R. nivalis* may be a consequence of the accumulation of deleterious mutations on Y-sex chromosomes (Charlesworth 2002; Vyskot & Hobza 2004). According to this hypothesis, deleterious genes expressed during both the gametophytic and sporophytic phases result in poor male performance and an amplification of female bias during the sporophytic life cycle.

Environmental influences on the sex ratios of gender dimorphic plants are commonly reported as a result of variation in site quality or local resources (Case & Barrett 2004; Ashman 2006). Inbreeding is also known to bias sex ratios in several animal groups (Hamilton 1967), although such effects have not been demonstrated in dioecious plants. Mulcahy (1967) reported that female frequencies in seed progenies of *Silene latifolia* were inversely proportional to the male-to-female flower ratio of the parental population. He proposed a group-selection hypothesis based on the “reproductive economy of the sexes” to explain this pattern (and see Lewis 1942; Kaplan 1972). The study presented here is the first to demonstrate an influence of the proximity of males on progeny sex ratios.
Natural populations of *R. nivalis* are characterized by the nonrandom distribution of sexes across snow-melt gradients (Stehlik & Barrett 2005). Less female-biased seed sex ratios produced in male-scarce environments could offset local male paucity leading to temporal oscillation in sex ratio. However, further work is required to determine whether environmental adjustment of primary sex ratios, mediated by pollination intensity, is an adaptive response for optimizing the fitness of parental plants in local environments characterized by heterogeneous flowering sex ratios. An alternative hypothesis is that female-biased primary sex ratios are simply nonadaptive consequences of the sex chromosome system of *R. nivalis*. 
CHAPTER SEVEN

HIGH OUTCROSSING IN THE ANNUAL COLONIZING SPECIES AMBROSIA ARTEMISII FOLIA (ASTERACEAE)

This chapter resulted from collaborations with Spencer C. H. Barrett. Spencer Barrett contributed to the ideas and writing of the manuscript published in Annals of Botany, 2008, 101: 1303-1309.

Summary

Variation in mating patterns may be particularly evident in colonizing species because they commonly experience wide variation in plant density. Here, I explore the role of density for the mating system of Ambrosia artemisiifolia (common ragweed), a wind-pollinated annual colonizing species previously reported as self-compatible. I examined the effect of population density on the proportion of self- and cross-fertilized seeds using allozyme markers and experimental arrays conducted over two seasons in the field. I also measured the reproductive success of isolated plants located in diverse habitats. I examined the potential occurrence of a physiological mechanism preventing self-fertilization, i.e. self-incompatibility, following controlled self- and cross-pollinations in the glasshouse. Outcrossing rates estimated using allozyme markers were uniformly high, regardless of the spacing between plants. However, when single plants were isolated from congeners they set few seeds. Observations of pollen-tube growth and seed set following controlled pollinations demonstrated that plants of A. artemisiifolia possess a strong self-incompatibility mechanism, contrary to earlier reports and assumptions. The maintenance of high outcrossing rates in colonizing populations of A. artemisiifolia are likely to be facilitated by the prodigious production of wind borne pollen, high seed production and extended seed dormancy.
Introduction

The immobility of plants exerts an important influence on mating which is commonly ‘context dependent’, varying with local ecological and demographic conditions and the availability of compatible mating partners. Density-dependent and frequency-dependent influences on rates of self- and cross-fertilization have now been widely reported (e.g. Farris & Mitton 1984; Kohn & Barrett 1994; Cheptou et al. 2002; Kalisz et al. 2004; Eppley & Pannell 2007). For species that grow in ephemeral habitats, and particularly those that are annual, demographic factors and mate availability can vary greatly among neighbourhoods and between years. This unpredictability can have important reproductive consequences with respect to mating and fertility and may explain the common occurrence of self-compatibility in many annual colonizing species (Baker 1955; Mulligan & Findlay 1970; Price & Jain 1981; Pannell & Barrett 1998; Cheptou 2004; although see Abbott & Forbes 1993; Sun & Ritland 1998; Cheptou et al. 2002 for exceptions).

Demographic factors, such as the local density of plants, are well known to influence mating and fertility. Both animal- and wind-pollinated species often show a positive correlation between plant density and outcrossing rate (e.g. Vaquero et al. 1989; Murawski & Hamrick 1991; Motten & Antonovics 1992; van Treuren et al. 1993; but see Karron et al. 1995; Herlihy & Eckert 2004; Brunet & Sweet 2006). For animal-pollinated plants this relation can arise because of density-dependent changes in pollinator behaviour (reviewed in Antonovics & Levin 1980). In contrast, because wind-pollinated plants do not rely on visits by pollinators, demographic effects on outcrossing are a feature of the interaction with local microclimatic conditions and the plants themselves. For example, Eppley & Pannell (2007) recently demonstrated that in *Mercurialis annua*, a wind-pollinated androdioecious annual, selfing rates were context-dependent depending on the local density and relative frequencies
of hermaphrodites and males. In general, context-dependent mating arises from either differences in the amount of pollen captured (pollen quantity) or the type of pollen captured (pollen quality).

Here, I investigate the effect of plant density on mating in *Ambrosia artemisiifolia* (common ragweed). This wind-pollinated monoecious annual is common in eastern North America as a weed of arable crops but has been introduced to Europe, Asia and Australia where it has become invasive (Bassett & Crompton 1975; Bass et al. 2000; Chauvel et al. 2006). There have been no studies of the mating system of *A. artemisiifolia* but most workers, probably because of its annual weedy habit, have assumed the species is self-compatible and capable of selfing (e.g. Genton et al. 2005). Indeed, Jones (1936) conducted genetic studies of sexual variation in *A. artemisiifolia* and reported all monoecious forms to be self-fertile. Similarly, Bassett & Crompton (1975) stated that *A. artemisiifolia* produces viable seed through both self- and cross-fertilization and McKone & Tonkyn (1986) and Lundholm & Aarssen (1994) both report that the species is self-fertile. I therefore motivated this study on the assumption that, like many annual colonizers, *A. artemisiifolia* was self-compatible.

This study had three objectives concerning the reproductive biology of *A. artemisiifolia*. First, I investigated using allozyme markers, the influence of plant density on mating patterns in field experiments conducted over two years. Second, I examined the reproductive success of isolated *A. artemisiifolia* plants in the field to assess to what extent low mate availability might reduce seed fertility. Finally, because the results from these two experiments suggested that *A. artemisiifolia* does not have the capacity for significant self-fertilization, I investigated the compatibility status of plants using controlled self- and cross-pollinations in the glasshouse.
Methods

Study system

*Ambrosia artemisiifolia* (Asteraceae) grows abundantly in disturbed habitats on a variety of soil types, including cultivated fields, roadsides, waste places and gardens. It is a successful pioneer in early successional ecosystems (Bazzaz 1974). In Ontario, Canada, where this study were conducted, plants flower in August-September with female flowers produced in the axils of bracts or upper leaves and male flowers in terminal racemes (Payne 1963). There is no vegetative propagation, so that all reproduction occurs through seed (Bassett & Crompton 1975).

Density arrays

During summer 2005, I transplanted 750 ragweed seedlings from ten different sites at the Koffler Scientific Reserve (KSR), in Southern Ontario (44° 03' N, 79° 29' W). I selected seedlings that were uniform in size with between six and eight leaves. I then grew the seedlings in a common environment in a glasshouse at the University of Toronto in 4-inch pots until developing buds were visible. In early August, just before flowers opened, I transported plants back to KSR and planted them in three freshly tilled fields each separated by approximately 500m. Plants were randomly assigned to one of three density arrays (plants 30cm, 90cm, 150cm apart). Each array was represented twice in each field for a total of 6 replicates per density array. In each density treatment, a total of 37 plants were used, with 18 focal plants with neighbours on all sides. Plants were positioned in hexagonal arrays so that all inter-plant distances were equal. In 2006 I repeated the experiments using the same general protocols but with two modifications. First, I used density arrays of 90cm, 450cm, 900cm, and only one array per field. Second, I germinated plants from seed and set up the
arrays 3 weeks earlier in the season so that flowering in the experiment did not overlap with
flowering of local ragweed populations. Both of these modifications were undertaken in an
effort to reduce the potential influence of background levels of pollen on outcrossing rates.

For the 2005 experiment, I collected stigmas to assess stigmatic pollen loads from one
of the three density treatments in each field once a week for four weeks. At the end of the
season, I collected 20 seeds from each maternal plant to assess mating patterns in each array.
Seeds were collected at two time intervals two weeks apart, to span the range of maturation
(and stigma exposure) times. I analysed stigmatic pollen loads with repeated-measures,
general linear models (SAS PROC MIXED; 9.1; SAS 2002; Neter et al. 1996). The
dependent variable was log-transformed to assure normally distributed residuals.

I used allozyme markers and gel electrophoresis to determine the outcrossing rate of
each density array (2005: 18 arrays, six of each density treatment; 2006: six arrays, two of
each density treatment). For each density treatment, I used 20 seeds from each of the 18 focal
plants to estimate outcrossing rates for that array. Variation at four isozyme loci was resolved
using horizontal starch gel electrophoresis. Seeds from each maternal plant were separately
ground in three drops of 0.1M Tris-HCl extraction buffer (Soltis et al. 1983), and the extract
absorbed onto 3-mm chromatography paper wicks and placed directly onto 11–12% starch
gels. Using a lithium-borate buffer system (pH 8.3), I resolved four variable loci from three
enzyme systems: alcohol dehydrogenase (Adh), glutamate oxaloacetate transaminase (Got),
and phosphoglucomutase (Pgm). I stained gels for enzyme activity following recipes in
Wendel & Weeden (1991). Four alleles were detected at an Adh locus, 3 alleles at Got-1, 4
alleles at Got-2, and 3 alleles at Pgm.

I inferred genotypes based on segregation patterns characteristic of either dimeric or
monomeric codominant enzymes. Single and multi-locus outcrossing rates ($t$ and $t_{ms}$,
respectively), and pollen \((p)\) and ovule \((u)\) allele frequencies were jointly estimated using the program MLTR (vers. 0.9, Ritland 1986). This program uses maximum-likelihood to infer the genotypes of the maternal parents, allele frequencies in the pollen pool, and the proportion of progeny that are the result of outcrossing versus selfing. I obtained standard errors of the estimates from the standard deviation of 1000 bootstrap values, using the seed family as the unit of re-sampling. Within each field, separate outcrossing rates were estimated for the density arrays (2005: six arrays per field; 2006: one array per field) using a common pollen allele frequency pool for each field.

To determine whether estimates of the multi-locus outcrossing rates were significantly different from 1.0, I examined the distribution of 1000 bootstrap values following methods outlined in Eckert & Barrett (1994b). Using this method, parameter estimates for each array were considered to be significantly less than 1.0 if \(100[1-(\alpha_{PC}/2)]\) percent of the bootstrap values were all less than 1.0 (where \(\alpha_{PC}\) represents the Type 1 error rate per contrast). Similarly, for each density array, I tested whether the difference between the multi-locus and single-locus estimators of mating was significantly different from zero. I tested overall differences in outcrossing rate between the density treatments in SAS using ANOVA. The outcrossing rates for each density treatment were weighted by the inverse of the bootstrap variance estimates, to account for the sampling error in each estimate (see Barrett et al. 1994).

*Isolation experiment*

To determine the reproductive success of isolated plants in the field, I planted solitary individuals at KSR during the summer of 2006. The seed for this experiment was collected in October 2005 from open-pollinated plants within the vicinity of KSR. Following
stratification and germination procedures suggested in Willemsen (1975), I grew seeds from 40 maternal families in June 2005 in a glasshouse at the University of Toronto. Before buds were visible, I transplanted 30 plants into isolated habitats at KSR. The individuals were planted in old-field habitat, in a small patch (approximately 0.5m x 0.5m), which was cleared of surrounding vegetation. Plants were watered upon transplanting, again three days later, and a week later to ensure successful establishment. The 30 sites were at least 300m from known *A. artemisiifolia* populations, and in almost all cases were separated by forest and other woody vegetation. Plants were visited throughout flowering to ensure both female and male flowers were present. At the end of the flowering season I harvested all seed from each plant to estimate seed production.

**Self-incompatibility experiment**

I tested for self-incompatibility in *A. artemisiifolia* by comparing self- and cross-pollinated flowers for both pollen-tube growth and seed set. During fall 2006, I germinated an additional 50 plants using the same batch of seeds and germination conditions as described above. I grew plants in a glasshouse using an artificial light regime to mimic the natural conditions during flowering in Southern Ontario (14 hour light: 10 hour dark). Just before flowering, I randomly assigned plants to one of three treatments: cross-pollen donors (30 plants), cross-pollen recipients (10 plants) and self-pollen recipients (10 plants). For each plant, five flowers were brushed with either self-pollen or a mixture of pollen from five donor plants. It was necessary to use separate plants for the self- and cross-pollen treatments, to prevent the possibility of cross contamination between pollination treatments. I maintained pollen donors in one glasshouse, cross-pollen recipients in a separate house, and each self-pollen recipient plant was isolated in its own glasshouse to prevent cross contamination. Cross-pollen recipients were emasculated continuously throughout flowering to avoid any
self-pollen deposition. For the self-pollen recipients, I created covers out of three layers of spun-fibre material and placed them over female flowers to control the time of self-pollination. Twenty-four hours after pollination, styles were fixed in alcohol and examined for pollen tube growth. I used the aniline blue staining method and fluorescence microscopy to examine and count pollen germination and pollen-tube penetration. The remaining flowers were allowed to develop seed. I compared seed set between plants in each treatment and analyzed the resulting data with generalized linear models (SAS PROC GENMOD; 9.1; SAS Inst. 2002; Allison 1999) with logit transformations to accommodate the binomial distribution of data.

Results

Density arrays

In 2005 pollen receipt varied significantly between plants in the three density arrays ($F_{2,62.2} = 22.60, P<0.0001$; Figure 7.1a). Stigmas on plants in low and mid density arrays captured equivalent amounts of pollen ($t_{54.3} = 2.03, P>0.05$). In contrast, stigmas on plants in high density arrays captured significantly more pollen than those in either low or mid densities (high vs. low $t_{63.1} = 6.54, P<0.001$; high vs. mid $t_{71.2} = 4.52, P<0.001$; Figure 7.1a). There was no significant difference between the same density arrays across the three different fields ($F_{6,60.9} = 1.17, P>0.3$).

Multi-locus outcrossing rates for all arrays were close to 1.0 in both 2005 and 2006 (Figure 7.1b). Based on the distribution of bootstrap values, one of the 26 arrays had an outcrossing rate that differed significantly from 1.0 (2005, one mid density array). For all arrays, the differences between the multi-locus and single-locus estimators of outcrossing rate were not significantly different from zero.
Figure 7.1. Influences of plant density (low, mid, high) on a) mean (SE) number of pollen grains on stigmas and b) multi-locus outcrossing rate (t, SE) for 2005 (●) and 2006 (▲) in experimental field arrays of *Ambrosia artemisiifolia*. Letters indicate the outcomes of Dunn-Šidák multiple comparisons. See text for statistical details.
There were no significant differences among the outcrossing rates of the three density arrays in either year (2005: $F_{2,10} = 3.29, P>0.05$; 2006: $F_{2,7} = 0.44, P>0.6$; Figure 7.1b). In 2005 there was no significant difference in outcrossing rate between the same density arrays among the three fields ($F_{3,10} = 1.28, P>0.3$).

*Seed production of isolated plants*

Plants in isolated locations at KSR set between 0 and 254 seeds (mean = 58.04 SE = 15.11), well below the maximum potential seed set since observations indicated that the plants produced hundreds of female flowers.

*Testing for self-incompatibility*

I found a significant difference in successful pollen tube penetration of the stigmatic tissue between plants that received cross- versus self-pollen ($F_{1,11.1} = 1721.53, P<0.0001$; Figure 7.2). The two classes of unsuccessful penetration (no germination, and germination but no penetration) also yielded significant differences between the two treatments (no germination: self vs. cross $F_{1,10.4} = 23.00, P<0.001$; germination: self vs. cross $F_{1,9.82} = 157.73, P<0.0001$; Figure 7.2). Qualitative differences in the growth of pollen tubes between cross- versus self-pollen can be clearly seen in Figure 7.3, where cross-pollen tubes grow straight and long and penetrate the stigma (Figure 7.3a), whereas self-pollen tubes are unable to penetrate the stigma and there is formation of callose (Figure 7.3b) typical of a self-incompatibility reaction (de Nettancourt 1977).

There was a significant difference in the proportion of seed set between plants that received cross- versus self-pollen ($X^2 = 79.55, P<0.0001$). Plants that received cross-pollen set mean = 0.56 (SE = 0.03) proportion seed; whereas plants that received self-pollen set mean = 0.04 (SE = 0.02).
Figure 7.2. Relation of proportion of pollen grains (SE) that fall into each subclass (germination, penetration of the style) for *Ambrosia artemisiifolia* plants that received outcross pollen (●) and self pollen (▲). Letters indicate the outcomes of Dunn-Šidák multiple comparisons within each subclass between the two treatments, with capital letters for germination, and lower case letters for penetration. See text for statistical details.
Figure 7.3. Pollen grains and tubes of *Ambrosia artemisiifolia* under a fluorescence microscope showing pollen tube growth for a) cross-pollen and b) self-pollen at 200 x magnification. Self-pollen tubes do not penetrate the style due to the formation of callose, while cross-pollen tubes penetrate the style, growing long and straight towards the ovule.
Discussion

The results clearly demonstrate that *Ambrosia artemisiifolia* is a highly outcrossing, self-incompatible plant. Provided that population densities are sufficient, seed set is generally high. Isolated plants experienced reduced seed set, although they still set some seed. The glasshouse experiment demonstrated that plants exposed to only self-pollen set very few seeds relative to those that were cross-pollinated. Furthermore, fluorescence microscopy provided evidence that *A. artemisiifolia* is self-incompatible based on observations of pollen germination and pollen tube growth. These findings were unexpected and contrary to previous reports that the species is self-compatible (e.g. Jones 1936; Bassett & Crompton 1975).

I detected a significant effect of plant density on stigmatic pollen loads with plants in high-density patches capturing more pollen grains. However, because flowers of *A. artemisiifolia* are uniovulate, plants at lower densities still captured sufficient pollen for high seed set. Measures of stigmatic pollen loads did not differentiate between self- and cross-pollen, so it is likely that a substantial portion of the pollen captured was self-pollen. In contrast to most wind-pollinated plants where pollen is held in the anthers until it is removed by the wind, studies on the release of pollen in *A. artemisiifolia* indicate that as anthers swell and dehisce, pollen first falls passively downwards to vegetation and is then swept away by the wind (Bianchi et al. 1959). This process causes deposition of large quantities of self-pollen on stigmas within the same plant (geitonogamy). Opportunities for geitonogamous pollination are exacerbated by the inflorescence architecture of *A. artemisiifolia*. Male flowers are typically positioned above female flowers and despite some protandry there is considerable overlap between the sex functions. Self-incompatibility in *A. artemisiifolia* may
therefore function largely to limit the deleterious consequences of inbreeding that arise from high levels of geitonogamous pollination.

Controlled pollinations in the glasshouse demonstrated that \textit{A. artemisiifolia} has a self-incompatibility system. Seven of the ten plants set no seed following self-pollination, with three plants setting small amounts of seed. There may be some leakiness in the SI system, as is commonly observed in self-incompatible species (de Nettancourt 1977; Levin 1996; Stephenson et al. 2000) and has been reported in other species of Asteraceae (Ferrer & Good-Avila 2007). It is possible that previous reports of self-compatibility in \textit{A. artemisiifolia} (Jones 1936; Bassett & Crompton 1975) involved plants with weak self-incompatibility. However, neither of these studies was designed to specifically test self-incompatibility, and so I cannot evaluate the rigour of their experimental designs or the validity of the results. Surveys of Asteraceae indicate that many species are partially self-incompatible (Ferrer & Good-Avila 2007), and, in some cases, this may have resulted from selection for reproductive assurance in colonizing populations or other demographic factors (Hiscock 2000, Cheptou et al. 2001, 2002; but see Brennan et al. 2005). Further studies of the incompatibility status of \textit{A. artemisiifolia} populations would certainly be warranted, especially in the invasive range (see Genton et al. 2005) where demographic factors associated with repeated colonizing events may possibly favour the breakdown of self-incompatibility.

The relative costs and benefits of self-compatibility versus self-incompatibility in colonizing species depend on a variety of demographic and life-history conditions including plant density, propagule number, life-span, seed dormancy and the capacity for clonal reproduction. Pannell & Barrett (1998) investigated the effects of different life-history traits on the reproductive success of self-compatible and self-incompatible phenotypes in a
metapopulation context. Their results suggest that the advantage of self-compatible phenotypes through reproductive assurance is diminished if plants can survive unfavourable conditions in a dormant seed bank. Germination studies in *A. artemisiifolia* indicate that seeds can remain viable for 39 years or more when buried in the soil (Toole & Brown 1946). Pannell & Barrett (1998) also found that enhanced seed productivity can offset the disadvantage possessed by self-incompatible plants. According to Dickerson & Sweet (1971), a small *A. artemisiifolia* plant produces about 3000 seeds, while large plants can produce up to 62000 seeds. Thus, although *A. artemisiifolia* is an annual species with no vegetative propagation, its prolific seed production, seed dormancy and high population densities may offset the cost of being self-incompatible allowing successful colonization.

*Ambrosia artemisiifolia* is an aggressive weed in North America, and invasive in Europe. Records from Europe suggest an early origin in France during the 18th century, and substantial spread since introduction as a seed contaminant in crops from North America (Chauvel et al. 2006). Both herbarium records (Chauvel et al. 2006) and a recent molecular study suggest multiple independent introductions (Genton et al. 2005) with no loss of genetic diversity in introduced populations compared to native North American populations. This result would be unexpected in a selfing colonist because uniparental reproduction commonly leads to severe genetic bottlenecks during range expansion in invading species (reviewed in Novak & Mack 2005; Barrett et al. 2008). However, their finding of similar levels of genetic diversity in the introduced and native range of *A. artemisiifolia* is consistent with what one might expect for a highly outcrossing, self-incompatible colonist that has spread through multiple introductions. Extensive pollen dispersal through wind pollination is more likely to foster outcrossing among separate introductions and the maintenance of genetic diversity in the introduced range.
Self-incompatible plants may suffer reduced reproductive success when population density or size is low (‘Allee effect’, Allee 1951). Animal-pollinated species may be more prone to Allee effects through insufficient pollen transfer or through the transfer of different species’ pollen, particularly when rare plants are surrounded by other flowering species (Kunin 1993, 1997) or patches are quite isolated (Aizen & Feinsinger 1994). Because *A. artemisiifolia* is wind-pollinated and plants produce copious pollen, the effects of being relatively isolated may to some extent be diminished. Long-distance transport of pollen in *A. artemisiifolia* could alleviate some of the costs associated with growing sparsely. There are records of *A. artemisiifolia* pollen appearing in air samples hundreds of kilometers from the nearest population indicating long-distance transport of ragweed pollen (e.g. Lorenzo et al. 2006; Stach et al. 2007), although the duration of pollen viability is unknown. I recorded low levels of seed set in isolated plants of *A. artemisiifolia* positioned among forest patches. However, whether this seed resulted from self-pollination in plants with leaky SI or from long-distance pollen transport is not known.

Pollen limitation has been well documented and discussed in animal-pollinated plants (see Burd 1994; Larson & Barrett 2000; Ashman et al. 2004; Knight et al. 2005 for reviews), but there is much less evidence on whether pollen availability limits reproduction in wind-pollinated plants. The dispersal of wind-borne pollen from point sources has a leptokurtic distribution, although the direction, speed, and turbulence of wind and settling velocity of pollen also affects patterns of pollen deposition (Bateman 1947; Gleaves 1973; Dowding 1987; Okubo & Levin 1989; Giddings 2000). Recent empirical work suggests that pollen capture and the proportion of fertilized ovules may decrease rapidly with increasing distance from pollen donors in wind-pollinated plants (Knapp et al. 2001; Davis et al. 2004a; Stehlik & Barrett 2006; Eppley & Pannell 2007). The density experiments indicated that pollen
capture decreased with increasing distance, but this did not have an effect on outcrossing rates. Also, isolated plants set few seeds, suggesting that they may have suffered from pollen limitation. However, various features of pollen dispersal in *Ambrosia artemisiifolia* demonstrating long-distance transport (Raynor et al. 1968, 1970, 1973) suggest that pollen-limited reproduction may be relatively uncommon in *Ambrosia artemisiifolia*.

The study illustrates the importance of understanding both demographic and genetic influences on mating patterns in plant populations. Because *Ambrosia artemisiifolia* is a weedy, colonizing species, the demographic conditions in which it occurs should have important influences on mating and fertility. Although this study suggests that *Ambrosia artemisiifolia* may be obligately outcrossing due to self-incompatibility, various features of its ecology and reproductive biology may ameliorate the costs that are normally associated with self-incompatibility in colonizing annuals. Traits that could diminish costs associated with outcrossing include producing enormous quantities of wind borne pollen, prolific seed production and the presence of a seed bank.
CHAPTER EIGHT

GENETIC AND ENVIRONMENTAL CONTROL OF TEMPORAL AND SIZE-DEPENDENT SEX ALLOCATION IN A WIND-POLLINATED PLANT

Summary

Sex allocation in hermaphrodites can be affected by spatial and temporal variation in resources, especially in plants where size-dependent gender modification is commonplace. However, the evolution of sex allocation depends on variation in patterns of investment in female and male function and the relative importance of genetic and environmental factors in governing this variation. Theoretical models in wind-pollinated plants predict a positive relation between increasing size and greater male investment because of the fitness advantages associated with more effective pollen dispersal. Here, I grow maternal half-sib families of annual, wind-pollinated, Ambrosia artemisiifolia in the sun and shade to investigate the genetic and environmental components of sex allocation over time. There was significant genetic variation for female and male flower production in both the sun and shade treatments. After controlling for plant size, I found weak evidence for a negative genetic correlation in the sun treatment only. Size-dependent sex allocation occurred in the direction predicted by theory, with male flower production increasing more rapidly in large plants in the sun. The timing of flower production differed between the treatments, with significant genetic variation for dichogamy within environments and plasticity of this trait between environments. The occurrence in A. artemisiifolia of a heritable component to female and male investment and genotype x environment interactions indicate the potential for adaptive sex allocation under different ecological conditions.
Introduction

In hermaphrodites the optimal allocation to male and female sex functions can vary with environment, age or size (Charnov 1982; Lloyd & Bawa 1984; Klinkhamer et al. 1997). Thus, selection should favour a genetically determined allocation rule specifying allocation in relation to size or environment, rather than a fixed genetically determined allocation (Lloyd & Bawa 1984; Zhang 2006). There is some evidence for developmental plasticity in allocation patterns in both animals (reviewed in Schärer 2009) and plants (reviewed in de Jong & Klinkhamer 2005). Flexible sex allocation may be particularly feasible to implement in plants because of their modular growth and the production of multiple flowers, which can vary individually in sex expression. In this way, sex allocation has the potential to influence gender strategies and dichogamy with consequences for reproduction and mating.

Models of sex allocation in hermaphroditic plants have attempted to explain varying patterns of investment into female and male function (Charleworth & Charlesworth 1981, 1987; Charnov 1982; Lloyd & Bawa 1984; Charlesworth & Morgan 1991; Morgan 1992). The models typically use gain curves based on the shape of the relation between investment and fitness return from that investment. Nonlinearity in one or both curves can cause the optimal allocation to be unequal. Moreover, the female and male components of fitness can vary differentially between environments and between individuals of different sizes. Thus variation in the patterns of sex allocation may arise when individuals are selected to alter their allocation in response to environmental conditions, or in response to increasing age or size (Lloyd & Bawa 1984; de Jong & Klinkhamer 1989; Zhang 2006). A clear example of this occurs in organisms with sequential hermaphroditism, where female and male reproduction comprise discrete stages of the life-cycle, and studies have identified the optimal size at which individuals should switch sex (Ghiselen 1969; Charnov 1982;
However, the vast majority of plant species are simultaneous hermaphrodites in which size-related changes in the relative investment in female and male function and a more flexible modification of allocation can occur (Freeman et al. 1980; Lloyd & Bawa 1984; Klinkhamer et al. 1997; Wright & Barrett 1999).

Sex allocation models are phenotypic in nature and typically use an evolutionary stable strategy approach to determine the optimal allocation of reproductive resources to female and male functions. Thus, they implicitly assume that female and male sexual functions share a limiting resource. One predicted outcome of such resource sharing is the occurrence of negative genetic correlations among floral traits. However, few studies have found negative genetic correlations between female and male allocation, despite significant genetic variation for most floral traits (Ashman & Majetic 2006). The extent to which sexual traits are influenced by environmental conditions is seldom considered (but see Mazer & Schick 1991; Conner et al. 2003; Mazer et al. 2003; Caruso 2006), despite the importance of determining the relative importance of genetic and environmental factors for predicting selection response on sex allocation.

Negative genetic correlations and trade-offs in female and male traits may be rarely detected because variation in resource acquisition among plants can obscure relations (van Noordwijk & de Jong 1986; Worley et al. 2003). Larger plants will often have more resources to invest in reproduction, and so an overall positive correlation is found between investment in female and male function. However, if there are dissimilarities in fitness gain through female and male function with increasing size then size-dependent sex allocation is beneficial (Zhang 2006). In wind-pollinated species, plant size can affect fitness returns directly and represent one of the clearest examples of direct size-dependent effects on allocation (Niklas 1992; Burd & Allen 1998). Pollen dispersal is expected to be more
effective with increased plant height both because it will be dispersed more easily from the plant at greater wind speeds, and because it will travel further and thus have better access to mates. Although absolute plant height is probably more important in determining the distance that pollen grains are dispersed, relative plant height may be more important when considering mating opportunities and local mate competition. A relatively tall plant should function better as a pollen donor, while a relatively diminutive plant may function better as a pollen recipient. Thus, contrary to the traditional expectation of greater female allocation in larger plants (Lloyd & Bawa 1984), larger wind-pollinated plants should have greater male investment and indeed there is some evidence for this (e.g. Solomon 1989; Aizen & Kenigsten 1990; Ackerly & Jasienski 1990; Fox 1993; Dajoz & Sandmeier 1997; Pannell 1997; McKone et al. 1998).

Many plant species use decreased light to sense the presence of competitors and this can influence height growth (Dudley & Schmitt 1996; Donohue et al. 2000). A reduction in the ratio of red to far-red light can indicate the proximity of competing neighbours, enabling developmental strategies to either tolerate or avoid shade before canopy closure. Cues induce a suite of plastic responses known as the shade avoidance syndrome (Dudley & Schmitt 1996; Franklin 2008). This includes stem elongation and accelerated reproduction (Donohue et al. 2001; Franklin 2008). The presence of shade, sensed through a reduction in total light quantity, can indicate to a plant that it is diminutive relative to its neighbours, and this may affect sex allocation and the timing of reproduction.

Here, I investigate the effects of genetic and environmental factors on allocation to female and male flower production in Common Ragweed (Ambrosia artemisiifolia - Asteraceae). Sex allocation in this species is variable and there is evidence that ecological conditions influence the relative production of female and male flowers (McKone & Tonkyn
1986; Ackerly & Jasienski 1990, Traveset 1992; Lundholm & Aarssen 1994; Paquin & Aarssen 2004). However, to what extent genetic variation also contributes toward gender expression in populations of *A. artemisiifolia* is poorly understood (although see Jones 1936). *Ambrosia artemisiifolia* is particularly suitable for investigating the genetic and environmental regulation of gender because it is monoecious, self-incompatible and monocarpic (Chapter Seven). Monoecy allows the easy quantification of female and male allocation, and the presence of self-incompatibility simplifies interpretation of results because geitonogamous self-fertilization and its effects on fitness do not need to be considered. Finally, the annual habit enables an assessment of lifetime reproductive investment, without concern for future survival or reproduction.

Using an experimental approach, I examine the genetic and environmental components of temporal and size-dependent sex allocation in *A. artemisiifolia* in two environmental treatments (sun and shade). I investigate the predictions of sex allocation models that include size-dependent effects on the timing of female and male investment by addressing three specific questions: 1) Is there evidence for quantitative genetic variation in female and male flower production and are these components of sex allocation genetically correlated? Quantitative genetic variation in sex allocation, including any interactions with environment, would enable populations to adjust their sex functions to prevailing ecological conditions. 2) Do contrasting sun and shade environments affect plant size and allocation to female and male flowers? By imposing treatments that alter plant size, my objective was to separate environmental and genetic causes of plant-size variation to assess whether size-dependent sex allocation occurs in the direction predicted by theory (Lloyd & Bawa 1984; de Jong & Klinkhamer 2005). 3) Is there evidence for genetic and environmental regulation of temporal changes in female and male function with consequences for the expression of
dichogamy? Recent theory suggests that temporal changes in sex allocation may serve as an adaptive response to environmental stochasticity in resource availability (Zhang 2006). However, empirical support for this idea is lacking and flexible environmentally-induced dichogamy has not been reported in the literature.

Methods

Study species and sampling

*Ambrosia artemisiifolia* (Asteraceae) is native to eastern North America but has become invasive in several regions including Europe, Asia and Australia. It grows abundantly in disturbed habitats on a variety of soil types, including cultivated fields, roadsides, waste places and gardens and experiences a wide range of light regimes (Bazzaz 1974). Male flowers are born on terminal racemes on both the primary stem and on lateral branches, whereas female flowers occur in small clusters in the leaf and branch axils only (Payne 1963). Female flowers have a single ovule and plants are highly outcrossing with an estimated female outcrossing rate of $t=0.95$ (Chapter Seven). Because there is no vegetative propagation in *A. artemisiifolia* all reproduction occurs by seed.

I collected maternal seed families from an extensive population at the Koffler Scientific Reserve in Ontario (44° 03' N, 79° 29' W) in October 2006. The plants were located in several colonies occurring in abandoned fields, disturbed ground and roadside edges. At least 30 seeds were collected from each of 80 randomly selected open-pollinated plants. Following a cold-stratification period (4°C and 24-hour darkness), I germinated seeds in petri dishes at the end of May 2007 in a glasshouse at the University of Toronto under ambient sunlight.
Experimental sun and shade treatment and measurements

When seedlings had produced two fully opened cotyledons they were transplanted into 2.5cm peat pots. Pots were filled with a soil mix consisting of 75% Pro-Mix, 20% sand and 5% topsoil. Plants were then placed into randomly pre-determined locations in one of three blocks within either a sun or shade treatment. Two weeks later I transplanted plants into 10cm pots. Plants were watered as needed and treated with water soluble fertilizer once (20:20:20 N:P:K, concentration 1.5g/L). I sprayed plants with a pesticide (Avid) to control for spider mites twice during the experiment.

The shade treatment was implemented by erecting three 1.2m rectangular frames above glasshouse benches constructed using 3.5 × 3.5cm cedar posts, resulting in three blocks. I covered these frames with black neutral-density shade cloth that reduced light levels by ~80%. The sun treatment also involved three blocks that received ambient full sunlight in the rooftop glasshouse. All blocks were in the same glasshouse. Each family had equal numbers of individuals in each treatment, and all families were represented in each of the three blocks per treatment. There was a total of 1523 plants in the experiment, with 760 in the shade and 763 in the sun. The experiment ran for a total of 118 days, and was terminated when a majority of plants were fruiting and beginning to senescence. The duration of plant growth in the experiment was equivalent to the annual life cycle in field populations.

I recorded height and canopy width at the widest part of the plant bi-weekly, beginning 7 days after transplanting. For each plant, I recorded the date the first female and first male flower commenced anthesis and the number of days between the start of each sex function. I counted the number of branches on each plant just prior to senescence. Four times during the experiment I counted the number of female flowers and estimated the number of male flowers on every plant by measuring the length of each male inflorescence. For a subset
of 40 plants, randomly selected from each treatment and different families, I measured the length of inflorescences and counted the number of flowers and assessed the fit of the regression equation separately for each treatment. The fit of the regression was $R^2=0.89$ for sun plants and $R^2=0.87$ for shade plants. This relation was used to estimate the number of male flowers from the inflorescence length for each plant. Just prior to senescence, I harvested all plants and weighed the aboveground dry biomass.

**Environmental influences on sex allocation**

To examine if the environmental treatments affected the proportion of male flowers, I used generalized linear mixed models (SAS PROC GLIMMIX; 9.1; SAS Inst. 2002). GLMM is a pseudo-likelihood statistical procedure specifically designed to analyse mixed model data with categorical or binomial response variables (Wolfinger & O’Connell 1993). The data was fit to a binomial distribution with a logit-link function, with treatment and block treated as fixed factors and family as a random factor.

**Genetic influences on sex allocation**

To examine the effects of quantitative genetic variation and environmental conditions on the patterns of sex allocation, I first standardized female and male flower production to a mean of zero and a standard deviation of one. I performed this standardization because I used the number of flowers as the unit of investment, but there is no reason to assume that the costs of female and male flowers are necessarily equal. I used a mixed model analysis (SAS PROC MIXED; 9.1; SAS Inst. 2002) to determine the effect of treatment, family and family $\times$ treatment on the production of female and male flowers. I used restricted maximum likelihood (REML) to estimate the variance components of random effects involving family, and their significance was calculated from log-likelihood ratio tests (Saxton 2004). I obtained
family means for traits using the best-linear unbiased predictors (BLUP’s) and fitted a single
model that included both levels of the trait (female and male flowers), and allowed among-
family and within-family variances and covariances to differ between treatments. I estimated
variance components and heritability of traits following methods described in Lynch &
Walsh (1998). I estimated genetic variance as four times the family variance component, and
calculated the broad-sense heritability ($H^2$) as the genetic variance divided by the total
phenotypic variance. I used the covariance parameter estimates to calculate the genetic
covariance and genetic correlation between female and male investment in each treatment
separately. I tested whether genetic variance ($V_G$) was significantly greater than zero by
likelihood ratio tests and whether $COV_A$ and $r_A$ differed significantly from zero by
constraining them in the model and using a likelihood ratio test with one degree of freedom.

The estimates of genetic variance are based on seed from field-collected maternal
families and therefore maternal environmental effects could inflate the estimates. To assess
this possibility, I measured the size (length in mm) of a random sub-sample of eight seeds
from each family prior to germination. The mean seed size across families ranged from
2.9mm–4mm. For each treatment, I calculated correlation coefficients using family means
between seed size and height one-week post-germination, final height, flowering date,
number of branches, and final number of female and male flowers. In both treatments there
was a significant positive correlation between seed size and height one week post-
germination (shade: $r = 0.38$, $P<0.05$; sun $r = 0.23$, $P<0.05$). However, there were no
significant correlations with any of the other variables measured later in plant growth. This
suggests that maternal environment is unlikely to strongly influence measures of sex
allocation or its interactions with size and treatment.
The effect of plant size on sex allocation under sun and shade

To investigate the relation between measurements of plant size (number of branches, dry biomass, vegetative height and canopy width) and the number of female and male flowers in sun and shade treatments I calculated phenotypic correlations among the traits for plants in each treatment. I determined the least-squares means and examined the effect of treatment on each character separately using a mixed model analysis (SAS PROC MIXED). The models included block nested within treatment as a fixed effect and family as a random effect.

To determine the size-dependent production of flowers, I used a general linear model (SAS PROC MIXED) that included plant size, treatment and floral sex (and their pairwise and three-way interactions) as fixed effects and family as a random effect. Because the analysis involved repeated measurements of the same subject (female and male floral counts) I used repeated measures to account for the lack of independence (Jennrich & Schluchter 1986). I used Akaike’s Information Criterion to choose between pooled or unstructured variance models. I selected between the different measures of plant size using Akaike’s Information Criterion, and used number of branches as the measure of plant size as this gave the best fit.

Dichogamy and temporal changes in sex allocation

I used a random regression mixed model analysis in SAS PROC MIXED to investigate the production of female and male flowers in each treatment over time. This approach fits individual intercepts and slopes for each family and accounts for the repeated measures design. It allowed me to account for the tendency for increased dispersion in the residuals towards the end of growth when total floral counts are highest. Because the data did
not show a linear increase in flower production over time I included both the linear and quadratic effects of time.

To investigate the effect of treatment and family on the type and expression of dichogamy, I calculated the difference in days between the first female and first male flower for each plant. A score of zero indicates no dichogamy (i.e. female and male flowers opened on the same day for a particular plant) whereas negative numbers indicate that plants were protogynous, and positive numbers indicate that plants were protandrous. The number itself represents the number of days separating sex functions. I then used a mixed model analysis in SAS PROC MIXED to determine the effect of treatment, family, and family × treatment on dichogamy scores. I used restricted maximum likelihood (REML) to estimate the variance components of random effects (those involving family), and their significance was calculated from log-likelihood ratio tests. I estimated variance components and heritability of dichogamy traits following methods described in Lynch & Walsh (1998). I fitted a model that allowed among-family and within-family variances to differ between treatments. I estimated genetic variance as four times the family variance component and calculated the broad-sense heritability as the genetic variance divided by the total phenotypic variance.

To determine whether the degree of dichogamy (number of days between alternate sex functions) differed between protogynous and protandrous plants in each treatment, I classified plants as protogynous or protandrous and then examined the effect of this classification on the degree of dichogamy. I used a mixed model analysis in SAS with the absolute number of days separating sex functions as the independent variable and determined the effects of treatment, dichogamy class, and their interaction. I used estimate statements to understand the cause of significant interactions.
Results

Variation in sex allocation and indices of plant size

Phenotypic correlations among indices of plant size indicated that they were all positively correlated with one another, and positively correlated with allocation to female and male flowers in both treatments (Table 8.1). All measures of plant size differed significantly between the two treatments (Table 8.2), with greater allocation to female flowers, male flowers, dry biomass, width and branching in the sun treatment. Only final plant height was greater in the shade treatment.

The effect of treatment and family on sex allocation

Overall, plants in the sun produced more female and male flowers than plants in the shade (Table 8.2). The proportion of male flowers differed significantly between the treatments \( F_{1,1257} = 636.31, P<0.0001 \), with plants in the sun making a larger fraction of male flowers than plants in the shade (male flowers/total flower production shade: mean = 0.48, SE = 0.02; sun: mean = 0.61, SE = 0.02, adjusted for other factors in the model).

I found a significant effect of treatment, family and a family by treatment interaction in the allocation to female and male flowers (Table 8.3). Families differed significantly in the number of female and male flowers they produced across the two treatments (Table 8.3). There was more variation among family means in female allocation in the shade treatment. In contrast, in the sun treatment this pattern was reversed with a greater amount of variation in male allocation (Table 8.4; Figure 8.1). There was also a significant interaction between family and treatment, but only for allocation to female flowers (Table 8.3, Figure 8.2). Families that had higher allocation to female flowers in the shade treatment had lower allocation to female flowers in the sun treatment and vice versa.
Table 8.1. Phenotypic correlations among measurements of plant size and sex allocation traits in *Ambrosia artemisiifolia* grown in sun and shade treatments in the glasshouse.

|                      | Shade (n=668) |          | Shade (n=668) |          | Shade (n=670) |          | Sun (n=672) |          | Sun (n=674) |          | Sun (n=673) |          | Sun (n=678) |          | Sun (n=678) |          |
|----------------------|--------------|----------|--------------|----------|--------------|----------|------------|----------|------------|----------|------------|----------|------------|----------|------------|
| Dry biomass          | 0.45         | 0.73     | 0.61         | 0.76     | 0.74         | 0.26     | 0.47       | 0.56     | 0.46       | 0.51     | 0.51       | 0.51     | 0.51       | 0.51     |
| Vegetative height    | 0.36         | 0.23     | 0.35         | 0.31     |              | 0.18     | 0.05       | 0.16     | 0.03       |          |            |          |            |          |            |
| Branches             | 0.51         | 0.66     | 0.65         |          |              | 0.31     | 0.34       | 0.48     |            |          |            |          |            |          |            |
| Plant width          |              | 0.46     | 0.55         |          |              |          | 0.18       | 0.43     |            |          |            |          |            |          |            |
| Female flowers       |              | 0.57     |              |          |              |          |            | 0.25     |            |          |            |          |            |          |            |
Table 8.2. Least-squares means (SE) and test-statistics for treatment effects for seven traits in *Ambrosia artemisiifolia* grown in shade or sun treatments in the glasshouse. The value for dichogamy represents the mean number of days between the day of first male anthesis and the day of first female anthesis (i.e. negative values indicate protogyny, positive values indicate protandry). Values for female flowers, male flowers, number of branches, dry biomass, and vegetative height were recorded just prior to senescence. Values for plant width were recorded 2 weeks prior to senescence.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Shade</th>
<th>Sun</th>
<th>F-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichogamy (days)</td>
<td>-1.25 (0.19)</td>
<td>1.82 (0.19)</td>
<td>$F_{1,154} = 99.57^{***}$</td>
</tr>
<tr>
<td>Female flowers (#)</td>
<td>167.61 (4.02)</td>
<td>247.53 (3.74)</td>
<td>$F_{1,154} = 211.24^{***}$</td>
</tr>
<tr>
<td>Male flowers (#)</td>
<td>279.66 (6.45)</td>
<td>462.19 (11.27)</td>
<td>$F_{1,154} = 197.40^{***}$</td>
</tr>
<tr>
<td>Branches (#)</td>
<td>9.49 (0.28)</td>
<td>15.44 (0.20)</td>
<td>$F_{1,154} = 295.14^{***}$</td>
</tr>
<tr>
<td>Dry biomass (g)</td>
<td>1.11 (0.03)</td>
<td>2.77 (0.04)</td>
<td>$F_{1,154} = 1165.26^{***}$</td>
</tr>
<tr>
<td>Vegetative height (cm)</td>
<td>29.51 (0.54)</td>
<td>19.27 (0.41)</td>
<td>$F_{1,154} = 231.42^{***}$</td>
</tr>
<tr>
<td>Width (cm)</td>
<td>18.32 (0.21)</td>
<td>19.15 (0.32)</td>
<td>$F_{1,154} = 4.66^{*}$</td>
</tr>
</tbody>
</table>

*P<0.05, ** P<0.01, *** P<0.0001*
Table 8.3. Summary of REML general linear mixed-models of the influences on the number of female and male flowers produced by families of *Ambrosia artemisiifolia* plants in sun and shade treatments in the glasshouse.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Source of variation</th>
<th>Test statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # female flowers</td>
<td>Block (treatment)</td>
<td>$F_{4,1185} = 20.62$ ***</td>
</tr>
<tr>
<td></td>
<td>Family</td>
<td>$\chi^2 = 40.9$ ***</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>$F_{1,77} = 244.52$ ***</td>
</tr>
<tr>
<td></td>
<td>Treatment × family</td>
<td>$\chi^2 = 14.1$ ***</td>
</tr>
<tr>
<td>Total # male flowers</td>
<td>Block (treatment)</td>
<td>$F_{4,1185} = 56.29$ ***</td>
</tr>
<tr>
<td></td>
<td>Family</td>
<td>$\chi^2 = 81.3$ ***</td>
</tr>
<tr>
<td></td>
<td>Treatment</td>
<td>$F_{1,77} = 407.24$ ***</td>
</tr>
<tr>
<td></td>
<td>Treatment × family</td>
<td>$\chi^2 = 1.1$ NS</td>
</tr>
<tr>
<td>Time - female flowers</td>
<td>Treatment</td>
<td>$F_{2,122} = 149.74$ ***</td>
</tr>
<tr>
<td></td>
<td>Treatment × time</td>
<td>$F_{2,317} = 781.77$ ***</td>
</tr>
<tr>
<td></td>
<td>Treatment × time × time</td>
<td>$F_{2,122} = 2931.56$ ***</td>
</tr>
<tr>
<td>Time - male flowers</td>
<td>Treatment</td>
<td>$F_{2,231} = 70.78$ ***</td>
</tr>
<tr>
<td></td>
<td>Treatment × time</td>
<td>$F_{2,424} = 390.68$ ***</td>
</tr>
<tr>
<td></td>
<td>Treatment × time × time</td>
<td>$F_{2,673} = 1074.06$ ***</td>
</tr>
</tbody>
</table>

*P<0.05, ** P<0.01, *** P<0.0001
Table 8.4. Genetic variance ($V_G$), environmental variance ($V_E$), broad-sense estimates of heritability ($H^2$), genetic covariance ($COV_G$), and maternal family genetic correlation ($r_G$) for measured and size-adjusted (parameter followed by (adj)) allocation to female and male flowers in sun and shade treatments for *Ambrosia artemisiifolia* in the glasshouse. Standard errors are in parentheses and are calculated from the asymptotic covariance matrix. Asterisks represent values that are significantly different from zero, based on likelihood-ratio tests.

<table>
<thead>
<tr>
<th></th>
<th>Shade</th>
<th></th>
<th></th>
<th>Sun</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>$V_G$</td>
<td>0.42 (0.12)***</td>
<td>0.19 (0.06)***</td>
<td>0.16 (0.08) *</td>
<td>0.64 (0.17)***</td>
<td></td>
</tr>
<tr>
<td>$V_G$ (adj)</td>
<td>0.24 (0.07)***</td>
<td>0.11 (0.04)***</td>
<td>0.05 (0.04) NS</td>
<td>0.45 (0.12)***</td>
<td></td>
</tr>
<tr>
<td>$V_E$</td>
<td>0.29</td>
<td>0.23</td>
<td>0.62</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
<td>$V_E$ (adj)</td>
<td>0.17</td>
<td>0.24</td>
<td>0.65</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>$H^2$</td>
<td>0.59 (0.07)***</td>
<td>0.45 (0.04)***</td>
<td>0.21 (0.04) *</td>
<td>0.63 (0.10)***</td>
<td></td>
</tr>
<tr>
<td>$H^2$ (adj)</td>
<td>0.58 (0.06)***</td>
<td>0.42 (0.03)***</td>
<td>0.07 (0.04) NS</td>
<td>0.57 (0.11)***</td>
<td></td>
</tr>
<tr>
<td>$COV_G$</td>
<td>0.13 (0.06) *</td>
<td></td>
<td></td>
<td>0.11 (0.08) NS</td>
<td></td>
</tr>
<tr>
<td>$COV_G$ (adj)</td>
<td>-0.01 (0.03) NS</td>
<td></td>
<td></td>
<td>-0.04 (0.06) NS</td>
<td></td>
</tr>
<tr>
<td>$r_G$</td>
<td>0.45 (0.16) *</td>
<td></td>
<td></td>
<td>0.36 (0.24) NS</td>
<td></td>
</tr>
<tr>
<td>$r_G$ (adj)</td>
<td>-0.04 (0.19) NS</td>
<td></td>
<td></td>
<td>-0.30 (0.26) NS</td>
<td></td>
</tr>
</tbody>
</table>

* $P<0.05$, ** $P<0.01$, *** $P<0.0001$
Figure 8.1. Relation between family means (BLUP’s) for female allocation and male allocation in shade (●) and sun (○) treatments in *Ambrosia artemisiifolia* in the glasshouse.
Figure 8.2. Relation between family means (BLUP’s) for 10 random families for a) female and b) male allocation across the two treatments (sun and shade) in *Ambrosia artemisiifolia* in the glasshouse. The effect of family × treatment is only significant for female allocation.
There was significant genetic variation for female and male allocation in both treatments (Table 8.4; shade female: $\chi^2 = 37.8, P<0.0001$; shade male: $\chi^2 = 23.4 P<0.0001$; sun female $\chi^2 = 5.8, P<0.05$; sun male $\chi^2 = 46.1, P<0.0001$). Broad-sense estimates of heritability for allocation to female and male flowers were similar in the shade treatment (Table 8.4; $H^2 = 0.59$ and 0.45, respectively); however, in the sun treatment heritability was higher for male allocation ($H^2 = 0.63$; Table 8.4) and lower for female allocation ($H^2 = 0.21$; Table 8.4). I also found a positive genetic correlation between allocation to female and male flowers in both the sun and shade treatments ($r_G = 0.36$ and 0.45 respectively; Table 8.4). The genetic correlation in the shade treatment was significantly different from zero ($\chi^2 = 5.2 P<0.05$), but the correlation in the sun was not ($\chi^2 = 2.1 P=0.15$).

Size-adjusted heritability and genetic correlations

Heritability estimates for flower production were not greatly affected by accounting for plant size (number of branches). Although size adjustments reduced the variation attributable to maternal family, they also reduced the residual variation (Table 8.4). However, controlling for plant size drastically changed the genetic correlations between female and male flower production in both the sun and shade treatments and removed the positive genetic correlations. In the shade treatment, the size-adjusted genetic correlation was -0.04 and in the sun treatment it was -0.30 (Table 8.4). Neither of these measures are significantly different from zero based on likelihood ratio tests.

Plant size and sex allocation traits

Allocation to female and male flowers increased with plant size (number of branches) in both the sun and shade treatments (Table 8.5). A significant interaction was detected between treatment, sex, and number of branches ($F_{1,1310} = 35.41, P<0.001$), indicating that
Table 8.5. Significance tests for general linear models of flower production of *Ambrosia artemisifolia* plants in sun and shade treatments in the glasshouse, with number of branches as a covariate. Floral sex was treated as a repeated measure. See Figure 8.3 for depiction of three-way interaction.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Test statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block (treatment)</td>
<td>$F_{4,1274} = 17.76$ ***</td>
</tr>
<tr>
<td>Branches</td>
<td>$F_{1,1361} = 749.79$ ***</td>
</tr>
<tr>
<td>Family</td>
<td>$\chi^2 = 8.4$ **</td>
</tr>
<tr>
<td>Sex</td>
<td>$F_{1,1286} = 2.39$</td>
</tr>
<tr>
<td>Sex $\times$ branches</td>
<td>$F_{1,1369} = 173.53$ ***</td>
</tr>
<tr>
<td>Treatment</td>
<td>$F_{1,1291} = 0.43$</td>
</tr>
<tr>
<td>Treatment $\times$ sex</td>
<td>$F_{1,1261} = 20.77$ ***</td>
</tr>
<tr>
<td>Treatment $\times$ branches</td>
<td>$F_{1,1331} = 15.03$ ***</td>
</tr>
<tr>
<td>Treatment $\times$ sex $\times$ branches</td>
<td>$F_{1,1310} = 35.41$ ***</td>
</tr>
</tbody>
</table>

* $P<0.05$, ** $P<0.01$, *** $P<0.0001$
plant size affected female and male flower production differently in the two treatments (Figure 8.3). In both treatments, male flower production increased faster than female flower production with plant size (shade: female vs. male $t_{1338} = -4.55$ $P<0.001$; sun: female vs. male $t_{1383} = 2.92$ $P<0.01$). However, male flower production increased faster in the sun (male: shade vs. sun $t_{1004} = -4.46$ $P<0.001$), whereas female flower production increased faster in the shade (female: shade vs. sun $t_{1004} = 2.64$ $P<0.01$).

The growth of plants (vegetative height) during the experiment differed significantly between the two treatments (main effect of treatment $F_{2,143} = 1424.25$, $P<0.0001$; time × treatment $F_{2,573} = 4741.15$, $P<0.0001$; time × time × treatment $F_{2,1500} = 3602.25$, $P<0.0001$). Shade plants grew more quickly but stopped height growth earlier than sun plants (shade vs. sun for linear effect: $t_{1387} = 23.10$, $P<0.0001$; quadratic effect: $t_{1500} = 14.14$, $P<0.0001$). They were also taller than sun plants at each measurement point.

*Flower production and changes to sex allocation over time*

The number of female and male flowers produced over time differed significantly between the two treatments (Table 8.3; Figure 8.4). Plants in the shade treatment began flowering earlier and initially produced more female flowers than plants in the sun treatment. However, plants in the sun treatment accelerated female flower production towards the second half of the experiment (shade vs. sun for linear effect: $t_{808} = 32.18$, $P<0.0001$; quadratic effect: $t_{1250} = 39.16$, $P<0.0001$). Plants in the shade treatment also produced more male flowers early during growth but by mid way through the growth period male flower production ceased. Plants in the sun treatment initially produced fewer male flowers, but flower production accelerated during the latter phases of growth (shade vs. sun for linear effect: $t_{954} = 27.85$, $P<0.0001$; quadratic effect: $t_{1343} = 43.83$, $P<0.0001$).
Figure 8.3. The relations of female and male flower production to the size of plants in sun and shade treatments in *Ambrosia artemisiifolia* in the glasshouse. Symbols represent observed flower production by plants and lines illustrate the regression predictions based on number of branches. Shade – Female intercept: 82.65 ± 5.56; slope: 9.01 ± 0.51. Shade – Male intercept: 125.12 ± 11.27; slope: 16.35 ± 1.03. Sun – Female intercept: 136.87 ± 11.67; slope: 7.37 ± 0.73. Sun – Male intercept: 45.50 ± 24.64; slope: 26.57 ± 1.56. See Table 8.5 for statistical details.
Figure 8.4. Flower production over time for a) female flowers ($F$) and b) male flowers ($M$) in sun and shade treatments in *Ambrosia artemisiifolia* in the glasshouse. The fitted lines depict quadratic regressions based on a repeated-measures random regression analysis for four sampling times a) shade: $F = 5.07 + 1.73t + 0.02t^2$; sun: $F = 0.76 - 1.04t + 0.12t^2$; b) shade: $M = 32.34 + 6.21t - 0.07t^2$; sun: $M = 16.28 - 1.08t + 0.16t^2$. 

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**CHAPTER EIGHT: FLEXIBLE SEX ALLOCATION**

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**The effects of treatment and family on dichogamy**

There was a significant effect of treatment on the type of dichogamy exhibited by plants ($F_{1,154} = 108.12, P<0.0001$). Shade plants tended to be more protogynous with a mean $= 1.25$ (SE = 0.19, range 0–16) days between female and male flower anthesis. In contrast, sun plants were more protandrous with a mean $= 1.82$ (SE = 0.19, range 0–14) days between male and female anthesis. In both treatments, individuals that were protandrous, protogynous or non-dichogamous occurred (shade: proportion protandrous 0.23, protogynous 0.48, non-dichogamous 0.29; sun: proportion protandrous 0.53, protogynous 0.16, non-dichogamous 0.32). Shade plants flowered earlier than sun plants, regardless of their type of dichogamy ($F_{1,154} = 92.95, P<0.0001$), with the mean day of first flowering 63.55 (SE = 0.40) days post-germination, while the mean day of first flowering for sun plants was 69.13 (SE = 0.42) post-germination. The degree of dichogamy (number of days separating the sex functions) was affected by treatment ($F_{1,154} = 65.18, P<0.0001$), and there was a significant interaction between the type of dichogamy and treatment ($F_{1,878} = 12.96, P<0.001$, Figure 8.5). In each treatment, the prevalent type of dichogamy (protogyny in the shade, protandry in the sun) had a greater number of days between sex functions than the less common type.

There was a significant effect of family on dichogamy ($\chi^2 = 15.9, P<0.0001$), but no significant family by treatment interaction ($\chi^2 = 3.4, P>0.05$). I detected significant genetic variation for dichogamy in both treatments (Table 8.6; shade $\chi^2 = 52, P<0.0001$; sun $\chi^2 = 29.4, P<0.0001$) with families that were most strongly protandrous in the sun the least protogynous in the shade, and vice versa (Figure 8.6). The heritability estimate for dichogamy in the shade treatment was 0.45 and in the sun treatment was 0.34 (Table 8.6).
Table 8.6. Genetic variance ($V_G$), environmental variance ($V_E$), broad-sense estimates of heritability ($H^2$) for dichogamy (number of days separating first female and male flowers) in each treatment for *Ambrosia artemisiifolia* in the glasshouse. Standard errors are calculated from the asymptotic covariance matrix. Asterisks represent values that are significantly different from zero, based on likelihood-ratio tests.

<table>
<thead>
<tr>
<th></th>
<th>Shade</th>
<th>Sun</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_G$</td>
<td>7.69 (1.91)</td>
<td>5.59 (1.66)</td>
</tr>
<tr>
<td>$V_E$</td>
<td>3.80</td>
<td>6.61</td>
</tr>
<tr>
<td>$H^2$</td>
<td>0.45 (0.08)</td>
<td>0.34 (0.06)</td>
</tr>
</tbody>
</table>

* $P<0.05$, ** $P<0.01$, *** $P<0.0001$
Figure 8.5. Mean (SE) number of days separating first flower of each sex function for protandrous (○) and protogynous (●) individuals in sun and shade treatments in the glasshouse. In both treatments, there is a significant difference in the degree of protandry and protogyny, with the prevalent form of dichogamy experiencing the longer duration (protogyny in the shade, protandry in the sun).
Figure 8.6. Family means (BLUP’s) of number of days between first anthesis of each sex function in sun and shade treatments in the glasshouse. The dotted lines indicate no dichogamy, values to the left or below the lines indicate protogyny, and values to the right or above the lines indicate protandry. Families differed significantly in their degree of dichogamy within each treatment. There was a consistent relationship among families in the degree of dichogamy across the two treatments (i.e. no significant Family × Treatment interaction).
Discussion

The primary results of this study are the findings of genetic and environmental control of sex allocation in *A. artemisiifolia* including adjustments in the allocation to female and male function over time and in individuals of different sizes. As predicted, plants grown in the shade allocated relatively more resources to female flowers, whereas plants in the sun produced proportionately more male flowers. Surprisingly, I found a reversal in the magnitude of genetic variation for female and male allocation between sun and shade treatments. In the shade, there was significantly more genetic variation in female than male flower production, while this relation was reversed in the sun (Figure 8.1). As predicted by theory on size-dependent sex allocation, I found a significant effect of plant size, with the strongest effect on male allocation in the sun treatment (Figure 8.3). Finally, I detected significant genetic variation for dichogamy and a plastic response to environment. Plants in the shade tended to produce female flowers first in contrast to plants grown in the sun, which produced male flowers first (Figure 8.6). To my knowledge this represents the first empirical study to demonstrate a link between genetic and environmental components of temporal sex allocation. I now consider the ecological and evolutionary consequences of these environmental and size-dependent influences on sex allocation, and discuss how the presence of quantitative genetic variation for gender allocation may allow adaptive adjustments in the timing of allocation to the sex functions in hermaphroditic plants.

*Environment-dependent sex allocation*

I have shown that sex allocation in *A. artemisiifolia* is dependent on environmental conditions and can be adjusted plastically. The finding of relatively higher male flower production in the sun treatment supports predictions based on the optimal sex allocation for
wind-pollinated plants growing in open environments. In open habitats male function should experience greater returns resulting from proficient pollen dispersal. This allocation pattern contrasts with studies of animal-pollinated species that have more often reported that shaded plants produce more male flowers and sun plants produce more female flowers (e.g. Gregg 1975; Bertin 1982; Solomon 1985; reviewed in Korpelainen 1998), presumably due to the greater resource requirements of female function. A second explanation may also account for the increase in male flower production in the sun despite the greater resources available, in addition to preceding aerodynamic argument above. Male allocation relative to female investment may not be as inexpensive as is often assumed, particularly in terms of nitrogen and phosphorous requirements (see Harris & Pannell 2008). Such costs could be especially important for a wind-pollinated species that invests heavily in pollen production such as *A. artemisiifolia* in which the pollen:ovule ratio can be as high as 60000:1 (Payne 1963). Thus, a consideration of the particular requirements of wind pollination alters the expected pattern of environmentally-induced changes in sex allocation.

At this stage it is not possible to know whether the flexibility in sex allocation that I have detected in *A. artemisiifolia* is indeed adaptive under field conditions without knowing the shapes of female and male fitness functions. Fitness gain curves, particularly for male function, are difficult to measure empirically and their shapes are likely to depend on the demographic and environmental features of populations. Because of these complexities there are virtually no published gain curves for wild plant populations (Brunet 1992; Klinkhamer et al. 1994; Campbell 2000). If the gain curves for the two sex functions of *A. artemisiifolia* do indeed differ between environments, such as in the sun and shade treatments, then the patterns that I found for female and male flower production may allow plants to optimally
adjust their sex allocation. I discuss this possibility further in the section below on size-dependent sex allocation.

*Genetic components of sex allocation*

I detected significant genetic variation among plants in the production of female and male flowers in both the sun and shade treatments. After I adjusted for size differences among maternal families, significant genetic variation persisted for all traits except for female allocation in plants grown in the sun. However, size-adjustments reduced the variation attributable to maternal family (Table 8.4). Significantly, I found a reversal in the magnitude of variation for female and male allocation between sun and shade treatments. In the shade, there was greater variation in female flower production than in male flower production, while this relation was reversed in the sun (Figure 8.1). This pattern indicates that the capacity of genotypes to adjust their allocation of resources to female versus male flower production depends on environmental conditions, and suggests that the lability of sex allocation is driven by opposite responses of the sex functions in the two environments. The observed result is consistent with adaptive arguments (i.e. plants in the sun vary their male allocation, plants in the shade vary their female allocation), and indicates that there is ample genetic variation to allow divergent selection on sex allocation in the two environments.

Genotypes of *A. artemisiifolia* responded differently in their allocation to female and male flower production in sun versus shade environments. I found a significant maternal family-by-environment interaction for female flower production, but not for male flower production. All families made fewer male flowers in the shade and the degree of this adjustment was similar across families. However, genetic variation in phenotypic plasticity was evident in the allocation to female flowers in the two treatments (Figure 8.2a). A significant G x E for sex allocation in the two environments resulted from differences in the
relative investment to female flowers among families, and differences in the magnitude of phenotypic variation among families. Few studies have examined the genetic and environmental components to sex allocation (but see Mazer & Schick 1991; Dorken & Barrett 2004). Given the important role that environmental factors play in governing gender variation in monoecious species (Freeman et al. 1981; Bierzychudek 1982; Delesalle 1992; Sarkissian et al. 2001; Dorken & Barrett 2003), establishing a genetic component to sex allocation variation is significant. It suggests that the phenotypic expression of genetic variation and consequently the response to selection may depend on habitat conditions.

If the phenotypes favored by natural selection are the same across environments, genotype x environment interactions slow the response to selection, because the selected phenotypes are produced by different genotypes in each environment (Via & Lande 1985). However in the case of *A. artemisiifolia* growing in sun and shade habitats, we expect the fittest phenotypes to differ across environments. In this way, genetic variation in phenotypic plasticity may accelerate the response to selection. If gender modification is to respond appropriately to changing environmental conditions, a population must not only have heritable genetic variation for gender expression but also heritable variation in the plasticity of allocation (Goldman & Willson 1986; Mazer 1992; Vogler et al. 1999). Therefore, the presence of significant heritability of plasticity in allocation in *A. artemisiifolia* indicates that there is the potential for adaptive evolutionary changes to sex allocation in different environments.

*Trade-offs in female and male flower production*

Negative trade-offs between female and male investment is a fundamental assumption of sex allocation theories (Charlesworth & Charlesworth 1981, 1987; Charnov 1982). However, only a few studies have provided evidence supporting this assumption (Rameau &
Gouyon 1991; Garnier et al. 1993; Delesalle & Mazer 1995; Ashman 1999), and recent reviews of empirical tests show mixed support for negative genetic correlations (Ashman 2003; Ashman & Majetic 2006). I found no evidence for a trade-off between allocation to female and male flower production in either experimental treatment. Rather, I found positive phenotypic and genetic correlations between female and male flower number (Table 8.4). Because trade-offs can be masked by genetic variation in ability to acquire resources (van Noordwijk & de Jong 1986; Worley et al. 2003), adjusting for plant size can reveal underlying negative correlations.

In this study, the strength of genetic correlations between female and male flower production was strongly influenced by genetic variation in plant size. First, the variation in allocation to both sexes decreased after accounting for variation in the number of branches on a plant. Also, there was a significant positive effect of branching (a surrogate of size) on allocation to female and male function indicating that larger plants produced more female and male flowers (Table 8.5). Finally, controlling for variation in size removed all positive correlations between female and male flower production (Table 8.4). In the shade treatment, the size-adjusted correlation was zero whereas in the sun treatment there was a negative correlation, although not statistically significantly different from zero. It is seldom considered how selection for size-dependent sex allocation might interact with genetic variation for size and its effects on trade-offs. For example, if the effect of size obscures negative genetic correlations then the ability of selection to favour a particular sex in large individuals will be reduced.

**Effects of size on sex allocation**

The results provide evidence for size-dependent sex allocation that is contingent on environmental conditions. In both experimental treatments, larger plants of *A. artemisiifolia*
produced more female and male flowers (i.e. $b > 1.0$). However, investment into male flowers increased more rapidly with size relative to female investment, particularly in the sun treatment (Figure 8.3). These results differ from those for animal-pollinated species, which generally show greater investment into female allocation with increased plant size (e.g. Wright & Barrett 1999; Ashman et al. 2001; Mazer & Dawson 2001; Sarkissian et al. 2001; Cao & Kudo 2008). However, they are in accord with several findings for wind-pollinated plants (Solomon 1989; Fox 1993; Pannell 1997), including other work on *A. artemisiifolia* (Ackerly & Jasienski 1990; Paquin & Aarssen 2004). These studies of wind-pollinated plants have relied on a correlative approach involving the detection of phenotypic correlations between plant size and sex allocation, thus confounding genetic and environmental sources of variation. By manipulating the growth environment and replicating families across environments, I have been able to demonstrate that plants adjust their sex allocation in response to size above-and-beyond any environmentally-induced changes to allocation.

The observed patterns of gender variation revealed by this study are those expected for size-dependent sex allocation models that predict greater male investment in wind-pollinated plants with increasing size (Sakai & Sakai 2003). However, in wind-pollinated plants direct effects of size are beneficial for pollen dispersal only when taller neighbours do not surround plants. Interestingly, I found that although sun plants were significantly shorter than shade plants (but larger in every other index of size – Table 8.2), their pattern of size-dependent sex allocation was consistent with the direct benefits that size would give to pollen dispersal. In herbaceous plants, like *A. artemisiifolia*, relative height may be a more relevant feature of plants than absolute height. Because all measures of size were highly correlated (Table 8.1), I used number of branches as an index of plant size in analyses since it gave the best fit to the data. Size-dependent sex allocation models have seldom considered the
distinction between height and other indices of size (but see Bickel & Freeman 1988), although the measures need not have similar consequences for resource provisioning, pollination, mating or seed dispersal. The various consequences of plant size are particularly relevant for wind-pollinated species. They may incur size-dependent benefits through both direct effects of increased pollen dispersal and effects due to more available resources for reproduction (Lloyd & Bawa 1984; de Jong & Klinkamer 1994; Zhang 2006).

It has been proposed that paternal fitness in wind-pollinated plants will be linearly related to male reproductive investment because wind is unlikely to be saturated with pollen, and each pollen grain has an independent probability of capture (Charlesworth & Charlesworth 1981; Charnov 1982; Lloyd 1984). There is some empirical evidence for a linear relation between pollen production and reproductive success from managed populations of White Spruce (Schoen & Stewart 1986). Larger wind-pollinated plants should be better able to disperse pollen both through increased pollen removal when anthers are presented further from the ground and through greater dispersal distances of pollen released at greater heights (Levin & Kerster 1974; Niklas 1985; Okubo & Levin 1989), which should give them better access to mates relative to smaller plants. Thus, we may therefore expect diminishing marginal returns on male investment because of the influence of plant height on pollen dispersal (Burd & Allen 1988; Sakai & Sakai 2003).

Restricted pollen dispersal in smaller plants should limit opportunities for siring success and increase local mate competition. Pannell (1997) observed increased female allocation in cosexual individuals of wind-pollinated Mercurialis annua under high densities. He attributed this result to the smaller stature of plants making them less suited for effective pollen dispersal than those growing at low density. Also, relative height may be more important than absolute height in affecting a plants success as a pollen donor and/or pollen
recipient (Lundholm & Aarssen 1994). If shade acts as a signal to a plant that it is relatively diminutive (Paquin & Aarssen 2004), then it would be adaptive for shade plants to allocate preferentially to female function. I found that the relative investment into female flowers of shade plants increased faster with size than that of sun plants, a pattern consistent with adaptive plastic sex allocation.

Temporal changes in allocation and the function of flexible dichogamy

Despite the widespread occurrence of dichogamy in the flowering plants (Lloyd & Webb 1986) there is relatively little theoretical work on this floral strategy (but see Lloyd & Yates 1982; Sargent et al. 2006), especially consideration of when plants should be protogynous versus protandrous. Comparative evidence suggests that abiotically-pollinated species are more likely to be protogynous, whereas biotically-pollinated species are more likely to be protandrous (Sargent & Otto 2004). Also protogyny is commonly associated with self-compatibility and protandry with self-incompatibility (Bertin 1993; Routley et al. 2004). The expression of dichogamy is seldom considered in the context of sex allocation theory and is usually treated as a fixed constraint (Zhang 2006). This may be, in part, because most plant species are either protandrous or protogynous with the exception of the small number of species that are polymorphic for the two conditions (heterodichogamy – see Gleeson 1982; Renner 2001; Pannell & Verdú 2006; Bai et al. 2007). In the literature *A. artemisiifolia* is reported as protandrous but the results of this study have revealed a more complex situation involving plasticity in dichogamy. Plants were more often protogynous in the shade and protandrous in the sun (Figure 8.6), although a smaller fraction of plants expressed the alternative condition in both environments. Significantly, I also found significant genetic variation for the expression of dichogamy in both environmental treatments (Table 8.6).
The flexible patterns of dichogamy I have discovered in *A. artemisiifolia* appear to be unreported and do not fit into the types of dichogamy typically described (Lloyd & Webb 1986). Because the duration of dichogamy is continuous and the overlap between sex functions is large it is unlikely that flexible dichogamy promotes disassortative mating between protandrous and protogynous morphs, as has been demonstrated for heterodichogamy (Bai et al. 2007). Flexible dichogamy is also unlikely to be an outcrossing strategy, as *A. artemisiifolia* is strongly self-incompatible (Chapter Seven). Thus alternative explanations are required to account for the plastic expression of dichogamy. It is possible that in *A. artemisiifolia* dichogamy may limit to some extent anther-stigma interference by minimizing the amount of geitonogamous pollen that is captured by stigmas. However, the large overlap in sex functions that is evident within most plants and the relatively short duration in which they function as either a female or a male (Figure 8.5) suggests that this is unlikely to be the sole factor responsible.

Dichogamy in *A. artemisiifolia* may be best interpreted as part of a continuum in temporal control of sex allocation. All size-dependent sex allocation models assume that plants can accurately assess their local conditions before they influence reproductive success (Lloyd & Bawa 1984). However, if a plant cannot accurately assess the total amount of resources available for reproduction, then resources at any time should be allocated to the sex that will yield the higher fitness returns. In the first study to link temporal displacement of sex functions to sex allocation theory, Zhang (2006) proposed that the duration of overlap in sex functions, and the timing of each sex function, will depend on the shapes and magnitudes of the fitness gain curves. Changes between female and male investment should occur when fitness returns are equal per unit of investment in each sex function. If the gain curve for one sex function is linear but the other sex has a concave function, then the transition between
them should be abrupt. If both sexes have saturating gain curves then the transition should be gradual and depend on the relative rate of saturation.

A key feature of fitness gain curves is that they are context-dependent, varying between environments and sometimes between individuals of different resource status (Charnov 1982). The quantity of light received by wind-pollinated plants may be an environmental cue that reflects the quality of local resources, and also provides information about the pollination environment. If the shade treatment signals to a particular plant that it is relatively diminutive and surrounded by larger neighbours (see Lundholm & Aarssen 1994), then it would be adaptive for that plant to enter female function first. The relative rates of female and male floral production should depend on their fitness returns at each level of investment. In sun plants the production of flowers of both sexes is similar over time. However, in shade plants the production of female flowers over time is near linear, while male flower production saturates about two-thirds of the way through flowering. This pattern is consistent with the hypothesis that plants are using the available light to indicate their neighbourhood composition and that plasticity in female and male flower production is adaptive. Empirical estimates of fitness gain curves would be required to confirm whether plastic sex allocation of female and male function in plants is adaptive.
CHAPTER NINE
CONCLUDING DISCUSSION

In this thesis, I investigated the evolution of wind pollination, the functional significance of traits associated with anemophily, and their consequences for pollination and mating. My approach involved a combination of comparative and phylogenetic analyses, theoretical modeling, and field and glasshouse experiments involving floral manipulations, genetic markers and quantitative genetics. The major conclusions of my thesis are summarized and discussed in the individual chapters. Below, I draw general conclusions from my results and discuss their significance to our understanding of wind pollination. Because previous work on the ecology and evolution of wind pollination has been quite limited, in each section I include a discussion of possibilities for future research.

The nature and evolution of wind pollination

The evolution of wind pollination from animal pollination has been considered a paradox because anemophily has appeared to some to be a random, hit-and-miss process that is wasteful of male gametes (see for example statements in Wessells & Hopson 1988 p.560; Campbell et al. 1999 p. 734; Maseuth 2009 p. 205). In my thesis, I explored this issue using two approaches. First, I addressed the common assumption that wind pollination is more inefficient than animal pollination by assessing the proportion of pollen captured by 19 wind-pollinated herbaceous species (Chapter Two). I showed that pollen-transfer efficiencies in wind-pollinated species are not substantially lower than in animal-pollinated taxa. Second, I used a comparative analysis to investigate the correlated evolution of reproductive traits and the order in which they were acquired in lineages (Chapter Three). This approach allowed me
to provide mechanistic explanations about traits that are associated with wind pollination. In particular, the association of dichlous flowers and uniovulate flowers with anemophily is particularly perplexing. In Chapter Three, I argued that in dichlous lineages wind pollination might evolve to provide reproductive assurance, particularly because the evolution of self-fertilization is mechanically precluded in the vast majority of plant groups with unisexual flowers (exceptions could potentially occur in Asteraceae, Begoniaceae, and Euphorbiaceae because unisexual flowers are in close proximity within inflorescences). In Chapter Four, I challenged the common assumption that wind-pollinated species typically have uniovulate flowers because they capture few pollen grains. Using a phenotypic selection model, I showed that when flowers are inexpensive, as they are in wind-pollinated species, it is beneficial to distribute ovules among numerous flowers. Because pollen capture per flower saturates, plants that make many flowers sample more of the airstream and maximize fitness.

The roughly equivalent pollen-transfer efficiencies and moderately high stigmatic pollen loads reported in Chapters Two and Four, respectively, are inconsistent with Cruden’s (1977) explanation of the very high pollen-to-ovule ratios in wind-pollinated species compared to animal-pollinated species. Why then do wind-pollinated plants produce so much more pollen if their transfer-efficiencies are not orders of magnitude lower than in animal-pollinated species? In wind-pollinated plants, male fitness has generally thought to be linearly related to investment in male function (Charnov 1982), because the wind cannot be saturated with pollen. Also, if pollen capture represents an indiscriminate sampling of conspecific pollen from the airstream then, on average, a plant’s fitness through male function would be proportional to its share of pollen represented in the air. This would impose selection for individuals to increase their pollen production and could be responsible for the high pollen-to-ovule ratios in anemophilous species.
Future research

Theoretical investigations into the evolution of wind pollination would be valuable and help guide our understanding of the selective forces responsible for the transition to anemophily. There is a growing theoretical literature on reproductive transitions in angiosperms, but remarkably there has been no theory developed for the evolution of wind pollination. Various theoretical approaches could be used, including ESS models based on sex allocation and gain curves, classical population genetic approaches, or simulation models that consider the timing and effectiveness of pollen dispersal for animal versus wind pollination under various demographic scenarios. These approaches might also provide insight on the stability of the mixed-pollination system (ambophily) involving both animal and wind pollination. Theoretical investigations could identify conditions that favour the evolution of wind pollination, which would then motivate detailed empirical studies.

Empirical studies on the transition from animal to wind pollination have been hampered by the limited number of taxa containing interspecific, and especially intraspecific, variation in pollination systems involving both animal and wind. For example, to my knowledge no species is known that shows “pollination ecotypes” involving populations that are on the one hand exclusively animal pollinated, and other populations that are primarily wind pollinated. Early progress in understanding the selective forces leading to wind pollination was made by studies of *Thalictrum* (Ranunculaceae) (e.g. Kaplan & Mulcahy 1971). This genus, with its range of sexual systems (hermaphroditism, androdioecy and dioecy) and both animal and wind pollination, may be a profitable group in which to explore the microevolutionary forces leading to the evolution of wind pollination. Current research on transitions between animal and wind pollination, and its correlated evolution with sexual systems, in *Schiedea* (Caryophyllaceae) in the Hawaiian Islands appears to be the best
contemporary case study on the evolution of wind pollination (e.g. Weller et al. 1998; Golonka et al. 2005; Weller et al. 2006). By incorporating experimental manipulations and common garden studies, the mechanisms involved in the evolution of wind pollination from animal pollination could be clarified. Future work aimed at identifying groups exhibiting both pollination systems at the intraspecific level would be a necessary prerequisite for conducting experimental studies on selective mechanisms.

Several other questions regarding the evolution of wind pollination deserve consideration, including the prevalence of ambophily and the type of animal-pollinated species that become wind pollinated. Theoretical and empirical studies are needed to explore whether ambophily is a stable strategy selected for its flexibility, or whether it represents a transitional state towards full insect or wind pollination. There is also the possibility that wind-pollinated lineages could revert to animal pollination through an ambophilous intermediate (Stelleman 1984). The frequency and nature of reversals is currently unknown, although it has been suggested in some groups (e.g. *Salix*: Peeters & Totland 1999; *Carex*: P. D. Wragg & S. D. Johnson pers. comm.) Ambophily occurs in families such as the Salicaceae and Arecaceae (reviewed in Culley et al. 2002) and involve generalist pollinators, but further work is needed to establish its frequency and the selective mechanisms that maintain ambophily. Some parallels between wind pollination and generalist pollinators have been discussed (Charlesworth 1993), but further empirical research on their similarities and how they might influence gain curves would be merited.

A surprising finding of the comparative analysis presented in Chapter Three was that wind pollination and geographical distribution do not evolve in a correlated way. This result is in contrast to the observation of much greater abundance of wind-pollinated plants in temperate regions (Regal 1982). Further studies may reveal that wind-pollinated lineages in
temperate regions are more likely to persist and undergo speciation. Additionally, the use of comparative phylogenetic analyses in particular floras or lineages might better explain biogeographic and ecological correlates of wind pollination. For example, wind pollination is very common on the Juan Fernandez Islands (Bernadello et al. 2001), but less frequent on the Galapagos Islands where selfing appears to be more common perhaps because of the early seral stages that characterize these relatively young volcanic islands (McMullen 1987).

**Controlled chaos: pollen dispersal in wind-pollinated plants**

Wind direction is often unpredictable, making the wind a relatively imprecise vector. Many wind-pollinated plants may be unable to avoid self-pollination when female and male gametes are produced simultaneously. The high incidence of dichogamy in wind-pollinated species has been interpreted as an anti-selfing mechanism (Lloyd & Webb 1986; Webb & Lloyd 1986). In particular, the common association between monoecy and protogyny in wind-pollinated plants (Lloyd & Webb 1986; Bertin & Newman 1993; Sargent & Otto 2004) may largely function to reduce the incidence of self-fertilization. I tested whether monoecy and protogyny function to limit selfing using manipulative field experiments and genetic markers to assess outcrossing rates in seven species of woodland *Carex* (Chapter Five). I found that neither monoecy nor protogyny were particularly effective at reducing selfing.

Instead, I proposed that when environmental conditions are inimical to proficient pollen transport (e.g. forest understory with low wind speeds) geitonogamy might provide reproductive assurance. Unlike in animal-pollinated plants where geitonogamy is unlikely to ever be adaptive (Lloyd 1992), in wind-pollinated species with monoecious flowers this mode of selfing might be beneficial because the wind cannot be saturated with pollen so that pollen discounting should be negligible. Experiments to investigate the extent to which
pollen discounting may occur in wind-pollinated plants would be valuable to confirm this suggestion.

Wind dispersal of pollen typically follows a leptokurtic distribution with most pollen deposited near the source and a long flat tail characterized by low deposition (Levin & Kerster 1974; Tonsor 1985; Rognli et al. 2000). This aerodynamic feature of wind pollination can have profound consequences for pollination, mating and offspring quality. In collaboration with Dr. Ivana Stehlik, I conducted a study of neighbourhood composition and its effects on pollination, seed set and sex ratio in the dioecious herb *Rumex nivalis* (Chapter Six). Sex determination in *R. nivalis* is governed by heteromorphic sex chromosomes where male plants are the heterogametic sex, and there is evidence that stigmatic pollen loads influence progeny sex ratios perhaps by differential performance of female- versus male-determining pollen tubes (Stehlik & Barrett 2005, 2006). The field study in natural populations of *R. nivalis* demonstrated that the maternal mating environment affects stigmatic pollen loads and results in biased progeny sex ratios. Thus neighbourhood composition can have profound consequences on pollination in wind-pollinated plants. In a second study on the demographic context of mating, I explored the consequences of variation in density for mating patterns in the hermaphroditic monoecious species *Ambrosia artemisiifolia* (Chapter Seven). Plants in high-density patches might be more highly outcrossed if they capture more pollen, in comparison with plants at low density where stigmatic pollen loads might contain more self-pollen (Eppley & Pannell 2008). I evaluated this idea using experimental arrays and genetic markers. Although I detected a relation between plant density and pollen load size, I found no significant differences among treatments in outcrossing rate. This result was quite unexpected because *A. artemisiifolia* had been reported in the literature as self-compatible, a common condition in annual colonizing
species. Subsequent studies of pollen tube growth demonstrated that previous reports appear to have been in error as the population that I investigated was strongly self-incompatible.

Wind pollination relies on efficient pollen removal and capture, which have different aerodynamic requirements (Niklas 1985). For example, pollen dispersal is expected to be more effective with increased plant height because pollen can be dispersed more easily from the plant because of greater windspeeds, and because pollen is also likely to travel further and thus have better access to mates. Thus, in wind-pollinated species plant size can affect fitness returns directly, representing one of the clearest examples of direct size-dependent effects on allocation (Burd & Allen 1998; de Jong & Klinkhamer 2005). Models of sex allocation in hermaphroditic plants have attempted to explain varying patterns of investment into female and male function. These models typically use gain curves to predict optimal sex allocation, which can vary by environment, age or size (Charlewsorth & Charlesworth 1981, 1987; Charnov 1982; Lloyd & Bawa 1984; Charlesworth & Morgan 1991; Zhang 2006). Few studies have explored the consequences of different environments on patterns of sex allocation, or how investment changes over time, or with variation in plant size. In addition, we know little about the interaction of genetic and environmental factors on sex allocation, and the extent to which male and female allocation is heritable.

In Chapter Eight I examined the genetic and environmental components of temporal and size-dependent sex allocation in *Ambrosia artemisiifolia* in two environmental treatments (sun and shade) in the glasshouse. Consistent with theoretical predictions, I found plants grown in the sun invested more in male function and there was a size-dependent adjustment with larger plants producing proportionally more male flowers. I also found a change in allocation over time between the two environments, in particular, plants in the shade tended to have female anthesis first, in contrast to plants in the sun, in which male anthesis was first.
Finally, I demonstrated significant genetic variation in sex allocation and a maternal family genotype-by-environment interaction in the patterns of investment. These results suggest that there is the potential for individuals with different genetically-determined patterns of sex allocation to respond adaptively to contrasting environmental conditions. Unfortunately, few studies have actually demonstrated that patterns of sex allocation are adaptive and this remains a major weakness of sex allocation theory.

*Future research*

It is often claimed that male fitness in wind-pollinated species should be an approximately linear function of resource investment into pollen production because wind is unlikely to saturate with pollen (Charlesworth & Charlesworth 1981; Charnov 1982; Klinkhamer & de Jong 1997). However, only one study has tested this idea (Schoen & Stewart 1986) and this involved measurements of male reproductive success in a plantation of White Spruce. Fitness gain curves, particularly for male function, are difficult to measure empirically (Brunet 1992; Klinkhamer et al. 1994; Campbell 2000). However, with the advent of hypervariable genetic markers such as microsatellites available for paternity analysis it should be possible to assess the male reproductive success of individuals with different levels of allocation. For example, use of artificial selection experiments in species like *Ambrosia artemisiifolia* could produce phenotypes with different allocations, which may then be used in gain curve analyses. Future work on wild populations of wind-pollinated plants would be valuable to confirm that the linear gain curve reported by Schoen & Stewart (1986) occurs more generally. It would also be beneficial to determine empirically whether male fitness depends on plant height as suggested by theory (Burd & Allen 1998). Manipulative experiments in which height variation is controlled could be used to test this idea.
One of the most recognized features of mating in wind-pollinated plants is the apparently low incidence of mixed mating leading to a bimodal distribution of outcrossing rates (Schemske & Lande 1985; Aide 1986; Barrett & Eckert 1990; Goodwillie et al. 2005), with species more frequently either predominantly selfing or outcrossing. However, in the most recent compilation of outcrossing rates (Goodwillie et al. 2005), all but 18 of the 76 wind-pollinated species belong to just two families (Poaceae and Pinaceae). Further effort is therefore required to broaden taxonomic representation to include a wider array of anemophilous families. The paucity of mixed mating may reflect different selective pressures in wind-pollinated species including a less stochastic pollination environment (Aide 1986; Vogler & Kalisz 2001; Goodwillie et al. 2005). This would imply that wind is a more predictable vector of pollen than animals and, provided population densities are sufficient, flowers should receive adequate outcross pollen. The data presented in Chapters Two and Four of this thesis provide some insight on this issue, but a more thorough investigation of pollen loads and pollen transfer efficiencies is warranted. My studies on this topic focused exclusively on wind-pollinated herbs, with an over-representation of members of the Cyperaceae and Poaceae. Clearly more extensive studies are required including more anemophilous families and in particular species of trees and shrubs since these were not included in my work. Additionally increased sampling of species with contrasting sexual systems (e.g. hermaphroditic species with perfect and unisexual flowers and dioecious species) would be valuable to confirm that the general trends I observed in Chapter Two are maintained.

A critical issue for our understanding of wind pollination is to determine the quantity and quality of pollen (conspecific outcross versus self; conspecific versus heterospecific) that is transported to stigmas by the wind. Obtaining quantitative estimates of these sources of
pollen on the stigmas of anemophilous species would greatly aid in assessing the efficiency of wind pollination in comparison with animal pollination. Moreover, despite a burgeoning literature for animal-pollinated species (Burd 1994; Larson & Barrett 2000; Ashman et al. 2004) information on the prevalence of pollen-limited reproduction in wind-pollinated groups is sparse. In Chapter Two I present data indicating that pollen limitation might be rare in herbaceous wind-pollinated species when population densities are adequate. However, much more empirical data is required on the frequency of pollen limitation in wind-pollinated plants, and especially the extent to which low population density results in insufficient pollination.

The enormous diversity of inflorescence architectures in different wind-pollinated species (e.g. Poaceae) is perplexing given that they are all subject to the same general aerodynamic constraints. The diversity suggests varied structural mechanisms to promote effective pollen dispersal and to reduce the costs of self-pollination, especially selfing and inbreeding depression, and pollen discounting. Virtually nothing is known about how different inflorescence architectures affect these key genetic parameters, which govern mating-system evolution. Wind-tunnel experiments have demonstrated that the mechanics of pollen receipt differ depending on whether species posses compact or diffuse panicles (Niklas 1987). And field experiments on grasses with compact and diffuse panicles have revealed that the oscillation of inflorescences can have contrasting effects on pollen removal and receipt (Friedman & Harder 2004). There is also preliminary evidence that wind-pollinated species may be able to discriminate between conspecific and heterospecific pollen prior to deposition on stigmas through different ‘pollen behaviours’ in the aerodynamic environments generated around stigmas (Niklas & Buchmann 1987; Linder & Midgley 1996). Following the seminal work of Niklas (e.g. 1985), future studies that link plant and
flower architecture to pollen dispersal and mating patterns may help to reveal the selective mechanisms leading to the diverse phenotypes that occur among wind-pollinated plants.

**Conclusion**

The puzzle of the evolution of wind pollination may be better understood if we discard the notion that anemophily is a chaotic, unpredictable process and a poor relative to the more efficient pollen dispersal that is thought to characterize many animal-pollinated systems. Instead, by accounting for the key differences between these two pollination systems in the mechanics of pollen dispersal and pollen capture, it is evident that a range of strategies enable wind pollination to be a viable alternative to animal pollination in particular lineages and environments. I hope this thesis contributes toward accomplishing this paradigm shift in plant reproductive biology.
APPENDIX ONE

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