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Pollen competition in style: Effects of pollen size on siring success in the hermaphroditic common morning glory, *Ipomoea purpurea*[1]

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PREMISE OF THE STUDY: Pollen size varies greatly among flowering plant species and has been shown to influence the delivery of sperm cells to the eggs. Relatively little is known, however, about the functional significance of within-species genetic variation in pollen size. This study tests whether pollen size influences the relative siring success of a pollen donor during in vivo pollen competition experiments.

METHODS: We used two groups of *Ipomoea purpurea* plants genetically divergent in their pollen sizes and applied equal number of pollen grains from one large-pollen and one small-pollen donor onto the same stigma. Using microsatellite genetic markers, we identified the pollen parent of each of the resulting progeny to determine the relative siring success of the competing donors. Competitions between donors of equal-sized pollen served as a control.

KEY RESULTS: Differences in pollen size significantly affected the relative siring success of a pollen donor; larger-grained individuals outcompeted smaller-grained competitors but not equal-sized competitors. Relative siring success, however, sometimes varied across different pollen recipients.

CONCLUSIONS: Pollen size can influence the relative siring success of different individuals competing on the same stigma during postpollination processes. However, other factors, such as pollen–pistil interaction and environmental conditions, are likely to influence these competitions as well.

KEY WORDS Convolvulaceae; *Ipomoea purpurea*; pollen competition; pollen size; siring success

The idea that males compete for access to females, as proposed by Darwin (1871) in his sexual selection theory, is well accepted as an important process shaping trait evolution in animals, particularly in species that have showy males such as peacocks and moose. However, male–male competition is not restricted to sexually dimorphic species, and it has been argued to occur even in species that do not have separate sexes (Willson, 1979; Andersson, 1994; Delph and Ashman, 2006; Birkhead and Møller, 1998), including many plants. The broader definition of sexual selection, as proposed by Arnold (1994a) and several others (e.g., Skogsmyr and Lankinen, 2002; Delph and Ashman, 2006; Moore and Pannell, 2011), includes all processes involved in sexual reproduction that can lead to differential mating success, either pre- or postpollination. In flowering plants, competition for pollinators could occur via floral traits or inflorescence displays. Competition could also occur postpollination via interaction between pollen grains that are deposited on the same stigma (Haldane, 1932; Mulcahy, 1979; Mulcahy and Mulcahy, 1987; Arnold, 1994b; Moore and Pannell, 2011). This latter form of male–male competition, often termed pollen competition, operates similarly to postcopulation sperm competition in animals and can lead to selection of floral traits that influence the male component of fitness even in hermaphroditic plants (Krauss, 2000; Lankinen and Skogsmyr, 2001). Researchers have extensively examined how floral traits help plants compete to attract pollinators (see reviews by Rathcke, 1983; Mitchell et al., 2009 and references therein); however, much less is known about how selection during postpollination processes is influenced by pollen (male-male) competition (Snow and Spira, 1991 a, b; Delph and Havens, 1998).

Pollen competition occurs when the number of pollen grains deposited on a stigma exceeds the number of ovules (e.g., Johnston, 1993; Delph et al., 1998; Erbar, 2003; Bernasconi et al., 2004). This condition is frequently reached after multiple visits from pollinators (Mulcahy, 1979; Mulcahy and Mulcahy, 1987; Winsor et al., 2000) but, in some species, a single pollinator visit can bring in more than sufficient pollen for all ovules (Snow and Roubik, 1987; King et al., 2013), setting the stage for pollen competition. Because pollen

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grains on the same stigma often come from multiple source plants, any traits that can enhance their ability to reach the eggs earlier than other grains will enhance the male fitness of the pollen source, assuming that the cost of such traits (e.g., postfertilization) does not exceed their benefit. For example, we know that pollen grains may vary in their arrival time, the probability of pollen tube germination, pollen tube growth rates, their interaction with the stigma and with other pollen grains, and/or pollen quality, all of which could potentially influence the outcome of pollen competition following pollination (Epperson and Clegg, 1987; Spira et al., 1992; Johnston, 1993; Herrero and Hormaza, 1996; Tejaswini, 2002; Mazer et al., 2010; Lankinen and Madjidian, 2011). Among these traits, pollen tube growth rate (PTGR) is considered to be the most important; faster growing pollen tubes tend to have higher siring success than slower growing tubes (Snow and Spira, 1991a, b, 1996; Delph et al., 1998; Snow et al., 2000; Lankinen and Io, 2002; Mazer et al., 2010). Unfortunately, PTGR can be difficult to measure because it often requires in vivo examination that is tedious and technically challenging. It is also a complex trait that likely represents a composite outcome of many factors whose contributions are hard to tease apart, including the genetic identity of the sporophytic pollen donors (Mulcahy, 1979; Walsh and Charlesworth, 1992; Lankinen and Io, 2002; Stephenson et al., 2003) and the gametophyic pollen grain (Mulcahy, 1979; Spira et al., 1992; Walsh and Charlesworth, 1992; Arthur et al., 2003), specific interactions between pollen and style environment or style attrition (Cruzan, 1990; Dresselhaus and Franklin-Tong, 2013), as well as the ecological conditions experienced by the pollen and ovule parents (Stephenson et al., 1992; Lankinen and Skogsmyr, 2001; Tejaswini, 2002). As a result, though we have evidence that siring success among males is nonrandom following pollen competition (e.g., Marshall and Folsom, 1991; Snow and Spira, 1991a, b, 1996; Spira et al., 1992; Delph et al., 1998; Marshall, 1998; Marshall and Diggle, 2001; Marshall and Oliveras, 2001) and that PTGR is likely to play an important role in this process, it is not clear whether variation in PTGR and associated siring success has a heritable genetic basis (but see Lankinen et al., 2009) or whether it is mostly a response to environmental conditions and unlikely to have any evolutionary consequences.

One effective way to study complex traits such as PTGR and male siring success is to focus on an easy-to-measure surrogate trait that is correlated with the trait or traits of interest (PTGR and male siring success), that has a clear underlying cause, and that is easy to measure. Pollen size has recently emerged as a good candidate for the following reasons. First, though measuring pollen size is tedious, it can be easily done either using microscopes coupled with imaging software (Costa and Yang, 2009) or particle counters (Kearns and Inouye, 1993). Second, it has long been recognized that pollen size is correlated with energy stores inside the pollen (Baker and Baker, 1979; Cruden and Miller-Ward, 1981; Lord and Eckard, 1984, Manicacci and Barrett, 1995). These energy stores support pollen germination and pollen tube growth, which start out as "autotrophic" processes, but tube growth later turns "heterotrophic" and draws the necessary nutrients from the stylar tissue (Stephenson et al., 2003). Higher energy stores in large pollen grains can thus provide a jump-start for these pollen grains during their competition against smaller pollen grains on the same stigma. Indeed, pollen size has been shown to be positively correlated with in vitro pollen tube growth rate following pollen germination in maize (Kumar and Sarkar, 1980) and in *Brassica rapa* (Sarkissian and Harder, 2001; see also Manicacci and Barrett, 1995; Lamborn et al., 2005), though not all species show this pattern (e.g., Cruzan, 1990; Tejaswini, 2002; Pietarinen and Pasonen, 2004). This early difference could theoretically convey an even larger advantage in the overall PTGR if it allows tubes from larger grains to use most of the resources in the styles before the slower tubes arrive.

Finally, it is generally believed that strong selection for optimal pollen size would have removed heritable genetic variation in this trait (Young et al., 1994; Delesalle and Mazer, 1995; Fenster and Carr, 1997) and that variation in pollen size would be largely caused by environmental conditions (Stephenson et al., 1992; Lau and Stephenson, 1993; Delph et al., 1997; Hersch, 2006; Distefano et al., 2012). This expectation implies that pollen size would not play an important role in determining the outcome of pollen competition. However, studies have shown that within-species pollen size displays substantial heritable genetic variation in several species, including *Phaseolus vulgaris* (common bean) (Montes-R and White, 1996), *Brassica rapa* (Sarkissian and Harder, 2001), and *Mimulus guttatus* (Lamborn et al., 2005). Furthermore, in all three of these species, there is evidence that genetically based pollen-size variation is associated with differential pollen performance. These recent results suggest that the influence of pollen size variation on male siring success and pollen competition may be more important than previously realized and can serve as a surrogate trait to study male–male competition in the context of sexual selection in flowering plants. To date, only a handful of studies have directly linked within-species pollen size variation to pollen performance (Kumar and Sarkar, 1980; Cruzan, 1990; Lau and Stephenson, 1993; 1994; Sarkissian and Harder, 2001; Lamborn et al., 2005), leaving a gap in our knowledge on the role that pollen size plays in postpollination sexual selection and how additive genetic variation is maintained for pollen size in natural populations.

Given that pollen size appears to harbor within-species additive genetic variation, and if pollen size is correlated with siring success during pollen competition as we argue above, this trait could be one of the targets that sexual selection can act upon. Our ultimate goal is to characterize the strength and direction of sexual selection enabling comparison with other forms of selection, such as fertility and viability selection, in hermaphroditic flowering plants. As a first step toward this goal, we conducted experiments to test whether pollen size, particularly genetically based pollen size variation, could influence siring success during pollen competition. Specifically, we focused on genetically based pollen size variation and tested the hypothesis that larger pollen grains were more competitive during pollen competition than smaller pollen grains. We used *Ipomoea purpurea* individuals previously bred to diverge in pollen size in pollen competition experiments that tested for an association between pollen size and siring success. By growing these plants in similar ecological conditions (common garden), we tested the hypothesis that genotypes with larger pollen grains would have a higher siring success when competing against those with smaller pollen grains on the same stigma. The specific questions addressed in this study are: (1) Does large pollen have a competitive advantage over small pollen? (2) Is the relative siring success between competing pollen donors consistent across different pollen-receiving plants? 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* (L.) Roth (Convolvulaceae), or common morning glory, is a climbing, self-compatible, weedy, annual species that occurs in most parts of the United States and is often found in crop fields and roadside ditches. Around Athens, Georgia, where this study was carried out, seedlings start to emerge in May and grow until the first hard frost kills the plants, which usually occurs in November. Flowers usually appear in late June or early July with funnel-shaped corollas and can be white, pink, or purple (Radford et al., 1968). Individual flowers last one morning, opening before sunrise and wilting by early afternoon 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 that can have up to six seeds. The predominant pollinators are bumblebees (*Bombus* spp.); but other bees (*Apis* spp, *Xylocopa* spp.), hummingbirds, and butterflies also occasionally visit these flowers (Radford et al., 1968; Clegg and Durbin, 2000; S.-M. Chang, personal observation).

Source of plants with divergent pollen sizes—Plants used in this study came from an artificial selection experiment that generated plants with increased (Large Pollen or LP line) or decreased (Small Pollen or SP line) average pollen grain size over six generations of selection. Founding population (Gen 0) consisted of 412 plants grown from seeds collected from two populations located in the town Watkinsville, Oconee County, Georgia. From this founding population, two replicate sets of selection lines (LP1 and SP1 and LP2 and SP2) were established; each consisted of plants with the largest or smallest average pollen sizes in the previous generation. Selection based on pollen size and the subsequent crosses between selected individuals within each line were performed to propagate the selection lines for five generations. Details of the selection design are reported elsewhere (S.-M. Chang, unpublished manuscript). Two corresponding Control lines (C1 and C2) were also established with randomly chosen individuals and propagated along with the selection lines. In Gen 5, crosses were carried out between replicate lines to reduce inbreeding in the resulting seeds, which were subsequently used for this study.

In January 2012, we planted 387 seeds from the crosses described above, and the plants started producing flowers in about 6 weeks. Before the pollen competition experiment, we double-checked the pollen trait divergence in the LP and SP lines by measuring the number and average size of pollen grains using a Beckman Multisizer II Coulter Counter (Beckman Coulter, Fullerton, California, USA). From these measurements, we calculated the average pollen diameter and the total number of pollen grains. Pollen grains of this species are spherical so pollen diameter is a good metric for pollen size. *Ipomoea purpurea* produces pollen grains that range from 70–125 μm. From our earlier studies, we found that some plants aborted a portion of their pollen grains, which can be distinguished from fertile pollen based on their significantly smaller size (average diameter ~80 μm for aborted pollen and ~110 μm for fertile pollen). Aborted pollen grains were found more often in the SP lines but also existed in LP lines. We recorded the number and average size of all pollen grains as well as just the fertile pollen and excluded individuals that consistently produced aborted pollen from this study. Because the machine-measured pollen number involved subsampling of the pollen collected from two flowers (10 anthers), we back-calculated the pollen count to get the per anther values to compare them with the hand-counted data. Pollen production (number) measured here was later used to determine the relative amount of LP vs. SP pollen grains on the same stigma during the pollen competition study (see details below).

Following pollen measurements, we ranked donors by their average pollen size and chose the largest 16 LP (designated as LP1 to LP16 pollen donors) and smallest 16 SP (designated as SP1 to SP16) plants to serve as pollen donor parents (donors) and an additional 16 control plants as ovule donor parents (i.e., pollen recipients, designated as R1 to R16 plants hereafter) for the pollen competition experiments. Pollen production in these 16 selected LP and SP donor flowers was verified by manually counting pollen number for three anthers per donor plant (1 anther/flower × 3 flowers/plant × 16 plants). Pollen grains were stained with fuchsin jelly (Kearns and Inouye, 1993) on a slide with grid lines and counted using a stereomicroscope. We carried out this step on two separate dates to check for any ontogenetic changes in pollen production during the course of this study. Control plants, genetically unrelated to any LP or SP donors, were used as the ovule donors (pollen recipients; R plants). 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.

In addition to pollen traits, we also examined whether LP and SP donors differed from one another in other floral traits. Before the experiments, we used digital calipers to measure the following traits on two flowers per plant: corolla depth, corolla width, longest stamen length, pistil 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; longest stamen was measured from the base of the filament to the top of the tallest anther; pistil length was measured from the base of the pistil to the top of the stigma; and stigma depth was measure from the bottom to the top of the stigma.

Pollen competition experiment—To determine whether pollen size influenced the competitive ability of a pollen donor, we allowed two pollen donors to compete for the ovules of a recipient (R) plant by simultaneously applying an equal number of pollen grains to the stigma. All pollen competitions (each referred to as a "race" hereafter) were carried out between 08:00 and 10:00 hours on the day of the race. In a preliminary study, we found that flowers in a natural population (in Watkinsville, GA) receive, on average, ~140 grains (with a range of 0 to 250 grains) of pollen before they wither. We had initially planned to apply a precise number of pollen grains (100 grains) from each competing pollen donor onto the stigma of an R plant, but that process proved to be detrimental to pollen fertility, and many early races failed to set seeds. Subsequently, we opted to directly apply pollen from freshly collected anthers onto the same stigma following a similar method described by Epperson and Clegg (1987). Specifically, we used two pairs of forceps to remove one anther from each of the two competing donors and touched these two anthers on the opposite sides of the recipient stigma three times simultaneously. We then rotated the anthers around the stigma 90° and touched the anthers to the stigma three more times. We made an effort to apply and mix both LP and SP pollen on every part of the stigmatic surface with as little time separating the application of these two pollen types as possible.

To examine whether this method was applying equal amounts of pollen from LP and SP anthers, we conducted test pollinations with

either two LP (designated as LP-LP) or two SP (designated as SP-SP) anthers and then counted the number of pollen grains that were deposited on the stigma, using the fuchsin jelly method. (Note that we could not use LP-SP crosses for this test, because their pollen grains cannot be unambiguously distinguished.) Results from a total of 40 test pollinations (20 LP-LP and 20 SP-SP pollen races) showed that this technique placed similar amounts of pollen grains on the stigmas between pollinations using LP and those using SP anthers (see data in the results section), validating the method for our main pollen competition experiments.

For the main pollen competition experiment, there were two types of pollen races: first, between two donors with very different average pollen size and second, two with similar pollen size. The first type of race included all possible pairings between LP1-8 and SP1-8 (64 combinations) as well as all possible pairings between LP9-16 and SP9-16. Each of these pairs competed on two recipient plants and on two flowers per recipient. Combined, this part of the study resulted in 128 different LP-SP donor-combinations, each on two different R plants, and resulted in a total of 2556 seeds (Appendix S1, see Supplemental Data with the online version of this article). These races allowed us to examine whether pollen size influenced the outcome of relative siring success between competing donors. The second type of race was between pollen donors of similar sizes from the same pollen group. This part of the study comprised 18 race combinations for LP-LP and 18 combinations for SP-SP (Appendix S1). Each combination was also performed on two recipients and on two flowers per recipient; resulting in 350 and 218 seeds for the LP and SP groups, respectively. All flowers on the recipient (R) plants were tagged with the information of the races performed, and fruits were collected when mature.

Genotyping and paternity analysis—To determine the outcome of pollen races, we identified the paternity of all seeds resulting from the pollen races using microsatellite markers. Seeds were scarified with a razor blade and planted in 96-plug trays in the University of Georgia Plant Biology greenhouse. Two weeks after planting, we collected leaf tissues to extract genomic DNA using a modified CTAB method (Doyle and Doyle, 1987; Cullings, 1992). DNA samples were stored in a −20°C freezer until used for PCR. With an overall germination rate of 88%, the numbers of total genomic DNA extracted from offspring per race type were 2324 for LP-SP, 232 for LP-LP, and 204 for SP-SP races.

We used a touchdown PCR program to amplify nine polymorphic microsatellite loci (see online Appendix S2 for PCR program and primer information), six of which were developed specifically for this study and three from Kuester and colleagues (Molecular Ecology Resources Primer Development Consortium, 2013). All LP, SP, and R plants used in the races were genotyped for all nine loci, but each progeny was only genotyped for the minimum number of loci required to identify its pollen parent (from 1 to 4 loci). Fluorescently labeled PCR fragments were analyzed using the ABI 3730 sequencing machine with a 500-bp size standard (by UGA-GGF). The genotype of each individual was manually assigned using Peak Scanner Software v1.0 (Applied Biosystems). This approach allowed us to determine paternity (which donor sired each seed) by comparing the microsatellite genotypes of the paired competing donors, the recipient and the resulting seed of that race.

For each race combination on a recipient plant, we calculated the relative siring success of the large-pollen plant as the proportion of seeds they sired to the total number of seeds resulting from that particular pollen race. This calculation was done for all pollen races including both LP-SP races and the within-group races. We plotted only the value for the larger-pollen donor since the value for smaller-pollen donor would be 1− the larger-pollen siring success. We excluded any races that had less than 8 seeds genotyped, resulting in one R plant having 13 races and the rest having 15 or 16 (full set) races. Conversely, each donor combination was raced on two different R plants. Specifically, as an example, all donor combinations raced on R1 were also raced on R9, and all combinations on R2 were also raced on R10, etc. This design allowed us to examine whether the relative siring success between two competing donors was consistent across R plants.

Statistical analysis—*Pollen size, pollen number, and floral traits*—Pollen size, pollen number per anther, and floral traits were analyzed in separate analyses of variance (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 correlations among floral traits and between floral traits and pollen traits (i.e., pollen size and number). Here we report Pearson's correlation coefficient (r). All statistical analyses were performed using SAS 9.3 (SAS Institute, 2013).

Siring success—We performed two analyses for siring success of the competing pollen donors in the races. The first analysis tested whether larger pollen grains were more likely to outcompete smaller pollen grains and to sire more ovules in the (LP-SP) races. As described earlier, outcome of pollen competition was recorded as the proportion of progeny sired by the LP donor in each LP-SP-R combination. This value represented the relative siring success of the larger pollen donor and was expected to be 0.5 under the null hypothesis that the two competing donors had equal competitive ability and that equal numbers of pollen grains were applied onto the stigma. A value significantly greater than 0.5 would indicate that larger pollen grains sired a higher than expected proportion of progeny and vice versa. We applied the same analysis to races between two LP and between two SP plants. These siring success values for all pairings were plotted against the difference in average pollen diameters between the two competing donors.

In the LP-SP races, we also tested the siring success of LP plants against a second null hypothesis that the slight and nonsignificant difference in the pollen number of LP and SP donors applied onto the stigma could, in fact, influence the outcome of the pollen races. To do so, we compared our siring success data against the expected value of 0.517, calculated as the portion of pollen on a stigma that belonged to LP plants, using data from our test pollinations (described above). We treated both 0.5 (equal siring success) and 0.517 (pollen-number based) as null hypotheses in this study. Student's t tests were used to compare observed values with their expected values.

Relative siring success on different recipient plants—To test whether the siring success of a particular pollen race was consistent across the two R plants used, we performed a Wilcoxon signed rank sum test between the two R plants that received the same set of races. This test was done for all pairs of R plants, resulting in eight tests total.

RESULTS

Pollen and floral traits—*Pollen size and number*—Our data confirmed that the average pollen size was significantly larger in LP than in SP plants, whether we considered all pollen grains or just the fertile pollen grains (Table 1). Mean fertile pollen diameters were 121.53 (±0.17 [SE throughout]) μm and 111.06 (±0.41) μm for LP and SP, respectively; and R plants had pollen size in between (115.42 ± 0.5 μm). These values did not change significantly when we included all pollen grains. When converting the diameter into volume ($V = (1/6) \times (\pi D^3)$, where V is the volume and D is the diameter of the pollen grain), the difference between LP and SP amounts to ~31% difference in pollen grain volume.

In contrast, no significant differences were observed for pollen number per anther (manual or machine count) among LP, SP and R, with mean fertile pollen numbers (machine counted) being 158.33 (±3.76), 149.12 (±6.84) and 157.84 (±7.3), respectively (p = 0.25). Similarly, there were no significant differences when all pollen grains were included in the counts. These pollen counts were similar but lower than the hand counts of number of grains per anther, which was only done for LP and SP plants. In our first sampling date (5/2), when including all pollen grains, the LP plants produced on average 13 more pollen grains per anther than SP plants (LP = 196.85 and SP = 183.54), but the difference was not statistically significant (Table 1). In the second sampling date (5/22), four plants in the SP line produced some aborted pollen grains. When these sterile pollen grains were excluded from our calculations, SP's pollen production dropped to 182.73 (±12.53), but was still not significantly lower than what the LP donors produced – 196.94 ± 9.33 ($F_{1, 29} = 0.84$, $p = 0.37$). Using these pollen production values, we would expect to observe ~51.87% [= 196.94/(196.94 + 182.73) × 100%] of the pollen grains from the LP plant when we mixed one anther from LP and one from SP plants.

Floral organ size—Of the five floral traits measured, the only trait that differed significantly between the two pollen donor groups was pistil length, with LP having slightly longer pistils than SP plants (difference = 1.56 mm; ~5.1%; Table 1). Floral traits of R plants were similar to those of LP and SP plants except the stigma depth, which was significantly larger in R (1.18 mm ± 0.02) when compared with LP and SP donors; but the latter two did not differ from each other (1.12 mm ± 0.02 and 1.09 mm ± 0.02, respectively) (Table 1). Of all pairwise correlations among the traits measured, only three were significant: between corolla depth and longest stamen (r = 0.44, p = 0.001, n = 48), between corolla width and style length (r = 0.38, p = 0.008, n = 48) and between style length and average pollen size (r = 0.41, p = 0.004, n = 48) (Table 2). These correlations remained significant after Bonferroni corrections.

Pollen competition studies—*Pollen deposition on the stigma*—In our test crosses using two LP or two SP plants, we were able to apply a similar number of pollen grains onto the stigma; 140.9 (±5.27) and 131.4 (±7.99) respectively ($F_{1, 18} = 0.98$, $p = 0.33$). Assuming that when we did an LP-SP race, half of each of the above values were applied to the same stigma, we calculated the expected proportion of LP pollen grains in an LP-SP pollination to be 51.74% [= 140.9/(140.9 + 131.4) × 100%], a value very similar to what was calculated using the pollen production data (51.87% as described in *Pollen and floral traits*), suggesting that our pollination technique was

TABLE 1. Mean pollen and floral measurements of donor and recipient plants.

	Mean diameter (μm) and number of pollen/anther by Multisizer II				Pollen number/anther by hand(SE)			Mean floral trait values (mm)				
Plant	Diameter fertile pollen †	Diameter all pollen (μm) †	No. fertile pollen	Total pollen	Sample 1 5/2-all fertile	Sample 2 5/22-all	Sample 2 5/22-fertile	Corolla depth	Corolla width	Longest stamen length	Pistil length†	Stigma depth†
LP donor	121.5^{a} (0.2)	121.5^{a} (0.2)	158.3 (3.8)	158.3 (3.8)	196.8 (6.4)	196.9 (9.3)	196.9 (9.3)	48.5 (0.7)	59.4 (1.0)	29.7 (0.4)	32.0^{a} (0.2)	1.1^{a} (0.02)
SP donor	111.1^{b} (0.4)	108.3^{b} (0.9)	149.1 (6.8)	165.3 (4.7)	183.5 (3.7)	195.8 (9.4)	182.7 (12.5)	48.2 (0.6)	57.2 (1.0)	29.3 (0.4)	30.5^{b} (0.3)	1.1^{a} (0.02)
Recipient	115.4^{c} (0.5)	114.7^{c} (0.9)	157.8 (7.3)	163.7 (5.8)	—	—	—	47.9 (0.8)	56.1 (0.8)	29.7 (0.3)	30.5^{b} (0.3)	1.2^{b} (0.02)
Welch's Pr > *F*	<0.001*	<0.001*	0.25	0.26	0.08	0.93	0.37	0.86	0.059	0.60	0.0003*	0.02*

Notes: † indicates hetergeneous variance among groups based on Levene's test. LP, Large pollen; SP, Small pollen. Different superscript letters after values indicate a significant difference in the means for a particular trait among the plant groups (LP, SP, and Recipient).

TABLE 2. Correlations among floral and pollen traits based on trait means. Pearson's *r* correlation coefficients are above the diagonal; *p* values are below. Statistically significant results at the 0.05 level are in boldface.

Trait	Fertile pollen size	Fertile pollen no.	Corolla depth	Corolla width	Longest stamen length	Pistil length	Stigma depth
Fertile pollen size		0.06	0.16	0.24	0.22	**0.41**	0.04
Fertile pollen no.	0.71		−0.12	0.25	−0.1	0.12	−0.06
Corolla depth	0.27	0.41		−0.005	**0.44**	−0.18	−0.13
Corolla width	0.1	0.16	0.97		0.15	**0.34**	0.09
Longest stamen length	0.13	0.51	**0.002**	0.3		0.23	0.001
Pistil length	**0.004**	0.43	0.23	**0.008**	0.12		−0.05
Stigma depth	0.8	0.66	0.37	0.53	0.99	0.72	

equally effective in placing pollen from both donor groups onto the stigma.

Siring success—Results from the paternity analysis revealed that when LP donors were competing against SP donors (with pollen diameter differences between 11 and 16 μm), LP donors sired, on average, 77.57% of the seeds produced (Fig. 1). This value 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 $t = 13.56$, $p < 0.0001$) or the expected value of 50%, assuming equal siring success. In contrast, when the competing donors came from the same group (i.e., in the LP-LP and SP-SP races), the mean siring success of the donor with slightly larger pollen size was 41.7% and 52.2% for LP-LP and SP-SP races, respectively. Neither was significantly different from the null expectation of 50% assuming equal siring success of the two competing donors (both $p > 0.1$) (Fig. 1). It is worth noting that there was a wide distribution of siring success in both types of races with a substantial portion of races resulting in siring success of either 0 or 1.

Wilcoxon signed rank sum tests revealed that among the eight sets of pollen race results, two sets showed significantly different relative siring success between the two R plants used ($p < 0.01$) and one set showed a marginal difference ($p = 0.08$) between R plants, while the other five pairs did not differ significantly (Fig. 2).

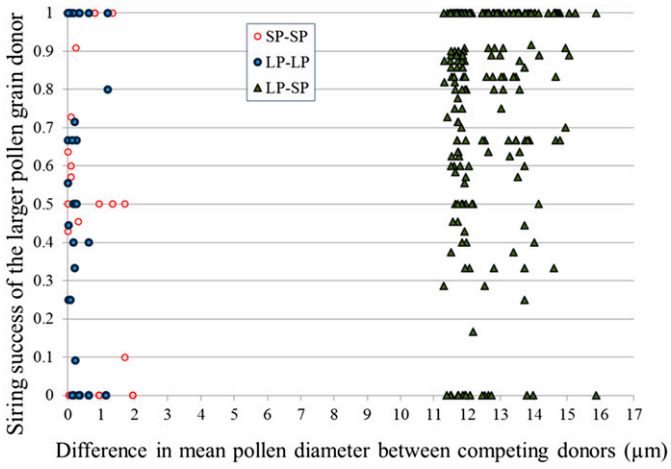

FIGURE 1 Siring success of the larger pollen grain donor in the three race types. Open circles: races between two small pollen donors (SP-SP), filled circles: races between two large pollen donors (LP-LP), triangles: races between one large and one small pollen donor (LP-SP). Values plotted are the siring success of the larger pollen grain donor. Values >0.5 indicate that the larger pollen grain donor sired more seeds, values of 0.5 indicate both donors contributed equally, values <0.5 indicate the smaller pollen grain donor sired more seeds.

DISCUSSION

The key finding of this study is that plants with large pollen grains were significantly more successful than those with small pollen grains when competing to fertilize ovules, providing direct support of the hypothesis that pollen size is an important trait in post-pollination male–male competition. However, the relative siring success between two competing pollen donors was not always consistent when examined with different recipient plants. In addition, we found that average pollen size was correlated with pistil length in this species, possibly due to selection favoring a correlation between these two traits. Other than pistil length, little correlation was found between pollen size and other floral traits that are likely to be important for pollinator attractiveness.

Floral trait differences between LP and SP plants—Our study plants are from an artificial selection experiment on pollen size; therefore, the difference in pollen size between the Large Pollen (LP) and Small Pollen (SP) lines used in this study is genetically based. Pollen size differed about 9% in diameter, which translates to a 31% difference in volume, between the two groups. This difference between the two donor groups is substantial, but still within the observed variation found in natural populations (S.-M. Chang, unpublished data). In contrast, the number of pollen produced did not differ significantly between the LP and SP groups. This lack of negative correlation between pollen size and number is consistent with results found in the artificial selection study that provided the seeds for this study (S.-M. Chang, unpublished data). This result is contrary to other studies that found not only a phenotypic, but also a genetic, negative correlation between pollen size and number (e.g., Vonhof and Harder, 1995; Worley and Barrett, 2000; Sarkissian and Harder, 2001; Lamborn et al., 2005). In fact, we also observed a negative phenotypic correlation (S.-M. Chang, unpublished manuscript) among field collected/measured plants in *I. purpurea*. Together, these results suggest that the negative phenotypic correlation was unlikely to be due to pleiotropic effects of the same genes controlling both pollen size and number but possibly due to either linkage disequilibrium, which was subsequently broken during the artificial selection process, or constraints on available resources that was not present in the experimental conditions. In addition to the differences in pollen size, SP plants were more likely to produce some aborted pollen grains while LP plants rarely showed this phenotype. The implications of this phenotype are discussed elsewhere (S.-M. Chang, unpublished manuscript). In this study, however, we made an effort to select SP plants that did not

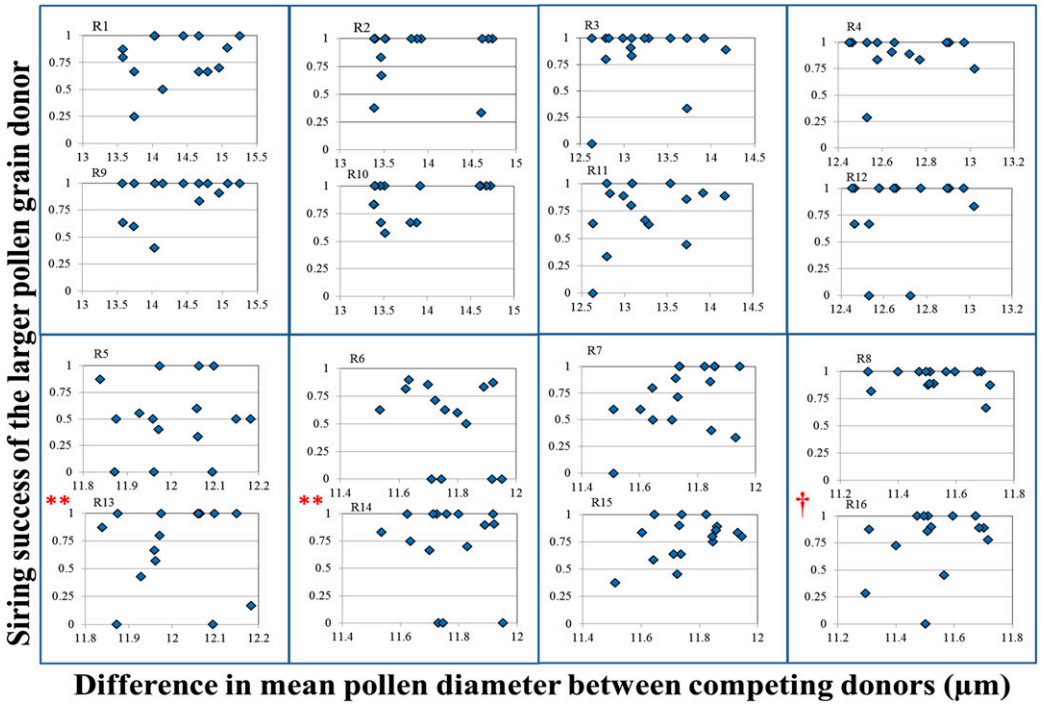

FIGURE 2 Siring success of the larger pollen donor in the eight sets of LP-SP combinations from the eight pairs of R plants. Each data point represents the outcome of one donor combination on one R plant. Results are plotted for every R plant, and the two R plants with the same set of race combinations are grouped in the same rectangle (R1&R9, R2&R10 and so on). Symbols between the two plots within one rectangle indicate the significance level of Wilcoxon signed rank sum tests between the two R plants. $^{**}p < 0.02$; $\dagger p = 0.08$.

abort or aborted only a small number of pollen grains; thus, we can attribute our results to pollen size differences and minimize the effect of differences in fertile pollen number (but see below).

The positive correlation between mean pollen size and pistil length in this experiment was also found in two of the six lines in a previous artificial selection study that gave rise to the study plants (S.-M. Chang, unpublished manuscript), providing some support that these two traits are genetically linked. Pistil length, as measured in this study, includes both stigma depth and style length, with the style representing ~96% of the pistil length in this species. Since we did not find differences in the stigma depth between the two groups, most of the variation can be attributed to variation in style length. Assuming that pollen tube growth rate is positively associated with pollen grain size in *I. purpurea*, then the positive correlation we found between pollen size and style length suggests that plants with more competitive (larger) pollen grains also provide a more competitive environment in their (longer) styles. Longer styles have been hypothesized by several researchers to be a more stringent environment for pollen competition where faster growing pollen tubes have a longer distance across which to "beat" slower growing pollen tubes and thus sire more seeds (Johnston, 1993; Williams, 2008, 2012; Mazer et al., 2010, 2016 [this issue]). The positive correlation between larger pollen and longer styles has also been found in several other species (Diaz and Macnair, 1999; Torres, 2000; Yang and Guo, 2004) and 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 driven by the correlated functions of these two traits (i.e., larger pollen grains have more energy stores and can grow faster to reach resources residing inside the style). In other words, correlational selection favoring the occurrence of large pollen and longer styles could have built up such genetic linkages (Sinervo and Svensson, 2002). Alternatively, long styles could be an evolutionary outcome of selection for stronger pollen competition, such as in highly outcrossing species (Williams, 2008, 2012). However, different opinions exist regarding whether pollen size is indeed functionally correlated with style length or, alternatively, with traits that are correlated with style length, such as stigma depth (Ganders, 1979; Mulcahy, 1979; Cruden and Lyon, 1985). Though our results indicate that stigma depth does not correlate with pollen size, our sample size is limited to the 48 plants used in this study. A larger sample size will be needed to draw any conclusion on this relationship and to fully understand the mechanism underlying the positive correlation between pollen size and pistil length.

Among other floral traits, we found only a couple of significant correlations: one between corolla depth and longest stamen length and the other between style length and corolla width; both were positive correlations. Genetic correlations among floral traits are not uncommon (e.g., Rosas-Guerrero et al., 2011), and it has been suggested that traits serving similar functions, for example, attractiveness (such as depth and width of corolla) or effective pollen placement (such as style and stamen length), might have been selected to be intercorrelated (Rosas-Guerrero et al., 2011). There is also some evidence that floral traits can be correlated with pollen traits, such as size and number (see Muller, 1979; Sarkissian and Harder, 2001). Whether such phenotypic correlations between pollen and floral attractiveness traits were due to correlational selection favoring a particular combination of traits remains to be tested.

LP plants outcompeted SP plants in pollen competition—Our study clearly shows that plants with larger pollen sired more seeds than those with smaller pollen when LP and SP pollen were competing on the same stigma. Because we applied pollen from both donors to the stigma simultaneously, we can reasonably assume that the nonrandom siring success we find is due to pollen competition on the stigma or inside the style and not pollen primacy, defined as the timing of pollen arrival (Epperson and Clegg, 1987). Several reasons, not mutually exclusive, could potentially explain the pattern we found. First, larger pollen may have more energy reserves and therefore are able to germinate faster and/or have a faster rate of early stages of pollen tube growth (Baker and Baker, 1979; Ganders, 1979; Snow and Spira, 1991b, 1996; Spira et al., 1992; Mazer et al., 2010). This explanation has been suggested

to underlie the effect of soil fertility on increasing pollen size (Stephenson et al., 1992; Lau and Stephenson, 1993, 1994; Delph et al., 1997) and subsequently greater siring success by larger pollen grains. If larger pollen grains in our study also contain more energy stores (starch and/or lipids) that facilitate pollen tube germination and growth through the stigma and style (Cruden and Lyon, 1985; Cruden, 2000), the greater energy reserves could explain the higher competitive ability when LP were competing against SP in this study. In *I. purpurea,* pollen grains do contain starch (S.-M. Chang, unpublished data), but no data are available on the amount of starch inside each grain and the role of other resource storage molecules, such as lipids, in this process. Analysis of the nutrient content of pollen grains of LP and SP will help to test this possibility.

A second possibility is that LP plants could be better competitors because the genes responsible for their larger pollen grains may be linked to genes that cause higher pollen tube growth rate. We are distinguishing this effect, which emphasizes the genetic differences between pollen donors, from the nutrient-based size effect discussed in the last paragraph, which underscores the paternal plant's ability to provision its pollen grains. An example of such an effect could be that genes expressed in pollen or pollen tubes enhance the growth rate of pollen tubes. Recent molecular genetic studies have identified a genetic basis for pollen tube growth rates in several model systems. For example, 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, Carlson et al. (2009) found that different genetic accessions of *Arabidopsis thaliana* showed nonrandom siring success during pollen competition. However, none of these studies linked pollen tube growth rate with any visible pollen phenotype, such as size or shape. In our study, the pollen donors came from genetically differentiated (selection) lines, and a faster pollen tube growth rate may have resulted from either pleiotropic effects of genes underlying pollen size variation or from genetic linkage between pollen size and pollen tube growth rate. If we consider that pollen size is likely to be a parental plant (sporophytic stage) provisioning trait and pollen tube growth rate is a pollen grain (gametophytic stage) genetic trait, such linkage could arise if there is a correlation between selection forces acting on both sporophytic and gametophytic stages of the plant's life cycle as found in some other species (Mulcahy, 1979; Delph et al., 1998; Lankinen and Skogsmyr, 2001; Stephenson et al., 2001; Bernasconi et al., 2004). The higher tendency of pollen abortion in the SP plants could be an indication of their weaker provisioning ability and lower general vigor than LP plants; however, further studies examining correlations between relative siring success and the sporophytic vigor of its progeny will be needed for a more direct test of this idea.

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, suggesting that our results are robust in that respect. 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; Tejaswini, 2002; 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 and Io, 2002).

Finally, LP donors and SP donors could have induced different interactions with the recipient pistils (donor × recipient effect) (Johnston, 1993; Mazer et al., 2010; Dresselhaus and Franklin-Tong, 2013)—similar to some animals systems, in which the relative success of a given male during sperm competition may be affected not only by the quality of the male but also by how compatible the male is with a specific female (Colegrave et al., 2002; Neff and Pitcher, 2005). Differential male–female complementarity, either due to genetic compatibility or environmentally induced interaction, has been suggested in a few plant systems (Stephenson and Bertin, 1983; Marshall and Fuller, 1994; Marshall and Diggle, 2001; Carlson et al., 2009). Whether such differential pollen–pistil interaction is occurring in our study system still requires more in-depth studies, but our results suggest that it is a viable hypothesis. Specifically, we found variable consistency between the outcomes of the same races on two different R plants. In three of the eight pairs of R plants, the relative siring success of the same donor combinations showed significantly ($p < 0.05$) or approaching significantly ($p = 0.08$) different patterns, with LP plants having siring success higher than 0.5 on one R plant (R13, R14, R8) but showing much more scattered siring success between 0 and 1 on the other R plant (R5, R6, R16, respectively). This inconsistency between R plants for the same set of races suggests that there might be other factors associated with certain stylar environments specific to the combination between donor and R plants, that can influence the outcome of the pollen competition (Havens and Delph, 1996). In several systems, stylar tissue could contribute to the outcome of pollen competition (Stephenson and Bertin, 1983; Marshall and Ellstrand, 1986), allowing the possibility of "female choice" in the context of sexual selection. Though we do not know in our study what females might be choosing, other than the larger pollen size, it is well documented that females could benefit from preferring a particular type of pollen tubes in the case of self-incompatibility systems (Charlesworth et al., 2005).

Two caveats in our study deserve some consideration. First, though we detected only one nonpollen trait that differed significantly between the LP and SP plants, we only measured a small set of traits. There could be other undetected responses to the original artificial selection process that were responsible for the differences we observed in siring success, but which have nothing to do with pollen or floral traits. This possibility cannot be dismissed especially given the fact that, in several cases, LP sired 0% of the progeny in the LP-SP races, suggesting that something other than pollen size was more important in determining siring success in those cases. We are currently carrying out genetic and biochemical comparisons between the LP and SP lines in hope of identifying other differences between these pollen size selection lines.

The second caveat involves potential differences between LP and SP plants in the postfertilization stage. Our results could be due to differential vigor between LP and SP progeny during seed development. For example, if progeny of SP tend to have a lower survival rate during the embryonic stage, the differential siring success we observed could be, at least in part, due to differential seed failure/abortion and not just pollen competitive ability. Our study design does not allow us to test this hypothesis. One way that this possibility can be tested is to use techniques such as fluorescent-protein labeling of pollen tubes (Huang et al., 2014; McDowell et al., 2015) to allow the differentiation of pollen tubes

(e.g., LP vs. SP donors) inside the stylar tissue so that we can determine whether competition in the postpollination–prefertilization stage can fully explain the siring success variation we see in seeds. Unfortunately, such techniques are still limited to model systems such as *Arabidopsis thaliana* (McDowell et al., 2015) and tomatoes (Huang et al., 2014) and not yet available in most other systems, including *I. purpurea*.

Evolutionary implications of genetically based variation in pollen competitive ability—Several findings in this and our previous studies have significant implications for the role that pollen size plays in postpollination processes and in sexual selection of *I. purpurea* in general. First, our previous results showed that there is significant additive genetic variation in pollen size in this species, with heritability estimated in the greenhouse to be between 0.3 and 0.5 (S.-M. Chang, unpublished data). This estimate is comparable to what was found in other species (Sarkissian and Harder, 2001; Lamborn et al., 2005). Second, in this study, we reveal that pollen size is important in male–male competition during postpollination process of sexual reproduction. Third, we have indirect evidence that though pollen size can be considered to be a "good-male" trait that helps males achieve higher siring success, pollen receiving plants (the females) still play a role in the outcome of pollen competition, i.e., there is potentially some female choice involved in the outcome of pollen competition. Combining these findings, we argue that pollen size is an excellent male "quality trait" for *I. purpurea* that has several utilities for studying sexual selection in hermaphroditic plants, as defined more recently by Delph and Ashman (2006) and Moore and Pannell (2011). First, it provides a clear mechanism underlying variation in the male component of fitness, through which we can investigate factors contributing to the maintenance of genetic variation in this trait and in male fitness in general. Second, pollen grain size is also likely to be important for pollen dispersal during the prepollination stage of reproduction (Sarkissian and Harder, 2001); hence, it can provide a link for understanding whether selection at different stages of sexual reproduction work in concert or in opposition to one another. Finally, there is a clear parallel between sperm competition in animals and pollen competition in flowering plants. With further investigation involving specific genotypic interactions between pollen and ovule donors, we will be able to broaden the test of sexual selection theories, particular regarding the hypothesis that some females may be choosing the "compatible males" rather than the "good males" (Colegrave et al., 2002; Neff and Pitcher, 2005).

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