FUNCTIONAL SIGNIFICANCE OF POLLEN SIZE VARIATION IN *IPOMOEA PURPUREA*,

COMMON MORNING GLORY

by

BRITNIE RENÉ FOLTZ

(Under the Direction of Shu-Mei Chang)

ABSTRACT

Variation in pollen size is prevalent among plant species. This variation also occurs within species. This study looks at the functional role of small and large pollen grains in *Ipomoea purpurea*. We hypothesize that 1) small pollen grains have an advantage during pollen transport, in that pollinators are likely to carry higher numbers of small pollen grains to a recipient stigma and 2) large pollen grains have a competitive edge over small pollen grains during pollen competition on the stigma. We found that similar quantities of both small and large pollen grains are removed from anthers and deposited onto stigmas by pollinators. However, we found that large pollen grains do sire more seeds than small grains when competing on the same stigma, suggesting that large pollen grains are better competitors. Thus, the question still remains regarding the functional role of small pollen in *I. purpurea*.

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by

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CHAPTER 1
INTRODUCTION

Variation in pollen size is widely prevalent across flowering plants, differing up to five orders of magnitude among species (Muller 1979). While there have been studies that show some of the variation in pollen size can be explained by pre-pollination factors (e.g. pollen transport) and post-pollination factors (e.g. pollen competition) (Muller 1979; Cruden and Lyon 1985; Johnston 1993; Harder 1998; Sarkissian and Harder 2001; Tejaswini 2002; Lankinen and Madjidian 2011; Jürgens et al. 2012), few studies have looked at intraspecific variation and the functional significance of this variation.

This thesis comprises two studies that investigate the role of pollen size in pollination by parsing the two stages of pollination: pre-pollination and post-pollination. We specifically are interested in the function of pollen size concerning pollen transport of the pre-pollination process and pollen competition of the post-pollination process using lines of *Ipomoea purpurea* that have been artificially selected for large and small pollen grains. The first study looks at pre-pollination—pollen transport by bumblebees, namely pollinator visitation patterns, pollen removal from anthers and pollen deposition on stigmas of *I. purpurea* at two field sites: one field site at the University of Georgia Plant Biology Greenhouse experimental plot and one field site in a field in Watkinsville, Oconee County, GA. The second study looks at post-pollination, particularly pollen competition between large and small pollen grains on the same stigma in *I. purpurea* plants at the University of Georgia Plant Biology Greenhouse.
Pre-pollination refers to pollen transport mechanisms, including what vector is carrying the pollen, and if the vector is biotic, the behavior of the pollinator. Although pollen transport is a pre-pollination component, it is a paramount factor affecting the post-pollination process of pollen competition. Patterns of pollen transport can determine where pollen comes from and how many pollen grains reach a stigma (Harder and Wilson 1998). Harder (1998) divides pollen transport into three parts: (1) pollen removal from anthers, (2) pollen loss during transportation, and (3) pollen deposition on the stigmas. Pollen removal from the anthers and deposition on the stigma are directly affected by floral morphology and the pollinator’s behavior on the flower while foraging. The combination of floral morphology and pollinator behavior then results in how much pollen is gathered or adheres to the pollinator and is subsequently available for potential pollination (Harder 1998). Additionally, pollen size variation related to vector has been known to exist for decades. Wodehouse (1935) gives the following examples: wind pollinated plants have pollen that ranges from 17-58µm, animal pollinated plants have pollen that range from 5-200µm and plants that have pollen carried in the water current can have linear pollen up to 6mm long.

This study focuses on pollen transport in *Ipomoea purpurea* (common morning glory), which is primarily pollinated by bumblebees. When bumblebees forage, they collect both nectar and pollen and are able to determine how much pollen is on their body. Studies have suggested the more pollen the bee removes from the anthers, the more the bee grooms and the less pollen that reaches the recipient stigma (Buchmann and Cane 1989; Harder 1990a; Harder and Barclay 1994). Bee grooming is made efficient by specialized body hairs located on the legs and mouth,
known as combs and brushes, which are specifically for pollen grooming and manipulation (Thorp 1979). Moreover, pollen size may be influenced by bee grooming because the combs and brushes are able to catch larger pollen easier and thus, groom it off easier, thereby allowing smaller pollen to remain and more grains to be transported (Thorp 1979; Harder and Barclay 1994; Harder 1998). Despite the importance of biotic pollen transport few studies have investigated how pollen transport may impose selection pressure on pollen size. This study attempts to fill this gap by investigating the effect of pollen size on pollen transport by bumblebees in *I. purpurea*.

**II: Post-pollination**

Early ideas of male-male competition proposed by Darwin have been demonstrated to be prevalent not only in sexually dimorphic animals, but also in other types of organisms such as plants. In plants, such competition could occur before pollination via floral or inflorescence display and attractiveness, or between pollination and fertilization through interaction between pollen grains that are deposited on the same stigma (pollen competition) (Winsor et al. 2000; Bernasconi et al. 2004). Pollen competition occurs when the number of pollen grains (pollen load) deposited on a stigma exceeds the number of ovules (Johnston 1993; Delph et al. 1998; Erbar 2003; Bernasconi et al. 2004). Characteristics of the pollen load - germination rate, pollen tube growth rate, interaction with the stigma, interaction with other pollen grains, deposition schedule, and/or quality - could potentially influence the outcome of pollen competition following pollination (Epperson and Clegg 1987; Spira et al. 1992; Johnston 1993; Mazer et al. 2010; Lankinen and Madjidian 2011). Among these, pollen tube growth rate has been
consistently shown to correlate with siring success; faster growing pollen tubes tend to have higher siring success than slower growing tubes (Snow and Spira 1991; Snow and Spira 1996; Delph et al. 1998; Lankinen 2002; Mazer et al. 2010).

Another pollen trait that has often been proposed as an important trait influencing the outcome of pollen competition is the size of the pollen grain due to its correlation with energy stores (Baker and Baker 1979; Cruden and Miller-Ward 1981). Pollen size has also been shown to correlate with style length (Baker and Baker 1979; Lord and Eckard 1984; Jürgens et al. 2012) or stigma depth (Cruden and Lyon 1985). Researchers often explain this correlation through the higher germination and higher pollen tube growth rate of larger pollen grains (Baker and Baker 1979; Lord and Eckard 1984). However, among the few studies that examined this trait within a species, mixed results were found (Kumar and Sarkar 1980; Cruzan 1990; Sarkissian and Harder 2001; Tejaswini 2002). Hence, our study will further explore this question, linking pollen size to pollen competition within a species where pollen size variation is genetically based.
CHAPTER 2

FUNCTIONAL SIGNIFICANCE OF POLLEN SIZE VARIATION IN *IPOMOEA PURPUREA*,
COMMON MORNING GLORY I: SMALLER POLLEN SIZE DOES NOT LEAD TO
INCREASED TRANSFER EFFICIENCY\(^1\)

\(^1\) Foltz, BRF and SM Chang. To be submitted to *American Journal of Botany*.  

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Abstract.—Pollen size variation occurs across angiosperm species and, in some cases, within species. Such is the case in the bumblebee pollinated *Ipomoea purpurea*, where pollen size varies by approximately 6-9% in diameter between the large and small grain sizes. Despite much literature documenting pollen size variation, there have been few studies that look at the functional significance of such variation, both among and within species. One hypothesis to explain pollen size variation is that pollen size is correlated with pollen transport efficiency, and in our study, specifically that small pollen grains are more efficiently transported by bumblebees to stigmas than large pollen grains. To test this hypothesis, we asked the following questions: 1) Do small pollen grains have a transfer advantage compared to large pollen? In other words, is more small pollen removed from anthers by pollinators and subsequently transferred to stigmas than large pollen? 2) Do pollinators preferentially visit large pollen producing plants over small pollen producing plants or vice-versa, thereby affecting pollen removal and deposition quantities? We found that small pollen grains and large pollen grains are removed from anthers and deposited onto stigmas in similar quantities. Additionally we found that pollinators do not preferentially visit either plant type, leading to the conclusion that pollen size is not correlated with pollen transport in *I. purpurea*.

INTRODUCTION

Variation in pollen size is widely prevalent across flowering plants, differing up to five orders of magnitude among species (Muller 1979). While there have been studies that show some of the variation in pollen size can be explained by pre-pollination factors and post-pollination factors, (Muller 1979; Cruden and Lyon 1985; Johnston 1993; Harder 1998;
Sarkissian and Harder 2001; Tejaswini 2002; Lankinen and Madjidian 2011; Jürgens et al. 2012) few studies have looked at intraspecific variation and the functional significance of this variation. Pre-pollination refers to pollen transport mechanisms, including what vector is carrying the pollen, and if the vector is biotic, the behavior of the pollinator. Pollen size variation related to vector has been known to exist for decades and is apparent given the following information from Wodehouse (1935): wind pollinated plants have pollen that ranges from 17-58µm, animal pollinated plants have pollen that range from 5-200µm and plants that have pollen carried in the water current can have linear pollen up to 6mm long. Post-pollination processes, such as pollen germination rate, pollen tube competition and ovule fertilization ability can also impose different selection pressures on pollen size (Muller 1979; Harder 1998; Sarkissian and Harder 2001; Tejaswini 2002). Further, ecological factors such as climate, altitude, latitude, light intensity, water and nutrient availability also have been shown to influence pollen size; for example, intraspecific pollen size tends to increase with increased light intensity, temperature, water availability, and nutrient availability in some species (reviewed in Muller 1979).

While several studies have looked at the role of post-pollination and ecological factors on selection for pollen size (Cruden and Lyon 1985; Stephenson et al. 1992; Sarkissian and Harder 2001; Tejaswini 2002; Lankinen and Madjidian 2011; Jürgens et al. 2012), fewer have investigated how pollen transport may impose selection pressure on pollen size. Pollen transport is necessary in outcrossing species to effect pollination and, in self-compatible species, to maintain genetic variation and reduce inbreeding depression. What is more, though pollen transport is a pre-pollination component, it is a paramount factor affecting the post-pollination process of pollen competition. Patterns of pollen transport can determine where pollen comes from and how many pollen grains reach a stigma (Harder and Wilson 1998). Harder (1998)
divides pollen transport into three parts: (1) pollen removal from anthers, (2) pollen loss during transportation, and (3) pollen deposition on the stigmas. While all aspects are important for effective pollination, only pollen loss during transportation is likely to be directly affected pollen size (and hence be a potential selective agent for pollen size) since pollen removal and deposition involve many pollen grains as the unit, whereas pollen loss or retention during transport has individual grains as a unit due to pollinator grooming habits.

Pollen removal from the anthers and deposition on the stigma are directly affected by floral morphology and the pollinator’s behavior on the flower while foraging. The combination of floral morphology and pollinator behavior then results in how much pollen is gathered or adheres to the pollinator and is subsequently available for potential pollination (Harder 1998). In our study, we were interested in pollen transport in *Ipomoea purpurea* (common morning glory), which is primarily pollinated by bumblebees. Other studies have suggested when bumblebees forage, they collect both nectar and pollen and are able to determine how much pollen is on their body. The more pollen the bee removes from the anthers, the more the bee grooms and the less pollen that reaches the recipient stigma (Buchmann and Cane 1989; Harder 1990a; Harder and Barclay 1994). Bee grooming may occur while still on the flower or during flight between visits (Buchmann and Cane 1989; Harder 1990a). Grooming consists of combing the pollen either off of the bee’s body or onto different areas of the body, which can either reduce the pollen’s chances of pollinating the next flower or improve the chances by being moved into a “safe site,” where the pollen is considered safe from being groomed off (Harder 1990b; Harder and Barclay 1994; Harder 1998; Harder and Wilson 1998). Bee grooming is made efficient by specialized body hairs located on the legs and mouth, known as combs and brushes, that are specifically for pollen grooming and manipulation (Thorp 1979). Pollen in safe sites is almost assured to reach
the next flower for pollination. Consequently, pollen size may be influenced by bee grooming because the combs and brushes are able to catch larger pollen easier and thus, groom it off easier, thereby allowing smaller pollen to remain and more grains to be transported (Harder and Barclay 1994; Harder 1998).

Our study system is a primarily bee pollinated annual, *Ipomoea purpurea* (common morning glory) that has been artificially selected for variation in pollen size. We use large and small pollen producing plants as pollen donors and control plants as pollen recipients to look at the effect of pollen size on two of the three categories of pollen transport: pollen removal and pollen deposition. We ask two main questions: 1) Do small pollen grains have a transfer advantage compared to large pollen? In other words, is more small pollen removed from anthers by pollinators? Also, is more small pollen subsequently transferred to stigmas than large pollen? 2) Do pollinators preferentially visit one donor type over the other, thereby affecting pollen removal and deposition quantities? Since the selected lines may also exhibit variation in floral traits that may affect pollinator preference, we also look at variation in floral morphology among donors and control plants.

**Materials and Methods**

**Study species**

*Ipomoea purpurea* (Convolvulaceae), or common morning glory, is an annual weedy species that can be found throughout the U.S. This species is commonly found in fields and roadside ditches, climbing up other vegetation or fences. Flowers are large and showy with funnelform corollas occurring in white, pink or purple (Radford et al. 1968), often with five
pigmented rays originating from the center of the corolla. Flowers are hermaphroditic and self-compatible, with five stamens and a single pistil. Reproduction occurs through both self-pollination and outcrossing. The predominant pollinators are bumblebees (*Bombus* spp.); but other bees (*Apis* sp., *Xylocopa* sp.), hummingbirds, and butterflies also occasionally visit the flowers (Radford et al. 1968; Clegg and Durbin 2000), personal observation). Flowers open at sunrise, when the primary pollinators become active, and senesce by late morning of the same day.

*Experimental plants*

Two replicate lines artificially selected for large pollen grains were generated in a previous study by selecting the individuals with the largest pollen grains to cross to generate the next generation. The replicate lines were maintained independently for five generations. The same procedure was carried out by selecting plants with the smallest pollen grains to generate two replicate lines with small pollen grains. By the fifth generation, the large and small pollen lines had diverged in their pollen grain size by 8.88 µm and 6.7 µm for the two replicate lines, with the average pollen diameter being 114.40 and 113.79 µm in the large pollen replicate lines and 105.52 and 107.04 µm in the small pollen replicate lines. In addition, two corresponding replicate control lines were also maintained by randomly selecting plants in each generation to produce seeds for the next generation.

We started 186 seeds from the generation five replicate lines in the University of Georgia Plant Biology Greenhouse in May 2012 before setting up field arrays in June 2012. Seeds were planted individually in 10” round plastic pots in the greenhouse. To ensure that we
had a wide array of plants to select from for the field study, we planted more than what we needed for the field arrays in order to select plants of similar size for this study. In total, 48 seeds from the control line (RP) (to serve as pollen recipient parents), 60 seeds from the Large Pollen (LP) line (to serve as large pollen donors) and 78 seeds from the Small Pollen (SP) line (to serve as small pollen donors) were planted.

Plants reached maturity in July 2012, at which point we chose 62 of the 185 plants to be placed in the field (31 plants per site x 2 sites): 12 LP, 12 SP and 7 RP per site for two sites. Plants were chosen as to ensure similar plant size and flower color among all plants used at both sites, thus most plants produced blue flowers and a few at each site produced pink flowers. We avoided plants that produced any sterile flowers (i.e. no pollen production) during our initial screening. We also measured corolla height and width, tallest stamen, and stigma height for three flowers of each plant to test for any differences in floral traits among pollen lines. In addition we measured pollen size and production for six flowers of each plant just before we put the plants in the field and another six flowers of each plant halfway through the experiment in order to get baseline pollen size and production numbers. To do this, we collected all five anthers from each of six flowers per plant split into three separate 1.5mL polypropylene tubes (only two flowers per tube=10 anthers for measurement purposes, 30 anthers total per plant) with 750 μL 70% ethanol and measured size and production with a Beckman Multisizer II Coulter counter (Beckman Coulter, Inc., Fullerton, CA). The production data was also used as the baseline “pre-pollination” pollen production number for the pollen removal calculations (see Pollen deposition and removal section of Material and Methods below).
Experimental Arrays

For the experimental arrays we placed 31 ten-inch “anchor pots” in a connected hexagon pattern (Figure 2.1) such that each plant was equidistant (127cm) from any of its immediately neighboring plants. Pots were secured in the up-right position with five-inch or eight-inch spikes to prevent tipping of the plants and deer fencing was also placed around plots. The ten-inch “plant pots” were placed in small plastic shopping bags to minimize water loss and then placed in the anchor pots. Each hexagon was made up of six plants with LP and SP in an alternating pattern and one RP in the center of each hexagon. This ensured that every RP was equidistant from three LP donors and three SP donors, allowing equal probability to receive pollen from either donor. The locations of all plants in the array were randomly re-assigned three times during the experiment, although the alternating LP/SP patterns was always maintained.

An array is defined as which donor plant group (LP or SP) was participating, thus there are two array types: Large array (LA) and Small array (SA). This gives 380 values for each plot-(12 donor plants and 7 recipient plants each in LA and SA array types = 19 data points per array type per replicate) and ten replicates each (10 LA and 10 SA), which equals a sample size of 760 total for both plots for the visitation rate. Replicates were individual observation days (temporal blocks) of each array type. For the visitation duration, because we could not get a value for a plant that was never visited during our observation, we had a lower sample size of 356 total.

Arrays were set up at two field sites: the University of Georgia Plant Biology experimental plot in Athens, GA (33.929652, -83.363721) (hereafter referred to as GH) and an open field in Watkinsville, GA (33.877328, -83.413525) (hereafter referred to as WV). Both plots were similar in light availability and topography. We also ensured that there were no other
populations of *I. purpurea* within one mile of either site. All plants were fertilized with Osmocote upon placement into the field. They were subsequently fertilized with liquid fertilizer (400 parts per million of Jack's Professional Water Soluble Fertilizer 20-10-20 Peat-Lite, JR Peters Inc., Allentown, PA) when flower production waned or leaves started to yellow in order to ensure continued flowering. We have previously found that fertilizer does not affect pollen size in this species (unpublished data).

**Experimental protocol and collections**

**Pollinator visitation**—Each day plants were manipulated such that six flowers of either the 12 LP or 12 SP plants served as pollen donors and each donor type participated for 10 days (20 total days of observations). The evening before each observation day all flower buds of non-participating donors were removed (i.e. on LP donor days, all SP buds were removed and vice-versa); all but six buds of each participating donor and RP were removed and these six remaining RP were emasculated (Figure 2.2). Remaining flowers were allowed to open naturally and received visitation naturally. We observed pollinators between 7AM and 9AM each morning for a total of one hour per the following observation schedule: six “snapshots” and four 15-minute observation periods were evenly dispersed throughout the two hours. For the snapshots, we quickly scanned the entire array and recorded all pollinators in the plot at that time. For the 15-minute observations we followed each pollinator that entered the array and recorded pollinator type, plants visited and visitation time at each flower (Figure 2.3).

**Pollen deposition and removal**—Each day at approximately 10:30AM, when pollinator activity had begun to wane, stigmas were collected to determine pollen deposition. We collected
stigmas from each of the six flowers of each RP for all days. All stigmas were collected in 96-well plates and placed in a refrigerator. Since only LP or SP were donors on any given day, the plates were labeled accordingly. Later in the laboratory, we rehydrated the stigmas by placing each one on a microscope slide with fuchsin jelly (Kearns and Inouye 1993). We then applied a glass cover slip and heated the slide on a hot plate until the stigma was fully embedded in the melted jelly. The stigmas were allowed to sit in the jelly for at least 24 hours to allow the pollen grains to imbibe the stain. After 24 hours, slides were reheated and the stigmas inside the melted jelly were squashed to allow visualization of all pollen grains on the stigma. Slides with squashed stigmas were examined under a stereomicroscope and pollen grains were counted by eye (Figure 2.4).

To get an estimate of pollen removal, each day we removed anthers from all six flowers of each donor and placed all 30 anthers in a 1.5 mL polypropylene tube and added 750 μL 70% ethanol to preserve the pollen grains. We counted remaining pollen grains on anthers using a Beckman Multisizer II Coulter counter (Beckman Coulter, Inc., Fullerton, CA). We then subtracted pollen remaining from our baseline pollen production data to get an estimate of pollen removal.

**Statistical Analysis**

The baseline pollen and floral traits measured were analyzed to determine whether they differed among the 3 groups of plants (LP, SP and RP) and whether such differences might affect pollination rate. We carried out two analyses. First, we tested to see if traits measured were correlated and, hence, may exhibit influence on pollination as a trait-complex rather than
individual traits. To do so, we carried out a large pair-wise correlation analysis among all traits measured (two pollen traits and four floral traits). Second, we expected that pollen size would be different between LP and SP donor groups, but we also wanted to know if there were any other traits that differ significantly between these two donor groups and could potentially be important for pollinator visitation. We carried out a one-way ANOVA for both pollen and floral traits using group as the sole predicting variable.

To determine if LP and SP flowers received different levels of pollination, we carried out ANOVAs for two pollination measures: visitation rate and duration. These data were obtained from our field observations, where visitation rate was calculated as the frequency of visits per plant per unit time (hour) and duration is the average duration of all of visits for each plant in each array. The unit in these analyses was each plant in each array. Because the visitation frequency varied greatly among the temporal blocks, we included replication (rep) along with array type and plant group. Plant group is nested under array type in our analyses so that the RP are only being compared to their corresponding donor plants. To evaluate whether other floral traits influenced the visitation pattern of bees on the study plants, we also included two floral traits as covariates that are least correlated – corolla height and corolla width as continuous variables and thereby represent the most variance in flower size. In order to satisfy the normality assumption of ANOVA, we log-transformed both visitation frequency and duration; however, back transformed values are reported in tables and figures.

To determine whether donor plant groups differed in their transfer efficiency to the RP stigmas and their pollen removal rate, we carried out ANOVAs on two dependent variables: pollen deposition on the stigmas of the RP and pollen removal from the donor plants’ anthers. The main predicting variable in these analyses was donor plant group (LP or SP).
evaluate how well pollinator behavior and other pollen and floral traits may influence pollen deposition and pollen removal, we included five additional factors as the predicting variables: visitation rate, visitation duration, pollen production per flower, corolla height and corolla width.

All statistical analyses were performed using SAS 9.3 (2013).

RESULTS

The size of pollen grains and number of pollen grains were significantly different between the two donor groups with the average pollen size being 117.84±1.25 µm for the LP and 102.50±1.25 µm for the SP donors (F$_{1,46}$ = 75.40, p<0.0001) and the average number being 370.80±17.05 for LP and 298.24±17.05 for SP (F$_{1,46}$=9.05, p=0.0042). Among floral traits, style length was the only one that was significantly different between LP and SP donors (31.65±0.28 mm and 29.86±0.28 mm, respectively) (F$_{1,46}$=20.63, p<0.0001). No other significant differences were found between LP, SP and RP in filament length, corolla height or corolla width (Table 2.1).

The correlation analysis, using all the LP and SP plants pooled, showed that pollen size and pollen numbers are significantly positively correlated (Pearson’s r=0.58, p<.0001). Floral traits are generally positively correlated with each other except for corolla width, which was not correlated with corolla height or filament length but was significantly correlated with style length (r=0.38, p=0.0072). The only correlation found between pollen and floral traits was between average pollen size and style length (r=0.46, p=0.0011) (Table 2.2).

Our preliminary analysis revealed that there was a significant interaction between plot and array type for visitation frequency (F$_{1,742}$=22.81, p<0.0001). Therefore, we analyzed the two
plots separately for all of the remaining data. Additionally, we only included bumblebee data, rather than all observed pollinators, because out of all flower visits, 91.75% were by bumblebees. For the visitation frequency and duration of bumblebees, we included the array type (LA or SA) and plant group (LP, SP or RP) as the predicting variables, with group nested under array type. Note that for each array type there are only two groups that participated: one of the donor groups, either LP or SP, and the recipient group (RP). In addition, and not surprisingly, replicates (basically the temporal block) for the arrays had a significant effect on bumblebee visitation frequency at both plots (GH plot: $F_{9,367}=38.94$, $p<0.001$; WV plot: $F_{9,366}=14.78$, $p<0.001$) and visit duration at the GH ($F_{7,109}=2.81$, $p=0.0099$) (Table 2.3). Thus, we included this factor in all of the pollination analyses to account for variation due to temporal fluctuation of pollinator abundance.

Overall, we found that the GH plot generally received fewer ($F_{1,742}=137.11$, $p<0.0001$) but longer visits than the WV plot ($F_{1,339}=91.08$, $p<0.0001$), though both plots shared bumblebees as the dominant pollinator. When considering the visitation rate and duration that each flower of each experimental plant received during each array trial, we found that both array type and replicate significantly influenced the visitation rate (visit/plant/hr), but not duration, at both plots (Table 2.3). At GH plot, LA arrays received significantly fewer visits ($1.39\pm0.057$ visits/plant/hour) than the SA arrays ($1.87\pm0.077$ visits/plant/hour) ($p<0.0001$) but the pattern was reversed in the WV plot ($3.33\pm0.199$ visits/plant/hour for LA and $2.70\pm0.162$ visits/plant/hour for SA, $p=0.0137$) (Table 2.4). When comparing the donor groups to the recipient groups within the same array (i.e. LP to RP in LA array or SP to RP in SA array), their visitation rates did not differ significantly, indicating that plants generally received similar number of visits in each array (Table 2.4).
within an array differed between donor and recipient, but only in the GH plot (Table 2.3).

However, when comparing between arrays, the duration differed only at the WV plot (Table 2.3, Table 2.4). The two floral traits included in this analysis, corolla width and corolla height, did not show any significant effects on the visitation rate or visitation duration in either plot.

Finally, we found that pollen deposition on the recipient stigmas did not differ between the array types, indicating that LP and SP donors transferred similar amount of pollen grains onto the recipient stigmas. At GH, corolla height and width had a marginally significant, but negative, effect on pollen deposition, in that the larger the flower, both in height and in width, the less pollen it received. However, neither visitation frequency nor duration appeared to influence pollen deposition at either plot (Table 2.5).

Pollen removal, which was measured as (sum of baseline pollen numbers of six flowers) - (sum of pollen remaining on the anthers of six flowers), was shown to differ between donor types. Small Pollen donors had more of their pollen removed (1562.96 grains) than the LP donors (1540.25 grains) but the difference was only significant at GH (p=0.0324) (Fig 2.5).

Unlike for pollen deposition, pollinator visitation rate was a significant factor that positively influenced pollen removal for both plots (GH: $F_{1,73} = 6.82$, $p = 0.0109$; WV: $F_{1,146} = 7.68$, $p = 0.0063$). Also, once again, corolla height had a significant negative effect on pollen removal only at GH, where the taller the corolla, the less pollen that was removed ($F_{1,73} = 3.71$, $p = 0.0579$). Additionally, at both plots, baseline pollen production had a significant effect on pollen removal, in that the more pollen a flower produced, the more pollen that was removed from the anthers (GH: $F_{1,73} = 11268.3$, $p < 0.0001$; WV: $F_{1,146} = 29733.3$, $p < 0.0001$) (Table 2.5).
While the selection processes to generate our experimental plants were only focusing on pollen size, we found LP and SP donors also differ with regards to pollen production and style length. The correlated responses to our selection processes suggest that pollen size and pollen number were correlated and, more specifically, LP donors produced, on average, more pollen than SP donors. While much literature points to a negative correlation between pollen size and number due to size-number tradeoffs (Cruden and Miller-Ward 1981; Stanton and Young 1994; Sarkissian and Harder 2001), this is not always the case (Stanton and Young 1994; Lamborn et al. 2005; Jürgens et al. 2012). It is possible that during selection for pollen size, any negative correlation between pollen size and number in the source population was broken or limited resources in the natural environment that placed a constraint on reproductive resource allocation (i.e. pollen size versus pollen number) was no longer present. Lastly, pollen size was also positively correlated with style length. This association has been show in several other studies and is often attributed to the hypothesis that larger pollen have more energy stores and are therefore able to grow their pollen tubes down longer styles (Baker and Baker 1979; Muller 1979; Lord and Eckard 1984; Torres 2000; Mazer et al. 2010; Jürgens et al. 2012).

More importantly, however, our results suggest that pollen size does not have an effect on pollen transport, neither removal nor deposition, by bumblebees. These results are consistent with results from a study done by Harder (1998), where he looked to see if pollen size corresponded to pollinator type, specifically by looking at nectar collecting versus pollen collecting bees in *Pedicularis* and bees versus birds in several other genera. He hypothesized that species pollinated by pollen collecting pollinators (bees), which groom more frequently, would have smaller pollen grains than species pollinated by nectar collecting pollinators (birds and
nectar collecting bees), which groom less frequently. However, the results of Harder’s study suggest, like ours, that there is not an association between pollen size and pollen transport. In addition, it has been shown that other closely related species that have the same pollinator can differ in both floral morphology and pollen size. For example, Muller (1979) showed that *Sonneratia* and *Duabanga* flowers are both pollinated by bats and have equal style lengths; however, *Sonneratia* has larger flowers and larger pollen than *Duabanga*. Our results also show that all of our experimental plants were visited with similar frequency and duration, suggesting that pollen size, pollen number and floral manipulations did not affect visitation by bumblebees in *I. pupurea*.

Following the same trend as pollinator visitation frequency and duration, there was not a consistent significant difference between array types in pollen deposition on stigmas. This indicates that both LP and SP donors contribute similar pollen numbers to recipient stigmas. On the other hand, pollen removal was different between donor types, where more pollen was removed from SP than LP donors’ anthers, but only at the GH plot. However, this was most likely due to SP having lower pollen production in the first place.

Although pollen size does not seem to affect pollen deposition or pollen removal, it is important to point out that other traits we measured did influence pollen transport data. First, regardless of significant differences between array types or plant groups, pollen production had a strongly significant effect on pollen removal. The more pollen an anther starts with, the more pollen that can be removed. This data is consistent with studies by Harder (1990b) and Young and Stanton (1990), who also found that most intraspecific among plant variability in pollen grain removal was due to variation in pollen production. Also, visitation frequency had a consistent significant effect on pollen removal from donor anthers at both plots, where more...
visits led to more pollen being removed, which intuitively makes sense— the more visits a donor plant gets, the more pollen that is removed from the anthers. Interestingly, visit duration did not affect pollen deposition or removal and visit frequency did not affect pollen deposition. These results counter those found by Harder (1990b). He found the opposite results in six different species, such that pollen removal increased with visit duration but visit frequency did not have an effect, suggesting that though pollinator behavior is important for pollen removal, the pattern of such a relationship depends on the specific interactions between pollinators and the flowers they visit.

Moreover, corolla height had a significant negative effect on both pollen deposition and pollen removal, but only at the GH plot. This data suggests that flowers with taller corollas have less pollen removed from anthers and less pollen deposited on stigmas. This also makes sense because in a taller corolla, the anthers and stigma would be located more internally, and thus more difficult for a pollinator to make full contact. In a shorter corolla, anthers and stigmas may be slightly more exerted and hence, more available for pollinator contact.

Despite our findings that pollen size variation does not seem to be associated with pollen transport, it was important to address this possibility since pre-pollination factors may play a role as selective agents for pollen size. It is evident that pollen size variation exists in *I. purpurea*, but it is not pollen transport that maintains the small pollen size.
Figure 2.1. Experimental array setup. Top-Diagram of experimental array setup. Center circles represent Recipient plants (RP). Squares and triangles represent donor plants, where the two shapes correspond to the two donor groups, Small pollen (SP) and Large pollen (LP). Each plant is equidistant (127cm) from its neighboring plants. Bottom-Photo of WV plot.
Figure 2.2. An emasculated Recipient Plant (RP). This photo shows a) the incision in the corolla, which we made in order to remove the anthers the afternoon prior to participation in an array and b) the recipient stigma.
Fig 2.3. Bumblebees visiting a donor (top) and recipient plants (bottom).
Figure 2.4. A stained stigma after being squashed on a microscope slide. Each dark pink circle is an individual pollen grain.
Table 2.1. Mean floral and pollen traits for all plant groups (top) and for donor groups only (bottom). For all plant group measurements, pollen data was only measured for donors. For donor group only measurements, the p-value is regarding the difference between donor groups for each trait measured. LP=Large Pollen, SP=Small Pollen. Statistically significant results at the 0.05 level are denoted with an *.

<table>
<thead>
<tr>
<th>All plant groups combined</th>
<th>Trait</th>
<th>n</th>
<th>Mean</th>
<th>s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pollen #</td>
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<td>334.52</td>
<td>13.05</td>
</tr>
<tr>
<td></td>
<td>Pollen size (µm)</td>
<td>48</td>
<td>110.17</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>Tallest filament length (mm)</td>
<td>62</td>
<td>28.30</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Style length (mm)</td>
<td>62</td>
<td>30.75</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Corolla height (mm)</td>
<td>62</td>
<td>51.64</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>Corolla width (mm)</td>
<td>62</td>
<td>49.73</td>
<td>0.62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Donor groups only</th>
<th>Trait</th>
<th>Donor group</th>
<th>Mean</th>
<th>s.e.</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
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<td>298.2</td>
<td>21.11</td>
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<tr>
<td></td>
<td></td>
<td>LP</td>
<td>370.8</td>
<td>11.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pollen size (µm)</td>
<td>SP</td>
<td>102.5</td>
<td>1.7</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LP</td>
<td>117.8</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tallest filament length (mm)</td>
<td>SP</td>
<td>28.07</td>
<td>0.25</td>
<td>0.2477</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LP</td>
<td>28.53</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Style length (mm)</td>
<td>SP</td>
<td>29.86</td>
<td>0.28</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LP</td>
<td>31.65</td>
<td>0.28</td>
<td></td>
</tr>
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<td>Corolla height (mm)</td>
<td>SP</td>
<td>51.74</td>
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<td>0.8483</td>
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<td></td>
<td>LP</td>
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<td>0.94</td>
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<td></td>
<td>Corolla width (mm)</td>
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<td></td>
<td>LP</td>
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<td>0.89</td>
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Table 2.2. Pollen and floral trait correlations with sample sizes (n). Pearson's correlation coefficient (r) is reported above the diagonal and p-values are reported below the diagonal. Significant values <0.05 are bolded with an *.

<table>
<thead>
<tr>
<th>Trait (n)</th>
<th>Pollen # (47)</th>
<th>Pollen size (47)</th>
<th>Tallest filament (62)</th>
<th>Style length (62)</th>
<th>Corolla height (62)</th>
<th>Corolla width (62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pollen # (47)</td>
<td>0.582*</td>
<td>&lt;0.0001</td>
<td>0.245</td>
<td>0.001</td>
<td>0.007</td>
<td>0.749</td>
</tr>
<tr>
<td>Pollen size (47)</td>
<td>0.22</td>
<td>0.457*</td>
<td>0.374*</td>
<td>0.266*</td>
<td>0.338*</td>
<td></td>
</tr>
<tr>
<td>Tallest filament (62)</td>
<td>0.068</td>
<td>0.003</td>
<td>0.036</td>
<td>0.164</td>
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</tr>
<tr>
<td>Style length (62)</td>
<td>-0.064</td>
<td>-0.16</td>
<td>-0.064</td>
<td>0.338*</td>
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<td></td>
</tr>
<tr>
<td>Corolla height (62)</td>
<td>-0.028</td>
<td>-0.079</td>
<td>0.041</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corolla width (62)</td>
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<td>-0.041</td>
<td>0.041</td>
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<td></td>
</tr>
</tbody>
</table>
Table 2.3. Summary of visitation data differences for each plot. Visitation data is given for each plot, with replicate, array type, and group within array type as the predicting variables. Group refers to the plant group: Large Pollen donor (LP), Small Pollen donor (SP) or Recipient Plant (RP). Array type refers to the participating donor type: LA (Large pollen Array) or SA (Small pollen Array). Replicate is the temporal block and is individual observation days. Each array type had 10 replicates. Statistically significant results at the 0.05 level are denoted with an *.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Visits per plant per hour</th>
<th>Visit duration</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>df</td>
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</tr>
<tr>
<td>Replicate</td>
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<td>38.94</td>
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<td>Array type (LA/SA)</td>
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<tr>
<td>Group(Array type) (LP/SP within LA/SA)</td>
<td>2</td>
<td>2.48</td>
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</tbody>
</table>

GH

<table>
<thead>
<tr>
<th>Variable</th>
<th>Visits per plant per hour</th>
<th>Visit duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>df</td>
<td>F-value</td>
</tr>
<tr>
<td>Replicate</td>
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<td>14.78</td>
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<tr>
<td>Array type (LA/SA)</td>
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<td>6.13</td>
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<tr>
<td>Group(Array type) (LP/SP within LA/SA)</td>
<td>2</td>
<td>0.22</td>
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</table>
Table 2.4. Mean visitation data for array types only (top) and group within array type (bottom). Statistically significant results at the 0.05 level are denoted with an *.

<table>
<thead>
<tr>
<th>Array type only</th>
<th>GH plot</th>
<th></th>
<th>WV plot</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Array type</td>
<td>Mean visits per plant/hour</td>
<td>s.e.</td>
<td>p-value</td>
<td>Mean visit duration (s)</td>
</tr>
<tr>
<td>SA</td>
<td>1.87</td>
<td>0.077</td>
<td>&lt;0.0001*</td>
<td>7.26</td>
</tr>
<tr>
<td>LA</td>
<td>1.39</td>
<td>0.057</td>
<td></td>
<td>7.09</td>
</tr>
<tr>
<td>SA</td>
<td>2.70</td>
<td>0.162</td>
<td>0.0137*</td>
<td>4.52</td>
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<tr>
<td>LA</td>
<td>3.33</td>
<td>0.199</td>
<td></td>
<td>5.15</td>
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<table>
<thead>
<tr>
<th>Group within array type</th>
<th>GH plot</th>
<th></th>
<th>WV plot</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Array type</td>
<td>Mean visits per plant/hour</td>
<td>s.e.</td>
<td>p-value</td>
</tr>
<tr>
<td>RP</td>
<td>SA</td>
<td>1.71</td>
<td>0.112</td>
<td>0.1329</td>
</tr>
<tr>
<td>SP</td>
<td>SA</td>
<td>2.04</td>
<td>0.103</td>
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</tr>
<tr>
<td>RP</td>
<td>LA</td>
<td>1.36</td>
<td>0.089</td>
<td>0.9612</td>
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<tr>
<td>LP</td>
<td>LA</td>
<td>1.42</td>
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<tr>
<td>WP</td>
<td>SA</td>
<td>2.81</td>
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<td>LA</td>
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<td>WP</td>
<td>LA</td>
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<td>0.246</td>
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Table 2.5. Summary of pollen deposition data (top) and pollen removal data (bottom). Statistically significant results at the 0.05 level are denoted with an *.

<table>
<thead>
<tr>
<th>Pollen deposition on recipient stigmas</th>
<th>GH</th>
<th>Variable</th>
<th>df</th>
<th>F-value</th>
<th>p-value</th>
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<td>1.42</td>
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<td>Visit duration (s)</td>
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<td>Corolla width (mm)</td>
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<td>3.92</td>
<td>0.0523*</td>
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</table>

<table>
<thead>
<tr>
<th>Pollen deposition on recipient stigmas</th>
<th>WV</th>
<th>Variable</th>
<th>df</th>
<th>F-value</th>
<th>p-value</th>
</tr>
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<tr>
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<td></td>
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<td>1.60</td>
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<td></td>
<td>Visits/plant/hour</td>
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<td>0.609</td>
</tr>
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<td>Visit duration (s)</td>
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<td>0.21</td>
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<td>Corolla height (mm)</td>
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<table>
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<th>F-value</th>
<th>p-value</th>
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<td>Donor type</td>
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<tr>
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<td></td>
<td>Visits/plant/hour</td>
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<td>Visit duration (s)</td>
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<td></td>
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</table>

<table>
<thead>
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<th>Pollen removal from donor anthers</th>
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<th>F-value</th>
<th>p-value</th>
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<td>1</td>
<td>29733</td>
<td>&lt;.0001*</td>
</tr>
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Figure 2.5. Mean pollen removal from donor anthers. Plot (GH or WV) is on the horizontal axis, mean number of pollen grains removed per plant (per six flowers) is on the vertical axis with standard error bars. Filled bars are Small Pollen donors and open bars are Large Pollen donors. Only GH values were significantly different, as denoted by different letters.
CHAPTER 3

FUNCTIONAL SIGNIFICANCE OF POLLEN SIZE VARIATION IN *IPOMOEA PURPUREA*,
COMMON MORNING GLORY II: POLLEN COMPETITION LEADS TO NON-RANDOM
SIRING SUCCESS\(^2\)

\(^2\) Foltz, BRF and SM Chang. To be submitted to *Evolution.*
Abstract—There are many studies that look at variation in pollen size across angiosperm species. However, few investigate the functional significance of pollen size variation, and even fewer consider this significance within a species. Here we use artificial lines of *Ipomoea purpurea* that have been selected for divergence in pollen size to examine one potential role of pollen size—pollen competition. We hypothesize that large pollen grains will have a competitive advantage over small pollen grains when competing on the same stigma. We measure competitive success by proportion of seeds sired. We test our hypothesis by considering the following questions: 1) Does large pollen have a competitive advantage over small pollen? and 2) Is there a correlation between siring parent pollen size and resulting fruit and seed weight (proxy for offspring quality)? Our study supports our hypothesis in that large pollen outcompeted small pollen the majority of the time and large pollen crosses produced larger seeds. Thus, it appears that one function of pollen size is to increase competitive ability, most likely through a positive correlation between pollen size and pollen tube growth rate.

INTRODUCTION

Early ideas of male-male competition proposed by Darwin have been demonstrated to be prevalent not only in sexually dimorphic animals, but also in other types of organisms such as plants. In plants, such competition could occur before pollination via floral or inflorescence display and attractiveness, or between pollination and fertilization through interaction between pollen grains that are deposited on the same stigma (pollen competition) (Winsor et al. 2000; Bernasconi et al. 2004). Pollen competition is an important population-level process as it allows for selection on traits that may directly increase pollen donor (male) fitness through differential
siring success (Krauss 2000; Lankinen and Skogsmyr 2001) or traits that indirectly impact male success through their correlation with pollen traits. While much has been done to understand how the morphological and architectural design and other attractiveness traits in flowering plants may influence male fitness of a plant, much less is known about post-pollination processes involved in male-male competition in flowering plants.

Pollen competition occurs when the number of pollen grains deposited on a stigma exceeds the number of ovules (Johnston 1993; Delph et al. 1998; Erbar 2003; Bernasconi et al. 2004). In natural populations, insect-deposited pollen loads often exceed the number of ovules available, especially after multiple visits (Mulcahy 1979; Winsor et al. 2000), which presents the opportunity for pollen competition. Characteristics of the pollen load - germination rate, pollen tube growth rate, interaction with the stigma, interaction with other pollen grains, deposition schedule, and/or quality - could potentially influence the outcome of pollen competition following pollination (Epperson and Clegg 1987; Spira et al. 1992; Johnston 1993; Mazer et al. 2010; Lankinen and Madjidian 2011). Among these, pollen tube growth rate has been consistently shown to correlate with siring success; faster growing pollen tubes tend to have higher siring success than slower growing tubes (Snow and Spira 1991; Snow and Spira 1996; Delph et al. 1998; Lankinen 2002; Mazer et al. 2010). Pollen tube growth rate is a complex trait that likely represents a composite outcome of the genetic identity of the pollen donors (Mulcahy 1979; Walsh and Charlesworth 1992; Lankinen 2002; Stephenson et al. 2003), the haploid genotype of the pollen grain (Mulcahy 1979; Spira et al. 1992; Walsh and Charlesworth 1992; Arthur et al. 2003), specific interactions of pollen donor and style environment or style attrition, as well as the ecological conditions for the pollen and ovule parents (Stephenson et al. 1992; Lankinen and Skogsmyr 2001; Tejaswini 2002).
Another pollen trait that has often been proposed as an important trait influencing the outcome of pollen competition is the size of the pollen grain due to its correlation with energy stores (Baker and Baker 1979; Cruden and Miller-Ward 1981). Pollen size often varies across even closely related species (Cruden and Lyon 1985; Tejaswini 2002; Jürgens et al. 2012) and has been shown to correlate with style length (Baker and Baker 1979; Lord and Eckard 1984; Jürgens et al. 2012) or stigma depth (Cruden and Lyon 1985). Researchers often explain this correlation through the higher germination and higher growth rate of larger pollen grains (Baker and Baker 1979; Lord and Eckard 1984). For example, using two inter-fertile species of the *Mimulus* complex, Diaz and Macnair (1999) showed that the species with larger pollen grains (*M. guttatus*) had a faster pollen tube growth rate than the smaller-pollen species (*M. nasutus*), and completely dominated the competition in *M. guttatus* styles. Though this pattern was found between two inter-fertile sister-taxa, it seems reasonable to expect a similar mechanism would also operate within a species. However, among the few studies that examined this trait within a species, mixed results were found. For example, Sarkissian and Harder (2001) found that larger pollen grains produce faster growing pollen tubes in *Brassica rapa* and Kumar and Sarkar (1980) also found a positive correlation between pollen grains size and in vitro pollen tube growth rate following pollen germination in maize. However, Tejaswini (2002) found that pollen size and pollen tube growth rate were not always correlated in *Dianthus caryophyllus* and Cruzan (1990) found that pollen size did not correlate with in vivo pollen tube growth rate at all in *Erythronium grandiflorum*.

The conflicting results between within-species and across-species studies might be due to confounding effects between genetically-based and environmentally-based pollen size variation. While the difference in pollen size among species is most likely genetically based, variation
within a species could be due to genetic differences and environmental conditions. Genetic variation in pollen size is generally considered to be low within a species but significant heritability has been found in several species such as *Phaseolus vulgaris* (common bean) (Montes-R and White 1996), *Brassica rapa* (Sarkissian and Harder 2001), and *Mimulus guttatus* (Lamborn et al. 2005); however, we know little about how genetically based pollen size variation may affect the outcome of pollen competition. Results from within species studies on pollen performance often involved ecological treatments (Stephenson et al. 1992; Lau and Stephenson 1993; Delph et al. 1997; Hersch 2006; Distefano et al. 2012), resource allocation/paternal provisioning, (Cruzan 1990; Delph et al. 1997; Stephenson et al. 2003) or unspecified differences (Snow and Spira 1991; Spira et al. 1992; Snow and Spira 1996). In addition, studies that used distinct genetic lines for pollen competition and showed consistent siring success variation among pollen donors did not examine the role of pollen size (Marshall and Folsom 1991; Snow and Spira 1991; Spira et al. 1992; Snow and Spira 1996; Delph et al. 1998; Marshall 1998; Marshall and Diggle 2001; Marshall and Oliveras 2001). We are then faced with the question of whether environment (soil fertility, presence of mycorrhizae, etc) or genetics is the predominant force underlying the results from within species studies. A critical next step is to examine how selection may act on genetically based pollen size variation (Walsh and Charlesworth 1992; Delph et al. 1998; Lankinen and Skogsmyr 2001; Sarkissian and Harder 2001) in order to understand how this trait may evolve in natural populations.

To isolate the effect of genetic based pollen size variation on the outcome of pollen competition, we need a study that can directly link different genotypes of distinct pollen size to their siring success during pollen competition. To do so, we used *Ipomoea purpurea* individuals that had been selected to be genetically divergent in pollen size. We grew these plants in similar
ecological conditions (common garden) to test whether genotypes of different pollen sizes show any difference in siring success when they compete on the same stigma. We used these artificially selected lines of *I. purpurea* to investigate two main questions: 1) Does large pollen have a competitive advantage over small pollen? and 2) Is there a correlation between siring parent pollen size and resulting fruit and seed weight (proxy for offspring quality)? In addition, we measured a suite of floral traits to rule-out any differences other than pollen traits that may confound our results.

**Materials and Methods**

*Study species*

*Ipomoea purpurea* (Convolvulaceae), or common morning glory, is a climbing, self-compatible, weedy annual species that occurs in most parts of the U.S and is often found in fields and roadside ditches. The corollas are funnelform and can be white, pink or purple (Radford et al. 1968), often with five pigmented rays. Flowers usually begin to open just before sunrise, are fully open just after sunrise, and they senesce by late morning of the same day. The androecium consists of five stamens with filaments of unequal lengths, while the gynoecium consists of a three-locular ovary, with two ovules per locule. The fruit is a dry, dehiscent capsule (Radford et al. 1968; Zomlefer and Knapp 1994). The predominant pollinators are bumblebees (*Bombus spp.*); but other bees (*Apis sp., Xylocopa sp.*), hummingbirds, and butterflies also occasionally visit these flowers (Radford et al. 1968; Clegg and Durbin 2000; personal observation).
Parent Plant Selection

The starting material for this experiment came from a selection experiment that selected for increased (Large Pollen or LP line) or decreased (Small Pollen or SP line) average pollen grain size over six generations starting in 2007. Founding population (Gen 0) consisted of 350 plants grown from seeds collected from two populations located in Watkinsville, Oconee County in GA. From this founding population, 21 plants with the largest and 21 plants with the smallest pollen grains were selected, and crosses were made among them to produce seeds for the following generation. Two replicate lines were created for both LP and SP lines and maintained entirely isolated from one another. In addition, two corresponding replicate control lines were also maintained by randomly selecting plants in each generation to produce seeds for the next generation. Each replicate, hence, consisted of three lines, one LP, one SP and one control. By the fifth generation, pollen size between the LP and SP lines was significantly diverged, with the average LP pollen size for the two replicates being 114.40 and 113.79 µm in diameter and the average SP pollen size being 105.52 and 107.04 µm in diameter. The overall divergence in the mean pollen size (diameter) between LP and SP was 8.88 µm and 6.7 µm for the two replicate lines. We carried out crosses between replicate lines to reduce inbreeding level in the resulting seeds that were subsequently used for this study. With the artificial selection targeting on pollen size, we did not detect any consistent correlated response in pollen number in any of the selected lines. Hence, the key difference between plants from the selected lines used in this study was in their average pollen size.

In January 2012, we planted 387 I. purpurea seeds resultant from the crosses described above, and these plants started producing flowers in March 2012. Among these plants,
we selected 16 individuals each from the LP and SP lines. Before we carried out pollen
competition, we first tested for the size divergence in the LP and SP lines by collecting anthers
from two flowers per plant. We repeated this confirmatory test twice during this study. Collected
pollen grains were sized and counted using a Beckman Multisizer II Coulter counter (Beckman
Coulter, Inc., Fullerton, CA). This machine both counts and measures the diameter of each
pollen grain simultaneously and produces data output that includes not only the counts, but also a
histogram of the pollen size distribution in the sample. From the output, we obtained average
pollen size and the total pollen number that falls within the possible range (70 – 125 µm) of I.
purpurea pollen size. The size criterion was used to remove the measurements of air bubbles in
our samples, which were much smaller than the pollen grains (generally smaller than 65 µm).
Because we had found aborted pollen grains in some of our samples during artificial selection
prior to this study, we distinguish aborted (<90 µm in diameter) from fertile (>90 µm in
diameter) pollen grains in our data. We recorded the number and average size of the fertile
pollen in addition to the total pollen counts and the overall average. In summary, there are a total
of four pollen measurements: total pollen number, average pollen size, fertile pollen number and
average fertile pollen size. Individuals with a consistent phenotype of pollen abortion were
excluded from the selection process.

After pollen measurements, we ranked donors by pollen size and chose the largest 16 LP
and smallest 16 SP plants to serve as pollen donor parents (donors) and an additional 16 control
plants as ovule donor parents (i.e. pollen recipients, designated as R plants hereafter) for the
pollen competition experiments. Large Pollen donors were labeled as LP1-LP16, with pollen size
progressively declining along this range. Small Pollen donors were labeled as SP1-SP16, with
pollen size progressively increasing along this range. Recipients were labeled as R1-R16. To
ensure that these 16 selected LP and SP donor flowers produced relatively similar numbers of pollen grains, we measured pollen production of each donor by hand counting all of the pollen on each of three anthers per donor plant (one anther x three flowers per plant x 16 plants) to confirm the machine (particle counter) estimates. To do this, we counted pollen grains stained with fuchsin jelly on a slide under a stereomicroscope. We repeated the counts two weeks later on one anther per donor to re-verify pollen production. During the second set of counts, we also recorded the number or aborted pollen grains, which are easy to distinguish since they are often extremely small and/or irregularly shaped. No aborted pollen grains were observed in the pollen donors when this study began, but we eventually found four plants in the SP line that expressed this phenotype. However, the proportion of aborted pollen grains was small. Control (R) plants were used as the ovule donors (pollen recipients) because they were unrelated to any LP or SP donors. This design allowed us to avoid any unexpected interactions between pollen tubes and the stylar tissues due to biparental inbreeding, i.e. mating between relatives.

**Pollen competition**

We originally attempted to apply precise numbers of pollen grains from each donor for each race by removing the pollen from an anther, counting 100 grains from each donor on a slide, mixing pollen from the competing donors and then applying the counted pollen to the recipient stigma with a paintbrush. However, this method caused pollen grains to become non-viable and, as a result, more than 71% of the first 567 pollinations that we carried out failed. This number is actually higher, but we stopped counting failed crosses after switching to less destructive methods. To avoid damaging the pollen grains, we directly applied pollen from
collected anthers onto the same stigma (following methods in Epperson and Clegg (1987), with more details below). This method is only valid for testing the effect of pollen size on pollen competitive ability if we can apply a similar quantity of pollen grains from both LP and SP pollen donors since unequal pollen numbers could contribute to increased competitive ability for a donor (Winsor et al. 2000; Lankinen 2002). To test this required condition, we performed ten LPxLP and ten SPxSP “test” crosses and compared the numbers of pollen grains applied from using two LP or two SP plants. Using LPxSP crosses would not be appropriate for this test, because their pollen grains cannot be readily distinguished when mixed on the same stigma. On May 2\textsuperscript{nd}, early in the pollen race experiment, we randomly selected ten LP donors and ten SP donors and split them each into two groups. Using forceps, we took one anther from each of two different LP donors and touched the two anthers on the opposite sides of the recipient stigma three times simultaneously. We then rotated the anthers around the stigma 90\degree and touched the anthers to the stigma three more times. Each combination (for both LPxLP and SPxSP combinations) was repeated twice. In addition, the entire procedure was repeated three weeks later on May 21\textsuperscript{st}, more than half way through the race experiment, for a different randomly selected set of LPxLP and SPxSP combinations. This resulted in a total of 20 LPxLP and 20 SPxSP pollinations. Following these pollinations, each stigma was immediately collected separately in a 1.5 mL polypropylene tube and fixed on a glass microscope slide with fuchsin jelly (Kearns and Inouye 1993). Pollen number on each stigma was counted using a stereomicroscope.

For pollen races, following Epperson and Clegg (1987), we took one anther each from an up donor and a down donor with forceps and applied pollen grains in the same way described in the test crosses each morning between 8am and 10am. We performed these crosses, henceforth
called “races,” with a total of 128 different LPxSP donor combinations. Each LP donor (LP1-LP16) was paired with eight different SP donors (SP1-SP8 or SP9-SP16) and each of these race combinations was performed on two recipients and repeated on two flowers per recipient. This gave us a total of 256 different LPxSPxR combinations and resulted in 2,556 seeds (Figure 3.1).

LPxSP combinations were chosen in order to cover the range of competing pollen size differences. For example, LP1 and SP1 are most different in size since LP1 has the largest pollen and SP1 has the smallest pollen. On the other hand, LP16 and SP16 are the closest in size since LP16 is the smallest LP donor and SP16 is the largest SP donor. In addition, we carried out 18 race combinations each for LPxLP and SPxSP. Similar to LPxSP races, each race was also performed on two recipients and on two flowers each. This resulted in a total of 36 different LPxLPxR combinations and 36 SPxSPxR combinations, and 350 LPxLP seeds and 218 SPxSP seeds. All flowers were tagged with the information of the cross performed, and fruits were collected when mature.

To see if LP and SP donors and recipients differed from one another in other floral traits besides pollen size, we conducted floral measurements on all participating plants about one week into the pollinations. Using digital calipers, we measured the following traits on two flowers per plant: corolla depth, corolla width, tallest filament length, style length, and stigma depth. Corolla depth was measured from the base of the filaments to the top of the corolla; corolla width was measured across the top of the corolla; tallest filament was measured from the base of the filament to the top of the anther; style length was measured from the base of the style to the top of the stigma; and stigma depth was measure from the bottom to the top of the stigma (Figure 3.2).
From June to July 2012, we collected the resulting fruits as they matured. Seeds were removed and counted from each fruit and placed in a labeled coin envelope. We then weighed each seed from each cross individually. Fruit weight was calculated as the sum weight of all seeds from an individual fruit. Fruit weight, mean seed weight per fruit and mean seed number per fruit were calculated by averaging the respective data within each of the three race types: LPxSP, LPxLP and SPxSP.

Genotyping and Paternity Analysis

In September 2012-April 2013, we planted seeds from all races: 2,556 from LPxSP, 350 from LPxLP, and 218 from SPxSP. Seeds were scarified with a razor blade and immediately planted in 96-plug trays in the University of Georgia Plant Biology Greenhouse. After the first true leaves emerged (usually around two weeks after planting), two sets of leaf samples were collected in 1.5 mL polypropylene tubes. One set of leaves was snap frozen with liquid nitrogen and placed in the -80°C freezer as backup tissue samples. The other set was ground with liquid nitrogen and whole genomic DNA was extracted using a modified CTAB method (Doyle and Doyle 1987; Cullings 1992). DNA samples were stored in the -20°C freezer until used for PCR. Numbers of total genomic DNA extracted from offspring per race type were: 2,324 LPxSP, 232 LPxLP, and 204 SPxSP. Differences in totals extracted from seeds planted were due to failure of all seeds to germinate.

We used a touchdown PCR program to amplify nine polymorphic microsatellite loci. These loci were chosen from an original set of 38, based on level of polymorphism among donors and recipients and amplification quality. We developed six of the loci and the other three
were developed by Kuester and colleagues (Molecular Ecology Resources Primer Development et al. 2013). All recipients and donors were genotyped for all nine loci, but each progeny was only genotyped for loci that were informative for the paternity analysis (at least one locus, but up to four). Microsatellite fragment sizes were analyzed using Peak Scanner Software v1.0 (Applied Biosystems). We determined paternity (which donor sired each seed) by comparing the microsatellite genotypes of each competing donor, the recipient and the resulting seed of that cross.

**Statistical Analysis**

**Pollen size, pollen number and floral traits**—Since the machine-measured pollen number was a subsample of the pollen collected from two flowers (ten anthers), we back-calculated the pollen count to get the per anther values in order to compare this value to the hand-counted data. All statistical analyses were performed using SAS 9.3 (2013). Pollen size, pollen number, and floral traits were analyzed in separate ANOVAs with group (LP, SP, or R) as the main predicting variable. A Levene’s test revealed that variance of pollen size was not homogeneous among groups, thus we used Welch’s ANOVA for pollen size. We also tested for correlation among floral traits and between floral traits and pollen traits (i.e. pollen size and number). Here we report the Pearson’s r correlation coefficient.

**Fruit weight, seed number and seed weight**—Fruit weight, seed number per fruit, and average seed weight per fruit were analyzed in separate ANOVAS with cross type (LPxSP, LPxLP or SPxSP) and the recipient plant as the predicting variables. We included the latter
variable to account for the variance among individual maternal plants, which had been shown previously to differ significantly in the size of seeds produced (Mojonnier 1998).

*Siring Success*—We carried out two analyses for siring success. The first analysis was to test whether larger pollen grains were more likely to outcompete smaller pollen grains and sire more ovules. In this analysis, outcome of pollen competition was recorded as the proportion of progeny sired by the donor with larger pollen grains. This value represented the relative siring success of the larger pollen donor, and was expected to be 0.5 if the two competing donors were equally likely to sire an ovule and if equal numbers of pollen grains were applied onto the stigma. A value significantly greater than 0.5 indicated that larger pollen grains sired a greater than expected number of progeny and vice versa. We applied this analysis to competitions between LP and SP plants as well as between two LP and between two SP plants. We then plotted this value against the difference in average pollen diameters between the two competing donors.

In the LPxSP races, we tested the siring success of LP plants against a second expected value that accounted for the slight pollen number difference we found in our test pollinations (described above). This was done because, even though the number of pollen grains we applied onto the stigma did not differ significantly between the test pollinations using LPxLP or SPxSP, the observed value was slightly lower in the SPxSP than in the LPxLP races. To account for any potential effects that this difference in the pollen numbers may cause in terms of the siring success, we also tested the observed value against the expected value calculated based on the relative LPxLP and SPxSP pollen grains on the stigma, which was 0.517. T-tests were used to compare observed values with their expected values.
Following Krauss (2000), a second analysis was carried out to determine whether the success of a pollen donor followed a specific “rank order” of competitive ability or whether it was dependent on the specific opponent against which they were competing. Because pollen size had the dominant effect on determining the outcome of competition in the LPxSP races, we applied this analysis only to the LPxLP and SPxSP races. For each race, we first determined the rank order between the directly-competing pollen donors. We determined the rank order by assigning whether donor 1 had a higher (i.e. siring success fell between 0.71 and 1), equal (0.31 – 0.7) or lower (0-0.3) value for each race and recorded the outcome as donor1>donor2, donor 1= donor 2 or donor1<donor2. We then combined outcomes from all races to try to reach a consensus rank order for donors within the same type of races (either LPxLP or SPxSP).

However, if there was a contradictory outcome (e.g. donor1>donor2 and donor1>donor3 but donor3>donor2), it was counted as one violation of the transitivity rule.

If there is a clear rank order among donors and the ranking follows the transitivity rule, it would suggest that pollen donors possess a particular competitive ability that can be ranked, and the outcome of competition is determined by this ranking. In that case, there is no evidence that interactions between specific competing donors, regardless of their rank order, are more important in determining the outcome of the competition. On the other hand, if the transitivity rule does not apply and we find many violations of this rule (i.e. conflicting outcomes), it would suggest that a pollen donor may win or lose the competition not necessarily depending on its rank order, but rather, depending on the specific interactions with a particular opponent (i.e. pollen-pollen interactions).
RESULTS

Pollen Data

There is a significant difference in pollen size among LP, SP and R plants and this was true regardless of whether we considered all pollen grains or just the fertile pollen grains and thus we only show data for fertile pollen grains. Mean fertile pollen sizes were 121.53 (±0.17) µm, 111.06 (±0.41) µm and 115.42 (±0.50) µm for LP, SP and R, respectively (F_{2,24.6}=310.34, p<.0001, Welch’s ANOVA). Mean pollen sizes for all pollen were 121.53 µm (±0.17), 108.26 µm (±0.88), and 114.66 µm (±0.88) for LP, SP and R, respectively (F_{2,21.4}=129.69, p<.0001, Welch’s ANOVA) (Table 3.1).

In contrast, no significant differences were observed for pollen number per anther (hand count or machine) among LP, SP and R. Mean fertile pollen numbers (machine counted) were 316.66 (±7.52), 298.24 (±13.68) and 315.68 (±14.6), and total pollen numbers were 316.66 (±7.52), 330.64 (±9.42) and 327.5 (±11.52) for LP, SP and R, respectively (F_{2,45}= 0.7, p=0.50; F_{2,45} = 0.58, p=0.56) . Hand counted pollen per anther was only done for LP and SP plants, but the same trend was found in those measurements. In the first round of collections, we did not detect any aborted pollen grains and the pollen production per anther was not significantly different between LP and SP donors (Table 3.1). In the second round of collections (May 21st), some aborted pollen grains were observed in the SP donors. When including all pollen grains, the LP and SP donors produced almost identical numbers of pollen grains per anther, 196.94 (±9.35) and 195.80 (±9.45) for LP and SP, respectively (p=0.9324). However, four plants in the SP line produced some sterile pollen grains, and when these were excluded their number dropped
to 182.73 (±12.53), but was still not significantly lower than the LP donors ($F_{1,29} = 0.84$, p=0.37).

If we take the face values of these pollen production data, when we mixed one anther from LP and one from SP plants, we would expect to observe ~51.87% $= (196.94/(196.94+182.73)) \times 100\%$ of the pollen grains from the LP plant.

Floral Measurements

Neither corolla depth nor tallest filament length differed significantly among plant types. Corolla depth ranged from 47.94 mm to 48.48 mm, and tallest filament length ranged from 29.28 mm to 29.75 mm. Corolla width was only marginally significantly different, with SP and R having similar widths (57.24 mm ±0.97 and 56.13 mm ±0.84, respectively), and LP plants having larger corolla widths (59.36 mm ±1.01) ($F_{2,45} = 3.01$, p=0.0591). Style length was significantly longer in LP donors (32.02 mm ±0.19) when compared to either SP or R, which did not differ from each other (30.46 mm ±0.27 and 30.51 mm ±0.35, respectively) ($F_{2,45}=9.59$, p=0.0003). Stigma depth was significantly larger in R (1.18 mm ±0.02) when compared to LP and SP donors, which did not differ from each other (1.12 mm ±0.02 and 1.09 mm ±0.02, respectively) ($F_{2,45}=4.02$, p=0.0248) (Table 3.1, Figure 3.2). We found expected significant correlations among floral traits. Corolla depth and tallest filament were positively correlated ($r=0.44112$, p=0.0017, n=48). The same was true of corolla width and style length ($r=0.37743$, p=0.0082, n=48). Interestingly, we also found a significant correlation between style length and pollen size among our donors ($r=0.40558$, p=0.0042 n=48) (Table 3.2).
Pollen Counts on Stigma

Our pollination method applied similar amounts of pollen onto stigmas during test crosses, regardless of the donor type. LPxLP crosses yielded a mean application amount of 140.9 (±5.27) pollen grains per stigma, and SPxSP crosses yielded a mean application amount of 131.4 (±7.99) pollen grains per stigma ($F_{1, 18} = 0.98$, $p=0.33$) (Figure 3.3). Using these values, we calculated the expected proportion of LP pollen grains in an LPxSP pollination to be 51.74% ($(140.9/(140.9+131.4)) \times 100\%$), which is very similar to what was calculated using the pollen production data.

Fruit and Seed Data

Fruit weight, seed number per fruit, and seed weight per fruit differed significantly overall among race types (LPxLP $p<0.0001$, LPxSP $p<0.0001$, SPxSP $p=0.0367$) and among recipients ($p<0.0001$ for all). In regards to total fruit weight, LPxLP races produced the heaviest fruit and SPxSP produced the lightest fruit (165.496 ± 3.664 mg and 118.974 ± 3.735 mg, respectively). This pattern was very similar for the seed number per fruit (Figure 3.4). However, LPxLP and LPxSP did not differ significantly ($p=0.0732$). Finally, mean seed weights per fruit were similar among the three race types though the difference between LPxLP and SPxSP was statistically significantly ($p=0.0360$) (Figure 3.4).
When LP donors were competed against SP donors, LP donors sired, on average, 77.57% of the seeds produced, which was significantly greater than the expected value of 51.87% calculated based on the average pollen numbers on the stigma from the test crosses (Student’s t=13.56, P<0.0001). When the competing donors came from the same group (LPxLP and SPxSP races), there was a wide distribution of siring success ranging from 0 to 1, with 0 being no seeds sired by the larger pollen grain donor and 1 being all seeds of a fruit were sired by the larger pollen grain donor. However, the mean siring success in both the LPxLP and SPxSP races were 0.417 and 0.522, respectively, and neither was significantly different from 0.5 (both p>0.1) (Figure 3.5). Further, the ranking analysis revealed no violation of the transitivity property among the donors, suggesting that there is a rank order among pollen donors in this study that can reasonably predict the outcome of pollen competition among pairs of pollen donors and that individual interaction had little influence on the outcome of pollen races in this study (Table 3.3).

**DISCUSSION**

One key finding of this study is that plants with large pollen grains outcompeted ones with small pollen grains when their pollen grains arrived simultaneously on the same stigma, providing direct evidence of pollen size being an important trait in male-male competition. We also showed that the average pollen size was correlated with the style length, possibly due to a functional correlation between these two traits. However, other than style length, little correlation was found between pollen size and other floral traits that are likely to be important
for pollinator attractiveness. We start our discussion with the similarity and differences among
the donor plants.

Our study plants were derived by crossing two replicate lines from an artificial selection
experiment on pollen size supporting that the difference between the LP and SP lines used in this
study was genetically based. To our surprise, pollen number did not show a correlated response
to selection targeting pollen size, despite a pattern of negative phenotypic correlation between
these two traits in the source population. These results suggest that the negative correlation
observed between pollen size and number in naturally collected seeds was unlikely due to
pleiotropic effects of the same genes but possibly due to be either genetic correlation, which was
subsequently broken during the selection process, or resource allocation that was no longer
present in the final selected lines (Stanton and Young 1994). These data are consistent with the
observation that there is not always a size-number tradeoff with pollen production (Lamborn et
al. 2005; Jürgens et al. 2012). This also suggests that increased siring ability of larger pollen was
not due to an increase in pollen number.

Interestingly, we found a positive correlation between mean pollen size and the style
length among plants. Mazer and colleagues (2010) hypothesize that a longer style creates a better
arena for competition, where faster growing pollen tubes have a further distance to “beat”
slightly slower growing pollen tubes and sire more seeds. If pollen tube growth rate is positively
associated with pollen grain size in our study species, then this positive correlation suggests that
plants with more competitive pollen grains also provide a more competitive environment in their
longer styles. Additionally, pollen tubes from pollen grains of lower quality, regardless of size,
are also more likely to experience pollen tube attrition when faced with a longer style to grow
down, even further assuring that the best competitors reach the ovules (Mazer et al. 2010).
Several other studies have also shown that larger pollen is correlated with longer styles and vice-versa. Torres (2000) found a positive correlation between pollen grain size and style length in several species of Asteraceae, and Yang and Guo (2004) found the same relationship in *Pedicularis*. This positive correlation is often attributed to the hypothesis that pollen grains store the energy they need to grow down the length of the style, and thus the relationship is functional (i.e. larger pollen grains have more energy stores and can grow down longer styles). However, while this may be the case in some instances, Ganders (1979) points out that this cannot always be the reason. In his review of floral characteristics of heterostyloous species, 50 of 55 genera investigated showed polymorphism in pollen grain size not only among flowers with different style types (pin and thrum), but also within flowers of a single style type. Furthermore, pollen size sometimes did not correlate at all with style length. His findings suggest that while pollen size-style length relationships may be a product of heterostyly, it is not a requirement, and thus larger pollen is not necessary to grow down longer styles and may simply be a phylogenetic relationship as suggested by Cruden and Lyon (1985). This finding of mixed results with regards to correlation between style length and pollen size is further reviewed in Muller (1979). Finally, pollen size was found to not be significantly correlated with stigma depth, a pattern that Cruden and Lyon (1985) suggested to be a more likely functional correlation in starchy pollen grains based on the amount of endogenous stored energy needed for the pollen grain to grow through the stigma and reach exogenous energy sources in the style. It should be noted that they did not find a correlation when looking at starchless pollen grains.

Among other floral traits, we found only a couple of significant correlations: one between corolla depth and tallest filament length and the other between style length and corolla width. Genetic correlations among floral traits are not uncommon. For example, Rosas-Guerrero
et al. (2011) show correlation among floral traits in *Ipomoea*, although combinations of traits differed in their correlation patterns in different species. They also show that traits within the same whorl are more highly correlated than traits between whorls due to developmental constraints. This can explain the correlation between corolla depth and filament length found in this study. There is also some evidence that floral traits can be correlated with pollen traits, such as size and number, among others (see Muller (1979). In a study done by Sarkissian and Harder (2001), selection on pollen size in *Brassica rapa* led to a correlated response in both pollen number and floral traits. Specifically, they showed that plants that produced larger pollen also produced larger flowers. In our study, neither corolla depth nor tallest filament differed significantly among LP, SP and R, but corolla width and style length were increased in LP plants and stigma depth was increased in R.

Our study clearly shows that larger pollen sires more seeds than small pollen when LP and SP pollen are competing on the same stigma. Since we minimized the effect of pollen primacy, the timing of pollen arrival, by applying pollen from both donors to the stigma simultaneously, we can then reasonably assume that the non-random siring success we find is due to pollen competition on the stigma or inside the style and not pollen primacy whose importance has been shown by Epperson and Clegg (1987). Several reasons, not mutually exclusive, could potentially explain the pattern we found. First, larger pollen has more energy reserves and therefore could be able to germinate faster and/or have faster pollen tube growth (Baker and Baker 1979; Ganders 1979; Snow and Spira 1991; Spira et al. 1992; Snow and Spira 1996; Mazer et al. 2010). This explanation seems likely since it is well known that larger pollen contains more energy stores (starch and/or lipids) for the pollen to use as it germinates and grows its pollen tube through the style. Second, larger pollen could be a better competitor due to
superior alleles of genes expressed during pollen tube growth. Pollen grains express genes during growth from the stigma to the ovary, and Bernasconi et al. (2004) and Arthur et al. (2003) found that specific alleles of ROP2 guanosine triphosphate in maize confer a competitive advantage to pollen tube growth. In addition, Delph et al. (1998) found that there is a correlation between pollen tube growth rate and sporophytic growth rate, suggesting similar natural selection might be operating in the gametophytic stage favoring large pollen grains (Mulcahy 1979; Lankinen and Skogsmyr 2001; Bernasconi et al. 2004). Third, small pollen grains might have lower viability than larger pollen grains. Though we tried to minimize the effect of pollen abortion by excluding pollen donors that showed such a phenotype prior to the start of this study, four of our SP plants still aborted a small portion of their pollen grains. When we excluded data from these four plants, our main findings did not change. Nonetheless, the presence of this phenotype in the SP group but not LP group suggests that it is possible that smaller pollen grains may be less vigorous or less viable than the large pollen grains (Dulberger and Horovitz 1984; Mayer and Gottsberger 2000; Jürgens et al. 2012). If this were the case, our outcome could be, in essence, due to the difference in effective number of competitors and not the competitive ability of the donors, as has been shown to affect competition outcome (Lankinen 2002). Our within group competitions provide some indirect support for this possibility; SPxSP crosses produced fruits that were, on average, only half full; whereas LPxLP competitions almost always resulted in full seed set, suggesting that small pollen grains may not be able to accomplish fertilization even when the more competitive opponents are absent. Finally, LP donors and SP donors could have different pollen-pistil interactions (donor x recipient effect) (Johnston 1993; Mazer et al. 2010). However, this scenario is unlikely to be very important in our study, because if it were true, we
would expect high variation of LP pollen performance in the LPxSP races across different maternal plants, but this was not observed in our results.

Though our results do not allow us to distinguish the first three possibilities discussed above, future studies examining pollen germination and pollen tube growth rate of LP and SP plants will help to determine the relative importance of these possibilities. For example, if small pollen grains tend to delay in their germination or in starting pollen tube growth, it would suggest that size is important in the early stages of pollen tube initiation into the style as Cruden and Miller-Ward (1981) suggest. On the other hand, if pollen tubes of the large and small pollen both reach the ovules, but at different speeds, it will suggest that pollen tube growth rate contributes, at least partially, to our results. Finally, if the small pollen grains tend to grow short tubes that terminate before reaching the base of the style or ovules, this would suggest that small pollen grains are associated with less overall vigor of the pollen tube and, as a result, lead to lower siring success. We are currently carrying out studies to test these possible explanations.

Our results from the same-group competition suggest that factors other than pollen size also contribute to the outcome of pollen competitions. In these races (LPxLP or SPxSP), siring success ranged from either one donor siring all of the seeds, to each donor siring half. Since pollen size is similar among the pollen grains on the stigma, this result suggests that size is not the only factor influencing the outcome of competition. Though it is not clear what this/these factor(s) might be, our rank order analysis suggested that there exists a ranking order among donors that was rarely violated in the competition outcome. In other words, among pollen of similar sizes, some donors are just better competitors than others. This has also been shown to occur in *Persoonia mollis* (Krauss 2000) and *Hibiscus moscheutos* (Snow and Spira 1996).
In addition to siring success, we also found that fruits resulting from crosses involving LP plants (LPxLP or LPxSP) were heavier and had more seeds. Higher fruit weight in these crosses than the SPxSP races could be due to a higher number of seeds per fruit, heavier individual seeds within these fruit or both. Our results supported the former possibility (higher number of seeds per fruit), because the average seed weight per fruit in SPxSP was similar to LPxLP and LPxSP races. A positive correlation between pollen size and seed number per fruit was also found in *Erythronium grandiflorum* (Cruzan 1990). Despite similar average seed size, progeny quality of large and small pollen donors may still differ. Several previous studies have shown a connection between pollen competitive ability and progeny quality and vigor. The prevailing hypotheses for these superior offspring are preferential maternal provisioning to first fertilized ovules and overlapping gene expression between the gametophytic and sporophytic phases (Spira et al. 1992; Delph et al. 1998; Bernasconi et al. 2004; Lankinen and Madjidian 2011). More specifically, Spira and colleagues (1992) showed that in *Hibiscus moscheutos*, seeds and resulting offspring were more fit when they were produced when the stigma was subjected to high pollen loads. They also suggested that the genes in pollen conferring faster pollen tube growth will not only cause these pollen to fertilize ovules first, but also transmit these genes to the seeds, potentially resulting in better seed quality. On the other hand, Delph and colleagues (1998) showed that in *Silene vulgaris*, pollen with faster growing pollen tubes resulted in higher quality progeny than slower pollen only when both pollen types were applied to the stigma simultaneously. It will be informative for our study with a follow up experiment that examines the fitness of the seeds resulting from large versus small pollen grains, to directly test for any progeny quality difference between the two types of pollen donors.
In summary, *I. purpurea* exhibits genetic based variation in pollen size. This variation in pollen size leads to variation in siring success that is non-random. When larger pollen is competed against smaller pollen on the same stigma simultaneously, large pollen sires over 75% of the seeds in a fruit. The increased competitive ability of larger pollen could be due to several factors, with the most likely being increase pollen tube growth rate. Additionally, large pollen not only sires more seeds, but crosses involving at least one large pollen donor result in heavier and fuller fruits. Beyond this study, we need more data on the variation in pollen size among natural populations of *I. purpurea* in the field to achieve a more complete understanding of how genetic variation of this important male trait is maintained in nature.
Figure 3.1. Diagram of all crosses (races) performed. Each cross was replicated on two flowers each recipient. The top row of races are LPxSP races. Small Pollen donor (SP) IDs are to the left of the grid. Large Pollen donor (LP) IDs are listed above the grid. Each color block inside the grid represents a Recipient Plant (RP), with the ID listed. Each Recipient was used for 16 different races (i.e. LPxSP combinations) for all 16 Recipients. The bottom two rows of races are LPxLP and SPxSP. The competing donor IDs are listed to the left and above the grids. Each Recipient was used for four or five of these crosses, and they are indicated by their ID and a color.
Figure 3.2. Diagram of floral measurements taken. a) corolla diameter, b) corolla depth, c) tallest filament + anther, d) style length and e) stigma depth.
Table 3.1. Mean pollen (top) and floral (bottom) measurements of donor and recipient plants. Standard errors are in parentheses. Superscripts of different letters indicate a significant difference in the means for a particular trait among the plant groups (LP, SP and RP).

^: Heterogeneous variance among groups based on Levene's test

<table>
<thead>
<tr>
<th></th>
<th>Mean pollen measurements (by Multisizer II)</th>
<th>Pollen number/anther (hand count)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fertile pollen size^</td>
<td>All pollen size^</td>
</tr>
<tr>
<td>Large Pollen Donor</td>
<td>121.53^a (0.17)</td>
<td>121.53^a (0.17)</td>
</tr>
<tr>
<td>Small Pollen Donor</td>
<td>111.06^b (0.41)</td>
<td>108.26^b (0.88)</td>
</tr>
<tr>
<td>Recipient</td>
<td>115.42^c (0.50)</td>
<td>114.66^c (0.88)</td>
</tr>
</tbody>
</table>

Welch's test Pr > F <.0001^* <.0001^* 0.5 0.56 0.0829 0.9324 0.3718

<table>
<thead>
<tr>
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<th>Mean floral trait values (mm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Corolla depth</td>
</tr>
<tr>
<td>Large Pollen Donor</td>
<td>48.48 (0.71)</td>
</tr>
<tr>
<td>Small Pollen Donor</td>
<td>48.24 (0.65)</td>
</tr>
<tr>
<td>Recipient</td>
<td>47.94 (0.77)</td>
</tr>
</tbody>
</table>

Welch's test Pr > F 0.864 0.0591^t 0.5993 0.0003^* 0.0248^*
Table 3.2. Correlations among floral and pollen traits based on trait means. Reported data are Pearson’s r correlation coefficient above and p values below (shaded area). Statistically significant results at the 0.05 level are in bold and denoted with an *.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Corolla depth</th>
<th>Corolla width</th>
<th>Tallest filament</th>
<th>Style length</th>
<th>Stigma depth</th>
<th>Fertile pollen #</th>
<th>Fertile pollen size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corolla depth</td>
<td>-0.00524</td>
<td>0.44112*</td>
<td>-0.17606</td>
<td>-0.13272</td>
<td>-0.1225</td>
<td>0.1627</td>
<td></td>
</tr>
<tr>
<td>Corolla width</td>
<td>0.9718</td>
<td></td>
<td>0.15314</td>
<td>0.33743*</td>
<td>0.09331</td>
<td>0.20504</td>
<td>0.23855</td>
</tr>
<tr>
<td>Tallest filament</td>
<td>0.0017*</td>
<td>0.2987</td>
<td>0.22661</td>
<td>0.00174</td>
<td>-0.09747</td>
<td>0.21911</td>
<td></td>
</tr>
<tr>
<td>Style length</td>
<td>0.2313</td>
<td>0.0082*</td>
<td>0.1214</td>
<td>-0.05387</td>
<td>0.11574</td>
<td>0.40558*</td>
<td></td>
</tr>
<tr>
<td>Stigma depth</td>
<td>0.3685</td>
<td>0.5282</td>
<td>0.7161</td>
<td></td>
<td>-0.06429</td>
<td>0.03703</td>
<td></td>
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<tr>
<td>Fertile pollen #</td>
<td>0.4066</td>
<td>0.1621</td>
<td>0.4334</td>
<td>0.6642</td>
<td></td>
<td>0.0557</td>
<td></td>
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<tr>
<td>Fertile pollen size</td>
<td>0.2692</td>
<td>0.1025</td>
<td>0.0042*</td>
<td>0.8027</td>
<td>0.7069</td>
<td>0.0557</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3. Distribution of number of pollen grains applied to stigmas in test crosses.
Figure 3.4. Mean fruit and seed data per race type. Mean fruit weight (upper left), mean seed number per fruit (upper right) and mean seed weight per fruit (bottom left) were calculated for each race type.
Figure 3.5. Siring success of the larger pollen grain donor for each race type. Values >0.5 indicate that the larger pollen grain donor sired more seeds, =0.5 indicate both donors contributed equally, <0.5 indicate the smaller pollen grain donor sired more seeds.
Table 3.3. Pollen donor rank orders based on the outcomes of LPxLP and SPxSP races. A donor was considered to (>) outcompete another donor if it sired 71-100% of seeds, (=) have equal ranking if it sired 31-70% of seeds, and (<) lower ranking if it sired 0-30% of seeds in a fruit. A denotation of (>=) means that sometimes the ranking was higher and sometimes it was equal. When two numbers are linked by "&" it indicates that these two plants did not race directly but occupied the same ranking with information derived from races with other individuals.

<table>
<thead>
<tr>
<th>LPxLP races</th>
<th>LP Donor ID</th>
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<tbody>
<tr>
<td>&gt; 1 &gt; 6</td>
<td>2&amp;5</td>
</tr>
<tr>
<td>&gt; 7=4 &gt; 3 &gt; 8</td>
<td>2</td>
</tr>
<tr>
<td>&gt;= 9 = 10 = 15&amp;12 &gt;= 11 &gt;= 16</td>
<td>14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SPxSP races</th>
<th>SP Donor ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 7 &gt; 2 &gt; 1 &gt; 6 &gt; 5</td>
<td>4</td>
</tr>
<tr>
<td>&gt; 8 &gt; 7</td>
<td>4=3</td>
</tr>
<tr>
<td>&gt; 10 = 15 = 16</td>
<td>9</td>
</tr>
<tr>
<td>&gt; 13 &gt; 14</td>
<td>9</td>
</tr>
<tr>
<td>&gt; 15 = 16 = 10</td>
<td>12&amp;11</td>
</tr>
</tbody>
</table>
Chapter 4

Conclusion

This study has two key findings. The first is that large pollen outcompetes small pollen when competing on the same stigma, supporting our post-pollination hypothesis. The second key finding is that, at least in *I. purpurea*, small pollen and large pollen are transported (removed from anthers and deposited onto stigmas) in similar quantities by pollinators, failing to support our pre-pollination hypothesis.

Specifically, plants with large pollen grains outcompeted plants with small pollen grains when their pollen grains arrived simultaneously on the same stigma, providing direct evidence of pollen size being an important trait in male-male competition. Our study clearly shows that larger pollen sires more seeds than small pollen when LP and SP pollen are competing on the same stigma. Several reasons could potentially explain the pattern we found. First, larger pollen has more energy reserves and therefore could be able to germinate faster and/or have faster pollen tube growth (Baker and Baker 1979; Ganders 1979; Snow and Spira 1991; Spira et al. 1992; Snow and Spira 1996; Mazer et al. 2010). Second, larger pollen could be a better competitor due to superior alleles of genes expressed during pollen tube growth. Third, small pollen grains might have lower viability than larger pollen grains. Future studies examining pollen germination and pollen tube growth rate of LP and SP plants will help to determine the relative importance of these possibilities.

Additionally, our results from the same-group competition suggest that factors other than pollen size also contribute to the outcome of pollen competitions. In these races, siring success
ranged from either one donor siring all of the seeds, to each donor siring half. Since pollen size is similar among the pollen grains on the stigma, this result suggests that size is not the only factor influencing the outcome of competition. Though it is not clear what this/these factor(s) might be, our rank order analysis suggested that there exists a ranking order among donors that was rarely violated in the competition outcome. In other words, among pollen of similar sizes, some donors are just better competitors than others.

In addition to siring success, we also found that fruits resulting from races involving LP plants were heavier and had more seeds. Higher fruit weight in LP races than the SPxSP races was most likely due to higher seed number, since individual seeds from all races were of similar weight. However, despite similar average seed size, progeny quality of large and small pollen donors may still differ. It will be informative for our study with a follow up experiment that examines the fitness of the seeds resulting from large versus small pollen grains, to directly test for any progeny quality difference between the two types of pollen donors.

Our pre-pollination (pollen transport) results are consistent with results from a study done by (Harder 1998), where he looked to see if pollen size corresponded to pollinator type, in that there does not appear to be an association between pollen size and pollen transport. Our results also show that all of our experimental plants are visited with similar frequency and duration, suggesting that pollen size, pollen number and floral manipulations do not affect visitation by bumblebees in *I. pupurea*.

Following the same trend as pollinator visitation frequency and duration, there was not a consistent significant difference between array types in pollen deposition on stigmas. This indicates that both LP and SP donors contribute similar pollen numbers to recipient stigmas. Pollen removal was different between donor types, where more pollen was removed from SP
than LP donors’ anthers only at the GH plot. However, this was probably simply due to SP having lower pollen production in the first place. Regardless of significant differences between array types or plant groups, visitation frequency did have a consistent significant effect on pollen removal from donor anthers at both plots, where more visits led to more pollen being removed, which intuitively makes sense.

Despite our findings that pollen size variation does not seem to be associated with pollen transport, it was important to address this possibility since pre-pollination factors may play a role as selective agents for pollen size. It is evident that pollen size variation exists in *I. purpurea*, but it is not pollen transport that maintains the small pollen size. Beyond this study, we need more data on the variation in pollen size among natural populations of *I. purpurea* in the field to achieve a more complete understanding of how genetic variation of this important male trait is maintained in nature.


