Pollen Limitation of Reproduction in a Native, Wind-Pollinated Prairie Grass

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1. ABSTRACT

Pollen limitation occurs when insufficient quantity or quality of pollen hampers plant reproductive success. Pollen limitation is widely studied in animal-pollinated plants, but less so in wind-pollinated plants. According to theory, pollen limitation is not expected in wind-pollinated plants, but evidence suggests that it might be more prevalent than previously thought, especially in fragmented populations. I quantified pollen limitation using a pollen addition and exclusion experiment in a small and isolated remnant prairie population of *Dichanthelium leibergii*, a wind-pollinated native prairie grass. I hypothesized that seed set (proportion of ovules that developed into seeds) differed among inflorescences receiving different treatments on the same plant: 1) supplemented with outcross pollen from distant plants (pollen-added); 2) excluded from external pollen (self-pollen only); or 3) open-pollinated (unmanipulated). Seed set differed among treatments after accounting for differences among individual plants (generalized linear mixed model with binomial response, N = 77 inflorescences on 32 plants, p = 0.014). The model predicted higher seed set for pollen-added and self-pollinated inflorescences than open-pollinated inflorescences on the same plants. However, density and indicators of plant resource status such as plant height and diameter, did not explain variance in natural seed set among plants. My results provide evidence that pollen quantity, but not quality, limited reproduction in this *D. leibergii* population. This suggests that wind-pollination may be less effective in fragmented populations of native grasses. My study provides insight into habitat fragmentation in remnant *D. leibergii* populations, which will inform prairie conservation and improve understanding of pollen limitation in wind-pollinated species.

2. INTRODUCTION

2.1 Pollen Limitation

Pollen limitation occurs when insufficient quantity or quality of pollen reduces plant reproductive success, leading to ecological and evolutionary consequences for plant populations and demography (Ashman et al. 2004, Aizen and Harder 2007). A decline in seed production may lower population growth rates, increase risk of inbreeding depression, and affect community and ecosystem-level processes (Kearns et al. 1998, Ashman et al. 2004). Pollen limitation may also affect evolutionary processes such as selection for sexual traits in flowers, breeding systems, plant-animal co-evolution, strategies for reproductive assurance, and sex ratios in populations (Burd 1994). Given the widespread effects of pollen limitation on plant populations, a better understanding of pollen limitation will help the conservation of plants and the study of plant evolution.

Pollen can be limited in quantity or quality due to a variety of reasons, such as: lack of pollinator visitations in animal-pollinated plants, inefficient pollen transfer, lack of pollen donors, and lack of compatible pollen (Byers 1995, Ashman et al. 2004). In wind-pollinated plants, concentrations of dispersed pollen decrease sharply with distance from the source (Gleaves 1973). Consequently, population density may be an important factor determining pollen availability. Plants located in small or sparsely populated (low density) patches tend to receive less pollen than plants with many close neighbors (high density). Species with short pollen dispersal distances relative to their size, such as the blue oak, *Quercus douglasii*, are especially vulnerable (Knapp et al. 2001). Lack of compatible pollen may be more important than low pollen loads, particular in self-incompatible species, which require outcross pollen for mating. Abiotic factors such as weather, topography, and physical barriers can also restrict pollen flow

(Holm, 1994). Furthermore, anthropogenic changes such as habitat fragmentation can increase the likelihood of pollen limitation in both animal and wind-pollinated plants (Knapp et al. 2001, Knight et al. 2005, Vranckx et al. 2012). Habitat fragmentation may reduce pollen quantity and quality directly by reducing the number of pollen donors or pollinators, or indirectly by decreasing plant population sizes such that inbreeding depression lowers pollen quality (Knight et al. 2005).

In particular, pollen limitation is widely discussed and documented in studies of animal-pollinated species. A review by Burd (1994) found evidence of pollen limitation in 62% of 258 species, while Ashman et al (2004) found evidence in 73% of 85 studies conducted on whole plants. Knight et al (2005) documented 63% of 482 studies that used fruit set data. However, pollen limitation in wind-pollinated species has received little attention. The reviews by Burd (1994) and Ashman et al. (2004) made no mention of wind-pollinated species, while Knight et al. (2005)’s meta-analysis included only a limited sample of abiotically-pollinated species. Wind-pollinated species are typically not expected to be pollen-limited, because wind-pollination itself may be an evolutionary adaptation for reproductive assurance (Friedman and Barrett 2009b). Nevertheless, there is little evidence for whether or not pollen limitation is prevalent in wind-pollinated plants (Davis et al. 2004). An exception to the case can be made for wind-pollinated trees, which have been the subject of interest in several studies (discussed in more detail in the literature survey following this section). Given the sparse information currently available, researchers have called for more studies to examine pollen limitation in a variety of wind-pollinated taxa (Friedman and Barrett 2009b). My research aims to answer this call by investigating pollen limitation in *D. leibergii*, a native wind-pollinated prairie grass in a highly fragmented remnant prairie landscape.

In the following sections of this chapter, I will: 1) review the literature on pollen limitation in wind-pollination plants, 2) describe the basic biology and reproductive system of *D. leibergii*, and 3) present my research questions which provide the framework for the subsequent chapters. With this, I hope to expand the scientific knowledge about pollen limitation in wind-pollinated species.

2.2 Literature Survey: Pollen Limitation in Wind-Pollinated Plants

I surveyed the literature for studies that provided empirical data on pollen limitation in wind-pollinated (anemophilous) or dually wind- and insect-pollinated (ambophilous) species. I identified 24 studies of wind-pollinated species representing a taxonomically diverse sample (28 genera across 20 families), which are summarized in Appendix IA and IB.

Experimental studies of pollen limitation in both animal- and wind- pollinated plants most commonly involved pollen addition by hand in a process termed pollen supplementation (Knight et al. 2005). In those types of studies, plants were considered pollen-limited if the flowers or individual plants that received supplemental pollen showed higher reproductive outputs than those that did not. Researchers most commonly measure plant reproductive output using percent fruit set, although other common metrics include percent seed set, number of seeds per fruit, number of seeds per flower, and number of seeds per plant (Ashman et al. 2004). According to Ashman et al. (2004), seeds per plant is the best indicator of reproductive success for pollen limitation studies because it specifically measures the effect on the maternal fitness of individual plants and is not affected by within-plant reallocation of resources. Most of the studies included in my survey conducted pollen addition experiments to measure fruit or seed set. Only two studies did not directly manipulate pollen. Knapp et al. (2001) measured pollen availability of *Q. douglasii* stands by counting the number of pollen-producing trees within 60m when the maternal tree was receptive, while De Cauwer et al. (2010) compared fruit and seed set of open-pollinated female sea beet plants (*Beta vulgaris* ssp. *maritime*) in populations with contrasting sex ratios (low versus high ratio of hermaphrodites to female plants).

Evidence of pollen limitation was found in 7 of 20 non-grass herbaceous species (35%), 5 of 7 grasses (71%), and 6 of 11 tree species (50%) (Appendix IA and IB). In 9 of these species, pollen was not significantly limited in a second population in the same study, and 6 were dually pollinated by wind and insects. The grass family (Poaceae) was represented by 7 species in 6 studies. Five of the grass species were significantly pollen-limited in at least one population (Appendix IA). Three of these studies were conducted in introduced or invasive populations (Ishii and Kadono 2002, Li et al. 2008, Davis et al. 2004). In terms of breeding systems, pollen limitation occurred at about the same rate in monoecious and dioecious plants (5 of 11 monoecious (45%); 6 of 12 dioecious plants (50%)). This was unexpected because dioecious plants are more likely to be pollen-limited than monoecious plants, as they cannot self-fertilize and not all plants produce pollen (Steven and Waller 2006). However, none of the three hermaphroditic (having bisexual flowers) species (*Coriaria arborea* (Coriariaceae), *Andropogon gerardii* (Poaceae), and *Sorghastrum nutans* (Poaceae)) were significantly pollen-limited. This result matches the expectation that pollen limitation is less likely to impact the reproductive fitness of hermaphrodites compared to unisexual plants (Maurice and Fleming 1995).

On average, the extent of pollen limitation in wind-pollinated taxa was less than in animal-pollinated taxa. Where pollen limitation was reported, it was usually under specific conditions such as low density (Allison 1990a, Gulias and Traveset 2012), high altitude (Holm 1994, Gulias and Traveset 2012), skewed sex ratios (De Cauwer et al. 2010), herbivory (Allison 1990b, Bertness and Shumway 2012), or a combination of several factors (Eppley 2005, Cázares-Martínez et al. 2010). Hence, pollen limitation mechanisms likely result from an interaction of biotic and abiotic factors affecting plant reproductive success via suppression of male reproduction.

Density was the largest contributor to pollen limitation. Low density populations are more likely to be pollen-limited than high density populations, because there are likely fewer compatible pollen donors in a low density population. The role of density was reflected in several studies (e.g. Nilsson and Wastljung 1987, Allison 1990a, Gulias and Traveset 2012, Steven and Waller 2006, Knapp et al. 2001, De Cauwer et al. 2011). Distance to nearest neighbors or nearest pollen donor was often negatively correlated with reproductive output (e.g. Allison et al. 1990a, Vandepitte et al. 2009). Nilsson and Wastljung (1987) found that stand density of beeches (*Fagus sylvatica*) negatively correlated to fruit set and pollination success. Gulias and Traveset (2012) attributed the difference in reproductive output between coastal and mountain populations of *Rhamnus lycioides* subsp. *oleoides* to the lower density of plants at higher altitudes. Although Steven and Waller (2006) reported non-significant results for pollen limitation in *Thalictrum dioicum*, they found a significant correlation between distance to males and seed set in lowest density populations. Similarly, pollen limitation led to reduced population growth rates of an invasive marsh grass, *Spartina alterniflora,* at low densities but did not significantly affect high density populations (Davis et al. 2004). Hence, it is not surprising that habitat fragmentation, which reduces population abundance, may increase the probability of pollen limitation (Knapp et al. 2001).

Several researchers also considered the role of resource availability in limiting seed set of plant populations. Two studies experimentally tested for resource effects with differing methods and results (Ishii and Kadono 2002, Cázares-Martínez et al. 2010). Cázares-Martínez et al. (2010) conducted a pollen and resource addition experiment, and found that both pollen and resources (water) limited seed viability of the evergreen shrub *Atriplex canescens* but not in the congeneric *A. canthocarpa*. They also found that in the absence of resource limitation, pollen availability limited seed number in *A. canescens.* On the other hand, Ishii and Kadono (2002) found no evidence of resource limitation, as inflorescence-cutting (alleviating resource limitation) did not increase seed set of the invasive common reed, *Phragmites australis.* Researchers also found indirect evidence suggesting an interplay between pollen and resource availability in limiting female reproduction in plant populations. Steven and Waller (2006) found that seed set was correlated with density of female *T. dioicum* plants but not male density, which supports resource limitation rather than pollen limitation. Gulias and Traveset (2012) suggested that lower resource availability in coastal populations limited fruit set of *R. lycioides,* while pollen availability affected mountain populations. Holm (1994) also compared seed production of silver birch (*Betula pendula*) in coastal and mountain populations, but he found no significant evidence for pollen limitation in either population type, and concluded that resource limitation was probably the cause of poor seed quality in mountain populations. Given the complex interactions between pollen and resource limitation suggested by these studies, it would be prudent for researchers to consider potential effects of resource limitation in pollen-limitation studies.

In summary, although there was no consistent trend across taxa, pollen limitation seems to be more prevalent in wind-pollinated species than predicted by theory for a pollination syndrome that is hypothesized to provide reproductive assurance (Friedman and Barrett 2009b). Population density was a major driver of pollen limitation. Interestingly, pollen limitation seems to occur frequently in grasses (5 of 7 species). Considering the limited number of studies available, there is a clear need for additional investigations of pollen limitation in wind-pollinated species.

2.3 Study Species: Dichanthelium leibergii

Leiberg's panic grass, *Dichanthelium leibergii* (Vasey) Freckmann, is a perennial cool season (C3) grass native to prairies in North America. It is found primarily on prairie remnants but may grow in sandy woodlands (Freckmann and Lelong 2003). Like many grass species, it is wind-pollinated (Spellenberg 1968). Individuals grow with clumps of culms 24-80 cm high (Freckmann and Lelong 2003). Its inflorescences are 6-10 cm long panicles, each producing as many as 40 spikelets, with one floret per spikelet. Florets are hermaphroditic, i.e. each floret contains both male and female parts (anthers and stigma).

Dichanthelium species, including *D. leibergii*, exhibit notable characteristics in their reproductive biology. Typically, the flowering of cool season plants is restricted to spring and early summer, but *Dichanthelium* species produce flowers over an unusually long period, from spring to early fall. In addition, *Dichanthelium* species have two distinct flowering phases, as hinted by its name (in Latin, "*dich*" means "twice" and "*anth*" is "flowering"). The two flowering phases are differentiated by flowering on primary panicles and secondary panicles (Freckmann and Lelong 2003). For *D. leibergii*, primary panicles are produced from mid-May through July, and secondary panicles from late June to September (Freckmann and Lelong 2003). Even more interesting is the occurrence of cleistogamy (reproduction in permanently closed, self-pollinated flowers) in many *Dichanthelium* species, including *D. leibergii* (Freckmann 1967). Secondary panicles are usually partially or totally cleistogamous, while primary panicles are at least partially chasmogamous (open flowers). If *D. leibergii* is typical for the genus, then we expect it to be self-compatible, which reduces its risk of being pollen-limited.

D.leibergii is a native prairie plant of conservation concern, because it tends to grow only in high quality prairies, making it a good indicator of prairie ecosystem health (Milburn and

Bourdaghs 2007). Due to severe habitat fragmentation of tallgrass prairie throughout North America, *D. leibergii* populations have become small and isolated. Seed germination rates for this species can be as low as 4-5% (M. Wang, unpublished data; Cathy Thomas, personal communication). Poor reproduction in remnant populations may be due to a combination of low rates of germination and seed set. This is alarming because poor reproduction may lead to population decline and eventual local extinction of the species. As *D. leibergii* only grows in high quality prairie remnants, resource availability is unlikely to limit its seed set. Considering that pollen limitation may be more prevalent in wind-pollinated grasses than predicted by theory, I suspect that pollen limitation may be the cause of low seed set in *D. leibergii*. Hence, investigating pollen limitation will provide valuable insight into effects of habitat fragmentation on *D. leibergii* populations, which will greatly aid conservation efforts for this important prairie species and contribute to greater understanding of pollen limitation in wind-pollinated species.

2.4 Research Questions

My research aims to quantify the mechanism of pollen limitation in a wind-pollinated species impacted by habitat fragmentation. Specifically, I examined how various aspects of pollen limitation affect seed set of *D. leibergii* in a remnant prairie. I address the following research questions through my project:

1) To what extent does pollen quantity limit seed set of *D. leibergii*?
2) To what extent does pollen quality (in terms of compatibility and viability) limit seed set of *D. leibergii?*
3) To what extent does the density of flowering plants affect seed set of *D. leibergii*?
4) Is there evidence for resource limitation in *D. leibergii*?

To address the first two questions, I conducted a field experiment in a remnant prairie population of *D. leibergii* and examined how seed set was affected by manipulating pollen quantity (pollen addition) and pollen quality (self vs. outcross pollen). I also assessed pollen quality in terms of its viability by determining pollen viability levels of *D. leibergii* plants in the lab. To address questions 3 and 4, I examined the relationship between seed set, the local density of *D. leibergii* plants, and indicators of their resource levels.

3. MATERIALS AND METHODS

3.1 Study Area and Sampling

The study area was a remnant population of *D. leibergii* in Hegg Lake State Wildlife Management Area, located in Douglas County, in rural western Minnesota, USA. Prior to conducting the experiment, I made field observations (June 21-June 29) to gain a better understanding of the process of anthesis (flower emergence) and anther dehiscence (pollen shedding) (see Appendix II for details).

I selected 32 plants along a 35 m transect and mapped individual plants using high precision GPS. I only selected plants that had two or more inflorescences that had not yet begun flowering. Any spikelet that had begun flowering before the experiment started was removed from its panicle. For each plant, I selected two inflorescences with similar heights and number of spikelets. I haphazardly assigned the two inflorescences to either the pollen addition group ("add"), or the open-pollinated, unmanipulated control group ("open"). Two days later, I selected a third inflorescence for the pollen exclusion treatment ("self"). Twelve plants did not have a suitable third inflorescence and thus did not receive the pollen exclusion treatment. Inflorescence heights and spikelet numbers are summarized in Table 1. Sample sizes at different levels and stages of the experiment are summarized in Table 2.

3.2 Pollen Addition and Exclusion Experiment

I performed field pollen manipulations in July 2012. Starting at 5:30 am daily, I checked inflorescences in the pollen addition treatment for spikelets with newly-emerged stigmas. The receptive spikelets were hand-pollinated on the same morning. To obtain pollen, I collected pollen-dehiscing spikelets from plants at least 2m away from the pollen recipient plant. Pollen grains were visible to the naked eye as white powder, although a magnifying eye-visor was helpful in confirming pollen availability. Immediately after collection, I brushed the pollen donor spikelets directly against the stigmas of the receptive spikelets (Appendix III, Figure S1). Although the flowering season was longer (May through August), selected inflorescences flowered synchronously so the pollen addition treatment was completed in 10 days (July 2 to July 11). For the pollen exclusion treatment, I bagged the assigned inflorescences with gusseted glassine bags (4” x 8.5” or 4.5” x 9.25”; from ULINE) and sealed the bags with twist-ties to keep out any outside pollen (July 3 – July 11) (Appendix III, Figure S2). I took off the bags for 1-2 minutes daily and used a wind-guard while the bags were off. I also replaced bags regularly because they tear easily and could weigh down the inflorescence when wet. I did not bag inflorescences in the control group, which were exposed to ambient pollen for pollination to naturally occur (“open-pollination”). I summarized the different types of pollen present in each pollination treatment in Table 3.

I recorded the flowering progress of individual spikelets for inflorescences in all three treatments daily for 8 days (pollen addition and open-pollinated: July 2 to July 9; pollen exclusion: July 4 – July 11). I recorded changes in the appearance of the anthers, stigmas, and the spikelet. When the terminal spikelets on an inflorescence had completed flowering, I bagged the inflorescence with a glassine bag to capture falling spikelets. I checked spikelets every other day, harvesting spikelets as they matured, and stored them in silica gel until weighing. Each spikelet contained a single ovule, which developed into a seed if successfully fertilized. I weighed spikelets individually using a Mettler Toledo Excellence Plus XP 105 Analytical Balance. The distribution of spikelet mass was strongly bimodal (Figure 1). I classified each spikelet as having successfully developed into seed (≥ 0.002 g, “heavy”), or otherwise (< 0.002 g, “light”). Seed set is expressed as the proportion of “heavy” seeds from total number of spikelets harvested per inflorescence.

3.3 Quantifying Density and Maternal Resource Status

To quantify the density of *D. leibergii* surrounding the focal plant, I counted the number of *D. leibergii* plants within a 1 m radius of each focal plant. To gauge the maternal plant resource status of each focal plant, I measured two traits that reflect both the overall plant size and floral display, namely 1) height of the tallest inflorescence (“height”); and 2) diameter of the plant clump at ground level, which represents the number of inflorescences put out by the plant (“diameter”). Table 4 shows summary statistics for the density, height and diameter.

3.4 Statistical Analyses

To test the hypothesis that seed set differed among pollination treatments, I used generalized linear models (GLM) with binomial response (spikelets as full or empty). I used stepwise backward elimination with likelihood ratio tests to select the minimal adequate model as described by Crawley (2005), with pollination treatments (“trt”) and individual plants (“plaID”) as predictors.

In the initial model, the residual deviance greatly exceeded the residual degrees of freedom. To account for overdispersion, I specified the model using the quasibinomial family. To reduce non-normality of data, I excluded six inflorescences from the analysis that had fewer than four seeds collected and weighed. A diagnostic plot of the residuals vs. fitted values in the selected model showed that the points were not evenly dispersed. Because the generalized linear model with only fixed effects was not appropriate, I used a mixed effect logistic regression model (GLMM) with inflorescence as a random effect (J. Ison and S. Wagenius, unpublished; Bates et al. 2011, Warton and Hui 2011). Again, I selected the minimal adequate model by stepwise backward elimination. The diagnostic Q-Q plot of fitted values from the GLMM model showed that the values were normally distributed.

To test the effects of density and maternal plant traits on seed set, I used generalized linear models (GLM) with the predictors “diameter,” “height,” and “density” as focal terms. To exclude the effect of pollination treatments, I compared the seed sets of inflorescences within the open-pollinated control group. Stepwise backward elimination showed that interaction terms were not significant, so I compared models containing a single focal term to a null model. Again, I specified the model using the quasibinomial family to account for overdispersion.

I conducted all statistical analyses in R, including the GLMM analysis using the glmer function in the lme4 package (Bates et al. 2011, R Development Core Team 2011).

3.5 Assessing Pollen Viability

To investigate another aspect of how pollen quality may affect seed set in *D. leibergii*, I assessed the viability of *D. leibergii* pollen from plants in the laboratory. I had grown these plants for a previous germination study in 2012, from seeds I had collected from Hegg Lake State Wildlife Management Area and other prairie remnants in Douglas County, Minnesota. I haphazardly collected spikelets from flowering inflorescences of four plants for the analysis. I tapped anthers from individual spikelets onto a glass slide and stained the pollen with 0.05% toluidine blue in water, following the protocol described by Bucciaglia et al. (2003). I viewed the slides under a compound microscope at 5, 10, or 20X and took digital images of the microscope fields using Remote Capture DC 3.1.0.5. I counted pollen grains from digital images of ten microscope slides of pollen collected from young and old spikelets (Appendix III, Figure S8). Viable pollen stained dark blue, while non-viable pollen was lightly stained. I classified stained pollen as dark, dark blue, squashed, light blue, empty, and small dark pollen. I counted the number of grains in each category, and calculated total pollen counts and pollen viability. I expressed pollen viability as the percentage of viable pollen over total pollen count. For the liberal estimate, I considered pollen in the light blue and empty categories to be inviable, and all other categories to be viable. For the conservative estimate, I also included the squashed and small dark pollen as inviable categories.

4. RESULTS

4.1 Pollen Addition and Exclusion

Supplemental hand-pollination (pollen addition) increased the seed set of inflorescences by 42% compared to controls (open-pollinated), according to a generalized linear mixed model (GLMM) with individual plants modeled as a random effect (Table 5, Table 6). Bagging inflorescences (pollen exclusion) also increased seed set of inflorescences by 33% compared to controls (open-pollinated), according to the same model (Table 5). Although seed set varied greatly among individual plants, effects of pollination treatments were significant ($p = 0.001$, $N = 77$ observations from 32 plants; see Figure 2, Table 6,).

4.2 Density and Resource Limitation

Natural seed set (as indicated by seed set of open-pollinated, unmanipulated inflorescences) varied greatly among individual plants, ranging from 0 to 0.86 (Figure 2). Median and mean natural seed set were 0.42 and 0.43 respectively.

Density of neighboring *D. leibergii* plants, plants height, and clump diameter did not explain the within-population variance in seed set, according to generalized linear models (GLM) ($p > 0.05$, $N = 28$; see Table 7). However, I found a marginally significant relationship between clump diameter and seed set ($p = 0.08$, Table 7), suggesting that a resources may influence seed production.

4.3 Pollen Viability

Pollen counts per anther ranged from 13 to 95, suggesting that pollen production varies widely among different spikelets and plants. I considered the lowest and highest pollen counts (6 and 264, respectively) to be outliers and omitted them from analyses of pollen viability. Pollen viability ranged from 68% to 96% (liberal estimate) or 42% to 77% (conservative estimate), indicating that *D. leibergii* plants produce high quality pollen.

5. DISCUSSION

5.1 Pollen Limitation in D. leibergii

Pollen addition significantly increased seed sets of inflorescences compared to controls, indicating pollen limitation in the *D. leibergii* population (Table 5, Table 6). Pollen exclusion also significantly increased seed sets of inflorescences compared to controls, indicating that pollen was limited by quantity but not quality of pollen (Table 5, Table 6). Although the pollen exclusion treatment did not involve hand-pollination, bagging inflorescences probably helped relieve limitation of pollen quantity by capturing and saturating pollen within the bag.

Pollen limitation often occurs in stochastic pollination environments, where pollen delivery or receipt is unpredictable (Ashman et al. 2004, Knight et al. 2005). Many factors affect pollen dispersal and capture, including aerodynamics, floral morphology, and plant architecture (Friedman and Barrett 2009b). The large variance in natural seed set among individual plants (Table 5, Figure 2) also indicates that that pollen dispersal or receipt by *D. leibergii* plants is highly variable.

Burd (1994) hypothesized that pollen limitation is the result of an adaptive resource allocation strategy in stochastic pollination environments, where plants produce more ovules per flower or inflorescence than are fertilized. By overproducing ovules, plants can take advantage of occasional abundance of pollen to maximize seed set in some inflorescences. Although not all ovules on all inflorescences may be fertilized, the peak in seed sets during favorable pollination events may outweigh the cost of producing additional ovules. In *D. leibergii,* many ovules (each spikelet contains a single ovule) are produced per inflorescence, and many inflorescences are produced over the course of the flowering season, which lasts from May to September. Hence, although seed set may be low in most inflorescences, the plant may still benefit from producing low amounts of seed, which adds up to many seeds over several months.

I did not expect resource reallocation to affect my analysis that compared seed set between pollen-added and control inflorescences because the control inflorescences flowered and set seed at the same time as the pollen-added inflorescences. One way to avoid resource reallocation issues is to apply pollen supplementation to whole plants (Ashman et al. 2004). However, it would be extremely difficult to do this for *D. leibergii* plants because they produce many inflorescences each bearing many spikelets. Furthermore, *D. leibergii* plants produce new shoots every few days throughout the flowering season. Hence, I could not exclude the possibility of resource reallocation trade-offs between inflorescences or other parts of the plant, or flowering in future years.

Seed set of *D. leibergii* may also vary with flowering phases and the occurrence of cleistogamy. Primary flowering panicles (inflorescences) are hypothesized to have lower seed set than secondary panicles, which have produce cleistogamous spikelets (L. G. Clark, personal communication). In other words, pollen limitation is more likely to affect primary panicles than secondary panicles which have cleistogamous spikelets that are self-pollinated within closed florets. However, the extent of cleistogamy and its precise occurrence in *Dichanthelium* species may be difficult to pinpoint. Lelong (1965) described terminal inflorescences as strongly cleistogamous, but Freckmann (1967) found many terminal inflorescences that were clearly chasmogamous and reports strong cleistogamy in axillary inflorescences growing close to the ground. The debate surrounding the occurrence of cleistogamy may also be in part due to vague terminology and the diversity of spikelets diagnosed as cleistogamous across different species of *Dichanthelium*. The study of cleistogamy, however, is beyond the scope of the present study.

My experiment showed that spikelets that received only self pollen successfully developed into seed, confirming that *D. leibergii* is self-compatible. Moreover, pollen-excluded inflorescences had higher seed set than the controls did. This result is consistent with results from Lelong (1965) who found that seed set of bagged inflorescences equaled or exceeded the seed set of open-pollinated inflorescences in several other *Dichanthelium* species. Although this study did not differentiate between autogamy or geitonogamy, my findings and the findings of previous studies (Lelong 1965) lead me to conclude that pollen compatibility should not limit seed set of *D. leibergii*.

Based on broad literature reviews, Burd (1994) as well as Larson and Barrett (2000) found evidence that self-compatible plants were less likely to be pollen-limited than self-incompatible plants, because self pollen provides an additional, more reliable source of pollen. This was not the case for *D. leibergii*, because inflorescences in the naturally-pollinated control group (which received ambient self pollen) were pollen-limited. Because experimental additions of either outcross or self pollen relieved pollen limitation in *D. leibergii* inflorescences, it is evident that pollen was limited by quantity and not quality.

5.2 Density and Resource Limitation

Seed set varied greatly among individual *D. leibergii* plants in the same population, but the within-population variance could not be explained by density, height, or clump diameter (Table 7). My findings indicate that density and resource availability did not affect female reproduction of *D. leibergii* in this population.

Density played a major role in influencing pollen limitation in several studies (see Section 2.2 for detailed discussion). However, my analysis did not reveal such a relationship between density and seed set. This could mean that *D. leibergii* plants were evenly distributed across my study site, such that local density did not contribute to differences in seed set. Alternatively, it could also be a matter of scales. The localized and fine-scale density measure I used perhaps did not adequately capture the spatial variation among plants. The role of density may have been better captured at a larger scale, e.g. by comparing densities between patches or between populations.

Among-individual variation in seed set could be related to variation in resource availability (Allison 1990, Burd 1994), which may be indicated by plant fitness traits such as height, size, above-ground or below-ground biomass, and floral display (Knapp et al. 2001, Steven and Waller 2006). Larger plants with larger floral displays are assumed to have more resources, i.e. less resource-limited than smaller plants; hence, they can invest more resources into developing seeds (Steven and Waller 2006). In this study, I did not find a link between seed set and plant height or diameter. This suggests that seed set of *D. leibergii* was not resource-limited. Nevertheless, my result could be a false negative, if for example, my choice of maternal plant traits did not truly reflect resource levels of individual plants; or that the variation in the traits measured were too subtle to capture the inherent differences among plants in this study.

5.3 Pollen Viability

Pollen viability of *D. leibergii* was generally high (liberal and conservative estimates were 68-96% and 42-77%, respectively), indicating that pollen quality (in terms of viability) does not hamper reproduction of *D. leibergii*. Previous studies also found similarly high percent pollen viability for *Dichanthelium* species (Freckmann 1967, Spellenberg 1968). Freckmann (1967) found that terminal spikelets of *Dichanthelium* species in the Oligosanthes group showed pollen viability as high as 96%. In particular, herbarium specimens of *D. leibergii*, also a member of the Oligosanthes group, showed pollen viabilities of 66-88%. Spellenberg (1968) found pollen viabilities of 90-100% for *Dichanthelium* plants grown in the greenhouse. Nonetheless, the question remains as to whether these results can be generalized for plants in the field. Hence, future studies should consider assessing pollen viability of *D. leibergii* plants in the field, which is possible using the toluidine blue staining procedure and a small field microscope (A. Smith, personal communication).

Because my results were consistent with values reported by Freckmann (1967) and Spellenberg (1968), I believe that the toluidine blue staining procedure works equally well as the lactophenol-aniline blue solution used by Freckmann and Spellenberg. Because toluidine blue solution is less toxic and simpler to make, compared to the lactophenol-aniline blue solution, future researchers may consider using toluidine blue solution to determine pollen viability.

5.4 Conclusion and Future Directions

My finding that pollen limitation occurred in *D. leibergii* was surprising because it contradicted theoretical expectations. Its reproductive syndrome and breeding traits (wind-pollination, self-compatibility, autogamy) are hypothesized to reduce its risk of suffering pollen limitation. This suggests that habitat fragmentation may impact prairie plants in unexpected ways, and researchers should consider the conservation of plant populations from various aspects. From an evolutionary perspective, pollen limitation does not necessarily indicate reproductive failure in *D. leibergii,* but may actually be evidence of an adaptive “bet-hedging” strategy to maximize pollination successes in an unpredictable pollination neighborhood.

My experiment investigated a key aspect of the reproductive biology of *D. leibergii,* a prairie species representing a functional group of native cool season grasses that are commonly neglected in scientific studies of prairie species. As such, I focused on the middle of the flowering season and a small site with subtle spatial variation in the distribution of plants. Future research could build on this study by investigating seed production of *D. leibergii* over the entire flowering season, over a larger area, or between populations with different densities. These studies will reveal valuable insights into pollen limitation and other factors affecting reproduction of *D. leibergii*.

6. TABLES AND FIGURES

Table 1. Height and number of spikelets for each inflorescence in each treatment group (mean ± standard deviation).

Measurements by treatment	Height of inflorescence (cm)	No. of spikelets
add	56.1 ± 7.08	12.9 ± 4.49
self	54.8 ± 6.39	10.4 ± 3.70
open	55.2 ± 7.56	13.2 ± 3.48

Table 2. Sample sizes at different levels and stages of the experiment, after adjusting for lost or problematic inflorescences and spikelets.

Level	No. of inflorescences	No. of spikelets	No. of seeds weighed
add	30	383 (Hand-pollinated: 287)	313 (Hand-pollinated: 238)
self	20	209	168
open	29	364	298
All groups	79	956	779

Table 3. Types of pollen received by each pollination treatment group. Note that the pollen exclusion group likely received elevated levels of self pollen due to bagging.

Type of pollen received by treatment	Ambient outcross pollen	Ambient self pollen (autogamous + geitonogamous)	Supplemental outcross pollen
add	Yes	Yes	Yes
self	No	Yes *	No
open	Yes	Yes	No

Table 4. Summary statistics of density, height, and diameter measurements. *Density* is number of *D. leibergii* plants within 1 m radius of focal plant, *height* is height of the tallest inflorescence (cm), and *diameter* is clump diameter at ground level (cm).

	Mean	Std. Dev	Range
Density	15.5	5.95	7 – 28
Height (cm)	67.8	7.64	53 – 83
Diameter (cm)	10.8	4.43	3 – 18

Table 5. Mean seed set values, based on model and actual data. Predicted values were back-transformed from logit scale parameter estimates of the GLMM.

Treatment	Predicted mean ± std error*
add	0.572 ± 0.06
self	0.534 ± 0.06
open	0.401 ± 0.05

* when positive and negative standard errors differed, I reported the larger values.

Table 6. Analysis of deviance table comparing a generalized linear mixed models (GLMM). Seed set is the binomial response and pollination treatment is the fixed explanatory variable. Individual plants (“plaID”) is the random effect. N = 77 observations from 32 groups (individual plants). Deviance is twice the log likelihood ratio.

Model and focal term	Model DF	Log Likelihood	Chi-squared	Test DF	Test p-value
Ha: wwbinom8 ~ trt + (1 | plaID)	4	- 94.298	13.165	2	0.001
Ho: wwbinom8 ~ 1 + (1 | plaID)	2	- 100.880	-	-	-

Table 7. Results of generalized linear models (GLMs) with quasibinomial response, testing for the effects of plant height, diameter, and density on seed set of inflorescences within the open-pollinated group. Interaction terms were not significant (data not shown). All three models were compared to a null model. N = 28 plants.

Focal term	Model DF	Model Dev	Test Dev	Test p-value
height	27	83.314	- 0.0659	0.874
diameter	27	75.37	- 8.0097	0.079
density	27	83.375	- 0.005144	0.965

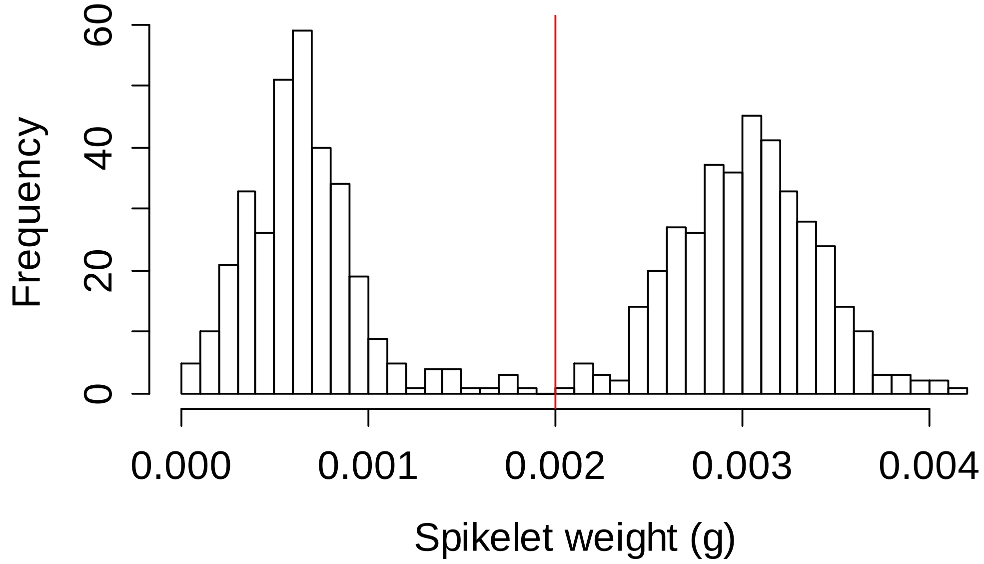

Figure 1. Bimodally distributed histogram of spikelet weights. The vertical line indicates the threshold value (0.002 g) I used to classify spikelets as having developed into seed (heavy) or otherwise (light).

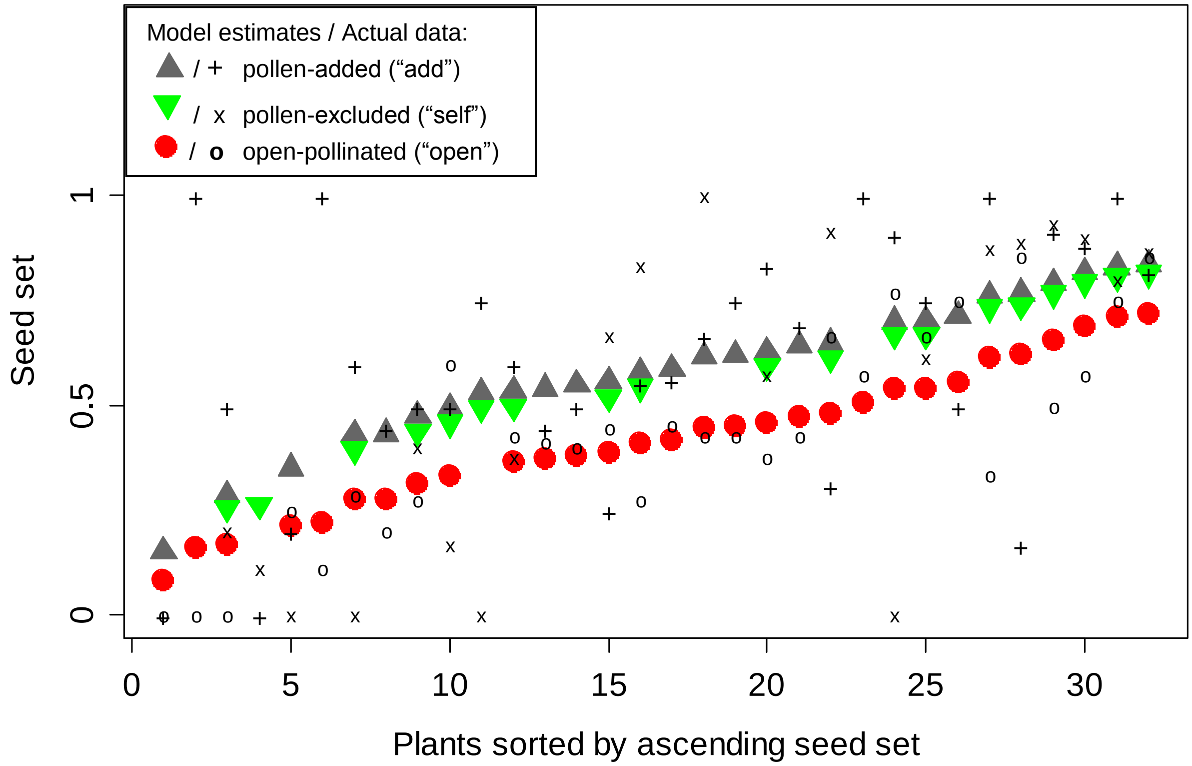

Figure 2. Seed set of inflorescences under different pollination treatments. Mean estimates from the GLMM model are shown as filled triangles or large circles. Actual data are shown as '+', 'x', and 'o'.

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9. APPENDICES

Appendix IA. Pollen Limitation Studies of Wind-Pollinated Herbaceous Plants

Summary of pollen limitation studies of herbaceous plants, sorted by plant family in alphabetical order.
(* indicates ambophilous species. PL = pollen limitation)

Species and Family	**Habitat/study site**	**Breeding system**	**Pollen-limited?**	**Reference**
Beta vulgaris ssp. maritima (L.) Arcang. (Amaranthaceae)	Coastal West Europe and around the Mediterranean basin	Gynodioecious	PL in populations with low ratio of hermaphrodites to female plants	(De Cauwer et al. 2010)
Atriplex acanthocarpa (Torr.) S. Watson (Chenopodiaceae)	Chihuahuan Desert, border of USA-Mexico	Dioecious	No	(Cázares-Martínez et al. 2010)
Atriplex canescens (Pursh) Nutt. (Chenopodiaceae)	Chihuahuan Desert, border of USA-Mexico	Dioecious	Yes	(Cázares-Martínez et al. 2010)
Coriaria arborea Linds. (Coriariaceae)	New Zealand	Hermaphrodite	No	(Merrett et al. 2007)
Carex arctata Boott ex Hook., *C. hirtifolia* Mack., *C. laxiflora* Lam., *C. pedunculata* Muhl. ex Willd., *C. pensylvanica* Lam., *C. plantaginea* Lam., *C. scabrata* Schwein. (Cyperaceae)	Mixed deciduous-evergreen forests; study site in Ontario, Canada	Monoecious (protogynous)	PL only in *Carex scabrata*	(Friedman and Barrett 2009a)
Mercurialis perennis L. (Euphorbiaceae)	European deciduous woodland	Dioecious	Weak quantitative PL	(Vandepitte et al. 2009)
Macropiper excelsum (G.Forst.) Miq. (Piperaceae)	New Zealand	Dioecious	No	(Merrett et al. 2007)
Andropogon gerardii Vitman (Poaceae)	North American tallgrass prairie	Hermaphrodite/ male only	No	(McKone et al. 1998)
Distichlis spicata (L.) Greene (Poaceae)	New England salt marshes	Dioecious	Yes (Bertness and Shumway 2012); PL in 2 of 3 populations (Eppley 2005)	(Bertness and Shumway 2012, Eppley 2005)
Phragmites australis (Cav.) Trin. ex Steud. (Poaceae)	Wetlands; introduced to Japan	Monoecious	PL in 1 of 2 populations	(Ishii and Kadono 2002)
Sorghastrum nutans (L.) Nash (Poaceae)	North American tallgrass prairie	Hermaphrodite	No	(McKone et al. 1998)
Spartina anglica C.E. Hubbard (Poaceae)	Salt marshes; introduced to coastal China	Monoecious (protogynous)	Yes	(Li et al. 2008)

Spartina alterniflora Loisel. (Poaceae)	New England salt marshes; invasive in estuaries in northeast America	Monoecious (protogynous)	Yes	(Bertness and Shumway 2012, Davis et al. 2004)
Spartina patens (Aiton) Muhl. (Poaceae)	New England salt marshes	Monoecious (protogynous)	Yes	(Bertness and Shumway 2012)
Linanthus parviflorus (Benth.) J.M. Porter & L.A. Johnson * (Polemoniaceae)	Grasslands and open woodland areas throughout California	Not mentioned	PL in low- lying, wind-protected site but not in wind-exposed site	(Goodwillie 1999)
Rumex acetosella L. (Polygonaceae)	Floodplains or riparian lands; study site in Ontario, Canada	Dioecious	No	(Friedman and Barrett 2009a)
Thalictrum dioicum L. (Ranunculaceae)	Mesic forests across eastern North America	Dioecious	No (but see discussion in Steven and Waller 2006)	(Steven and Waller 2006), (Friedman and Barrett 2009a)
Thalictrum fendleri Engelm. ex A. Gray (Ranunculaceae)	Montane open forest and shrub habitats across western North America and northern Mexico	Dioecious	No (but see discussion in Steven and Waller 2006)	(Steven and Waller 2006)
Rhamnus lycioides ssp. *oleoides* (L.) Jahand. & Maire * (Rhamnaceae)	Shrublands in Mediterranean basin	Dioecious	PL in mountain population but not in coastal population	(Gulias and Traveset 2012)
Coprosma spathulata A. Cunn. (Rubiaceae)	New Zealand	Dioecious	PL in 1 of 2 populations	(Merrett et al. 2007)
Melicytus novae-zelandiae (A.Cunn.) P.S.Green (Violaceae)*	New Zealand sand dunes	Dioecious	No	(Merrett et al. 2007)

Appendix IB. Pollen Limitation Studies of Wind-Pollinated Trees

Summary of pollen limitation studies of trees, sorted by plant family in alphabetical order.
(* indicates ambophilous species. ** indicates studies that did not involve pollen manipulation. PL = pollen limitation)

Species and Family	Habitat/study site	Breeding system	Pollen-limited?	Reference
Betula pendula Roth (Betulaceae)	Forests in Northern Sweden; Relatively warm and dry habitats with coarse-grained soils	Monoecious (dichogamous)	PL in mountain population but not in coastal population	(Holm 1994)
Betula pubescens Ehrh. ssp. ***pubescens*** (Betulaceae)	Forests in Northern Sweden; organic, mesic to moist soils; more humid local climate.	Monoecious (dichogamous)	PL in mountain population but not in coastal population	(Holm 1994)
Quercus douglasii Hook. & Arn. (Fagaceae)	Foothills of Central Valley, California	Monoecious	Fruit production positively associated with number of neighboring pollen producers when trees were productive. **	(Knapp et al. 2001)
Carya ovata (Mill.) K. Koch (Juglandaceae)	Mixed oak forests, New Jersey	Monoecious (herkogamous)	No	(Mccarthy and Quinn 1990)
Carya tomentosa (Poir.) Nutt. (Juglandaceae)	Mixed oak forests, New Jersey	Monoecious (herkogamous)	No	(Mccarthy and Quinn 1990)
Juglans mandshurica Maxim. (Juglandaceae)	Along brooks/rivers in mainly northern and north-eastern China; Dongling Mountain, Beijing	Monoecious (heterodichogamous)	No - Pollen supplementation did not increase fruit production in either protogynous or protandrous individuals.	(Bai et al. 2006)
Polylepis australis BITT. (Rosaceae)	Cordoba mountain range of central Argentina	Monoecious (protogynous)	No	(Seltmann et. al 2007)
Salix miyabeana Seemen * (Salicaceae)	Mixed willow stands riversides or wet places in lowlands	Dioecious	Yes	(Tamura and Kudo 2000)
Salix sachalinensis Fr. Schm. * (Salicaceae)	Mixed willow stands riversides or wet places in lowlands	Dioecious	Yes	(Tamura and Kudo 2000)
Salix lapponum L. * (Salicaceae)	Southwest alpine Norway	Dioecious	No	(Totland and Sottocornola 2012)
Taxus canadensis Marsh. (Taxaceae)	Moist deciduous and mixed deciduous coniferous forests in the northeastern United States and southeastern Canada	Monoecious	PL in deer-browsed populations, but no PL in unbrowsed populations (Allison 1990b). Pollination success and seed set positively correlated with pollen production and density (Allison 1990a).	(Allison 1990a, Allison 1990b)

Appendix II. Notes on Floral Biology of D. leibergii

In this section, I describe aspects of *D. leibergii* floral biology that I observed in the field and of plants growing in the laboratory, which will be useful for future studies of pollination and reproductive biology of *D. leibergii* and other *Dichanthelium* species. I also supplement my description with photos, which can be found in **Appendix III**.

Based on Spellenberg's (1968) account, anthers of *Dichanthelium* plants begin to exsert around 5:30 am in warm weather, and begin to dehisce within the next 30 minutes. From my observations during summer 2012, anthers and stigmas on some spikelets have fully exserted by 5:30 am, but other spikelets may still be undergoing anthesis (Figure S3). Some spikelets showed no visible signs of anthesis at 5.30am, but I found their anthers and stigmas in the process of emerging or fully exserted one or more hours later.

The reproductive parts are initially enclosed in the palea and second lemma of the spikelet. The stigmas emerge first, followed by the anthers. The palea and second lemma is open during this process, but usually closes up when stigmas and anthers are fully exserted. Sometimes the stigma and/or anthers never fully exsert, but I do not know whether this is an indication of partial cleistogamy or merely a failure of the spikelet to complete anthesis.

Stigmas vary in color, shape, and size (Table S1). Stigma colors range from bright purple to pink (Figure S4). The stigmas are feathery and usually resemble a bottle brush, but may appear roundish, or pointy at the tips (Figure S4). Although the second lemma and palea closes up a few hours after the onset of anthesis, the stigmas remain erect. Eventually, the stigmas dry out and turn dark brown. However, I do not know if the color of the stigma indicates whether it is still receptive to pollen.

Anthers also vary in shape, size, and their position relative to the stigma (Table S1). Anthers are borne in threes. Fresh anthers are dark purple in color, but turn dark brown when aged (Figure S5). Anthers may be well-exserted and dangling away from the stigmas; exserted just in between the stigmas; or remain within the spikelet, enclosed by the second lemma and palea (Figure S4).

Some spikelets produce an additional set of three anthers, which emerges from between the first lemma and the palea (Figure S6). Both types of anthers may be simultaneously present on a spikelet. Both types of anthers also dehisce white powdery pollen which is similar to other grass pollen. *D. leibergii* pollen is visible to the naked eye in large amounts. Anther dehiscence is highly weather-dependent. In sunny and windy conditions, anthers dehisce more quickly and little pollen is left on anthers by about 10am. Under the microscope, pollen grains look like clear spheres. Sometimes they are retained in aged anthers for several days, where their appearance resembles “cakey” plaster (Figure S5).

D. leibergii inflorescences, or panicles, take 2-3 days to fully exsert. The top spikelets may undergo anthesis before this process is over. In some inflorescences, the bottom-most spikelets remain partially enclosed in the leaf sheath even during anthesis. Some plants have panicles with branches that expand or spread out laterally, but some plants have panicles that remain appressed to the main stem of the inflorescence (Figure S7).

It is difficult to tell which spikelets would flower on a certain day based on their appearance alone. However, it is possible to make rough predictions of the flowering sequence of spikelets based on their positions. Flowering proceeds from top to bottom and centripetally (outermost to innermost spikelets).

Table S1. Sizes of *D. leibergii* floral parts. Measurements were obtained from spikelets from *D. leibergii* plants growing in the laboratory.

Floral part	Length (mm)	Width (mm)
Stigma	0.53 – 1.17	0.52 – 1.13
Anther (first set)	0.71 – 1.08	0.25 – 0.37
Anther (second set)	0.91 – 1.58	0.17 – 0.39

Appendix III. Photos of D. leibergii

Figure S1. Hand-pollination of *D. leibergii* spikelet.

Figure S2. Bagged inflorescences. These inflorescences were not involved in the experiment, but were bagged for a preliminary test of the protocol.

Figure S3. Primary reproductive parts of a *D. leibergii* spikelet. Note the variation in anther position (not exserted, exserted close to stigmas, and drooping away from stigmas). Not all spikelets exsert their florets at the same time. (This photo was taken at 6:20 am on June 28, 2012.)

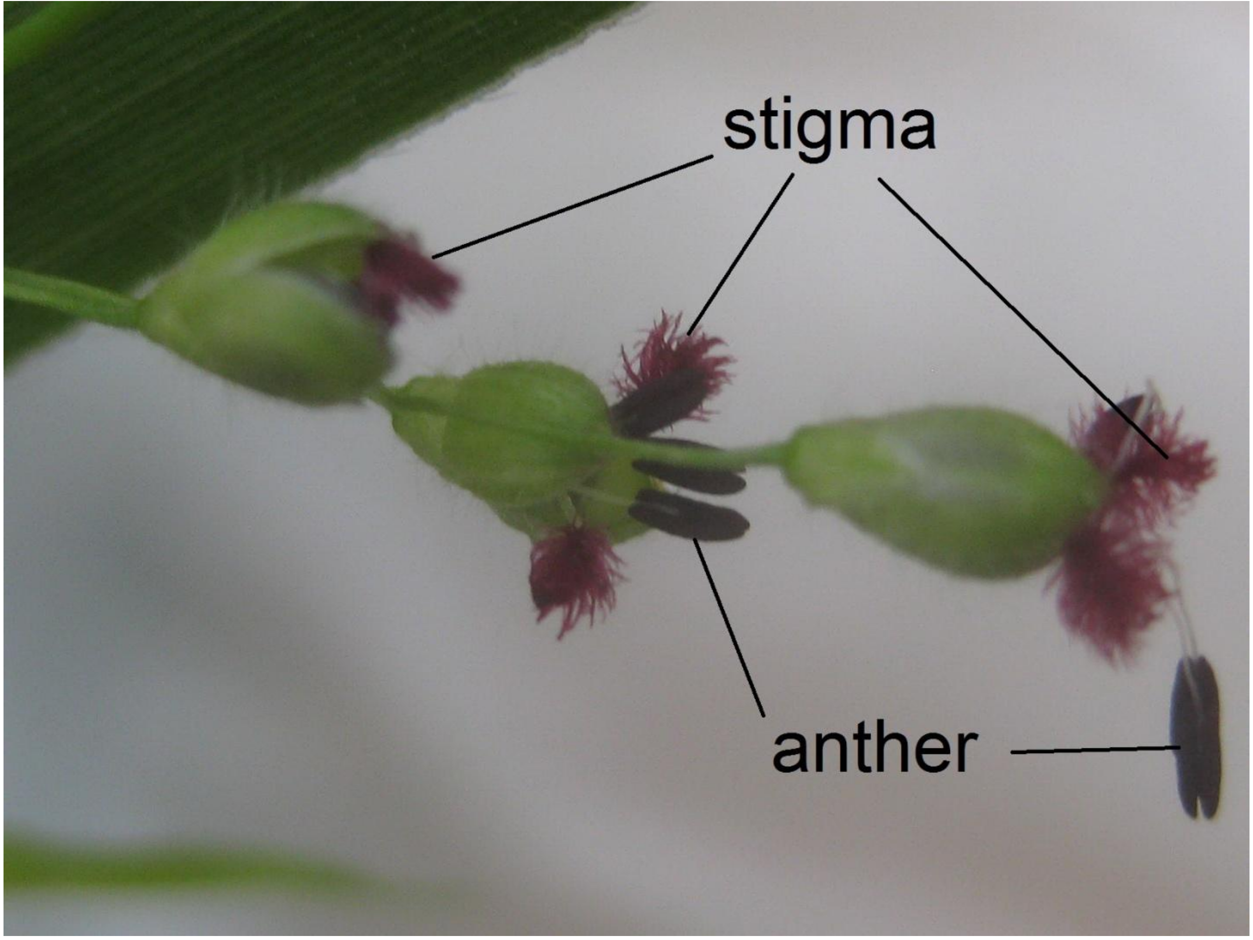


Figure S4. Variation in stigma color and shape.

Figure S5. Fresh or young anthers, on the first day of anthesis (left). Compare with aged anthers, with aged, “cakey” pollen (right).

Figure S6. *D. leibergii* spikelets may produce a second set of anthers, which are exserted from between the first lemma and the palea

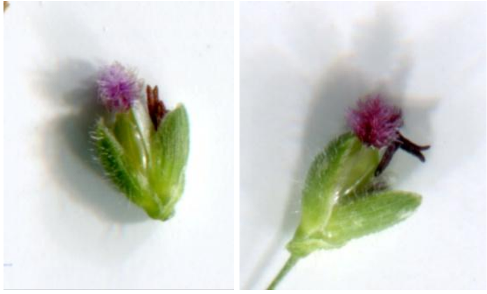
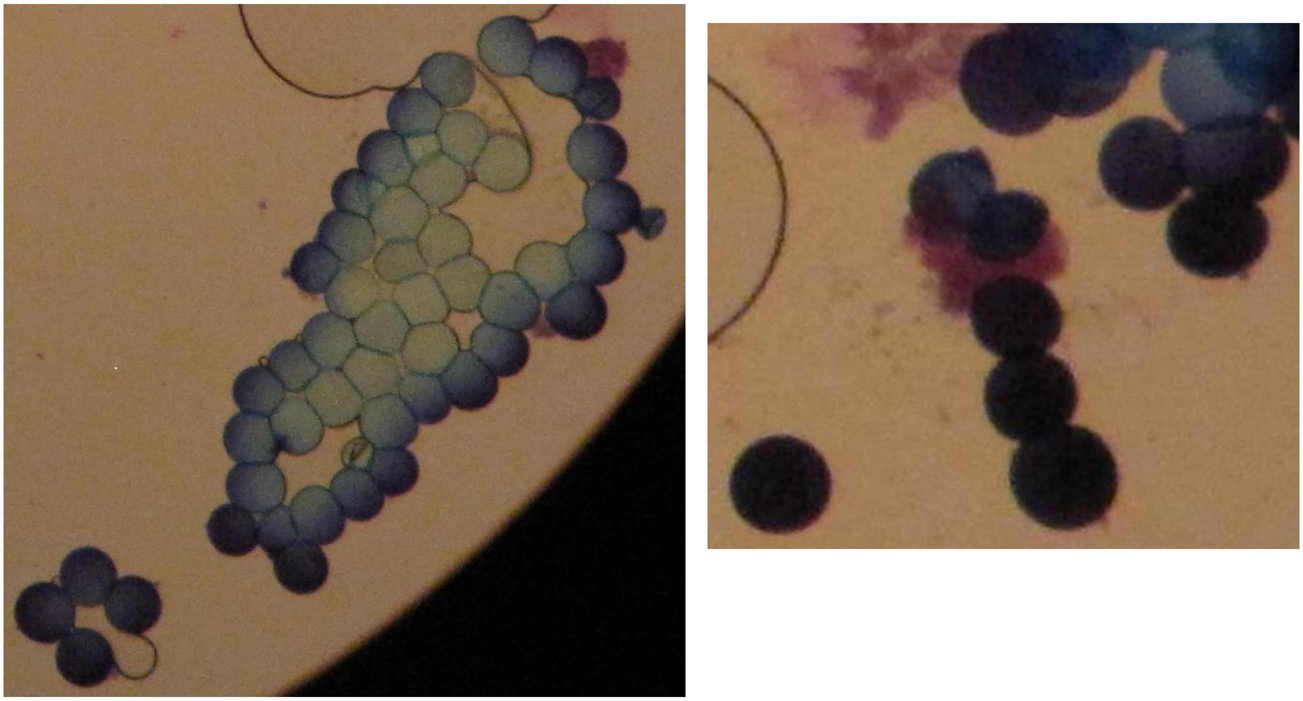


Figure S7. Variation in *D. leibergii* panicle structure. (Left) Panicle branches are expanded and spreading out laterally. (Right) Panicles branches are appressed to the main stem of the inflorescence.

Figure S8. *D. leibergii* pollen grains stained with toluidine blue, as viewed under a compound microscope. Staining levels of pollen grains indicate their viability. I classified pollen grains as: a) dark; b) dark blue; c) squashed; d) small dark pollen; e) light blue; f) empty.

For the liberal estimate of pollen viability, I considered pollen grains in the light blue and empty categories to be inviable, and all other categories to be viable. For the conservative estimate, I included the squashed and small dark pollen as inviable categories.

Examples of microscope slides with stained *D. leibergii* pollen grains.



10. CURRICULUM VITAE

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Permanent Address: Lot 1179, Jalan Lemal, 17000 Pasir Mas, Kelantan, Malaysia
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EDUCATION

M.S., Plant Biology and Conservation, Expected June 2014
B.A., Biological Sciences and Minor in **Environmental Policy and Culture**, Expected June 2013
Northwestern University, IL
GPA: 3.9/4.0

GRANTS, HONORS, & AWARDS

Full Scholarship for B.A. Studies in USA, Public Service Department of Malaysia, 2008-2013
Clara Carter Higgins Scholarship for Environmental Study, Garden Club of America, 2012
Academic Year & Summer Undergraduate Research Grant, Northwestern University, 2012
Katherine L. Krieghbaum Research Scholarship, Northwestern University, 2012-2013
Phi Beta Kappa, 2013

RESEARCH EXPERIENCE

Undegraduate Researcher, Northwestern University/Chicago Botanic Garden
Sept 2011 - present

- Determined techniques to maximize germination in a native prairie grass *Dichanthelium leibergii*
- Investigated the effect of habitat fragmentation on variation in seed germination and plant progeny fitness of *D. leibergii* within and among natural populations
- Designed and conducted experiment to assess pollen limitation of reproduction in a remnant prairie population in *D. leibergii*

Summer Field Research Intern, The Echinacea Project/Chicago Botanic Garden
June - Sept 2012; June - Sept 2011

- Collected data for long-term ecological and demographic study investigating the effect of habitat fragmentation on seed ecology of native prairie species, *Echinacea angustifolia*

LEADERSHIP AND SERVICE

CILIPADI (Malaysian Students Association), Northwestern University
President (2011 - 2012) and *Secretary* (2010 - 2011; 2012 - Present)

POSTER PRESENTATIONS

- Wang, M. 2012. Dormancy Breaking and Germination Techniques for *Dichanthelium leibergii*, a native cool season prairie grass. Poster: Midwest Ecology and Evolution Conference (MEEC) 2012. *Also at:* Undergraduate Research and Arts Exposition, Northwestern University.
- Wang, M. 2012. Examining pollen limitation in a native prairie panic grass, *Dichanthelium leibergii*. 12th Annual Summer Undergraduate Research Symposium, University of Minnesota.
- Wang, M. 2013. Does pollen availability limit reproduction in a native wind-pollinated prairie grass? Poster: Midwest Ecology and Evolution Conference (MEEC) 2013.
  Also at: Chicago Area Undergraduate Research Symposium (CAURS) 2013.