UNIVERSITY OF CALGARY

The Evolutionary Genetics and Adaptive Significance of Intraspecific Variation in Pollen Grain Size in *Brassica rapa* L.

by

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ABSTRACT

Pollen size commonly varies within and among angiosperm species, but the biological significance and genetic basis of this variation remain largely unknown. A pollen-mixture experiment in *Brassica rapa* revealed that donors producing large pollen enjoyed a 20% siring advantage over donors producing small pollen during post-pollination competition for access to ovules. For selection to respond to such fitness differences, inter-individual variation in pollen size must be genetically determined and heritable. Artificial selection for large and small pollen over three generations caused significant divergence in pollen diameter, with realized heritability ranging from 0.13 to 0.61. In addition, selection on pollen size led to correlated responses in pollen number (–), flower size (+), style length (+), and ovule number (+), suggesting that pollen size cannot evolve independently. The heritable genetic control of pollen size variation and consequent effects on siring success suggest that post-pollination processes provide the primary impetus for pollen-size evolution in angiosperms.

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1. INTRODUCTION

1.1 Size variation in angiosperm pollen

Pollen size varies extensively among angiosperm species, from diameters of approximately 5 μm to 250 μm (Wodehouse, 1935; Whitehead, 1969; Muller, 1979). This variation in diameter corresponds to differences in volume approaching five orders of magnitude. Such variation implies that natural selection effects evolutionary change in this trait, presumably in response to each species' particular pollination and fertilization environment (Harder, 1998). This process requires that the variation in pollen size evident within populations (e.g., Prentice et al., 1984; Stanton and Preston, 1986; McKone, 1989, 1990; Cruzan, 1990; Young and Stanton, 1990a, b; Vonhof and Harder, 1995) bear fitness consequences and be genetically determined.

The evolutionary consequences of inter-individual differences in pollen size depend, in part, on the relative importance of environmental and genetic controls on pollen-size variation. Development of angiosperm pollen depends completely on the nutrients provided by the sporophyte, so that the growing conditions of the parent plant influence both the quantity and quality of pollen produced. Light, temperature, humidity, water, root crowding, and mineral nutrition all affect pollen size (reviewed in Muller, 1979; Lau and Stephenson, 1993, 1994; but see Young and Stanton, 1990c). If environmental conditions during pollen development account for all variation in size among plants in a population, then natural selection does not occur, regardless of the fitness consequences of such variation.

Selection requires additive genetic control of some component of phenotypic variation in pollen size. The few estimates of the heritable component of pollen-size variation exhibit little consistency. For example, coastal *Raphanus sativus* populations, harbor significant additive genetic variance for pollen grain volume (h^2=0.40 ±0.21 at low density: Mazer and Schick 1991; also see Mazer, 1992), whereas inland populations do not (h^2=0.18 ±0.10: Young et al., 1994). Similarly, phenotypic variation in pollen size has a significant additive genetic component in *Phaseolus vulgaris* (Montes-R and White, 1996), but not in *Spergularia marina* (Delesalle and Mazer, 1995) or *Mimulus guttatus* (Fenster and Carr, 1997). Hence, prior to evoking any model of pollen-size evolution by natural selection for a specific species, it is necessary to first understand the genetic basis of pollen-size variation.

1.2 Functional and adaptive aspects of pollen size

The evolutionary consequences of intraspecific pollen-size variation also depend on the relation between pollen size and individual fitness. In general, differences in pollen size among plants in a population could influence siring success through effects on pollen transport and/or post-pollination processes. Hence, the overall fitness consequences of a particular pollen size depend on the joint effects during both phases of male function.

Dispersal ability seems to have influenced pollen-size evolution, at least at a very general level. Most wind-pollinated species possess grains 20 to 60 μm in diameter (Whitehead, 1969), whereas pollen diameter in animal-pollinated species ranges from 5-200 μm (Wodehouse, 1935). However, differences in transport conditions among animal-

pollinated species seem not to have affected pollen-size evolution consistently (Harder, 1998). Based on this finding, Harder (1998) suggested that the functional explanation of interspecific differences in pollen size likely lies in their post-pollination effects, although such effects have yet to be demonstrated unequivocally.

After pollen successfully reaches a stigma, its size may influence fertilization success (Cruzan, 1990; Lau and Stephenson, 1993, 1994; Aizen and Raffaele, 1996). Pollination creates a competitive environment for male gametophytes when stigmas receive more pollen than is needed to fertilize all available ovules from several individuals (reviewed in Snow, 1994). In such an environment, differences in pollen performance (speed of germination and/or pollen tube growth rate) can lead to nonrandom fertilization. Given that pollen accumulates diverse products during development, such as mRNAs, proteins, enzymes, ribosomes, starch, lipids, and phosphorus storage compounds (reviewed by Lau and Stephenson, 1993, 1994), pollen-grain size likely reflects the resources available for post-pollination processes. In particular, pollen grains use endogenous resources during the initial autotrophic phase of pollen germination and tube growth until the heterotrophic interactions between pollen tubes and the stylar tissue commence (reviewed in Heslop-Harrison, 1971). If rates of germination and tube elongation vary with pollen reserves, then pollen size may reflect the relative competitive ability of donors.

1.3 Objectives

In this thesis, I determine the magnitude of the heritable genetic component of pollen-size variation and its post-pollination fitness consequences using *Brassica rapa* L. (syn. *campestris*, Brassicaceae).

In Chapter 2, I describe an artificial selection experiment in which I applied divergent selection on pollen size in *B. rapa*. This experiment assesses the heritable basis of pollen-size variation and the correlated effects of selection on pollen grain size on other traits. I examined genetic correlations to evaluate the opportunity for natural selection to act independently on pollen size.

In Chapter 3, I assess whether pollen size influences the post-pollination competitive ability of male gametophytes. In this experiment, I competed large and small pollen from two donors on a stigma using controlled hand pollinations and measured relative competitive ability in terms of the proportion of seeds sired. In particular, this experiment tests whether large pollen outcompetes small pollen.

In Chapter 4, I summarize and synthesize the results and conclusions from Chapters 2 and 3. With this Master's project, I demonstrate that pollen size has a heritable basis and indicates competitive ability. Hence, natural selection can effect evolutionary change in this character when pollen competition is common. The demonstrated consequences of pollen size on siring success suggest that post-pollination processes provide the primary impetus for pollen-size evolution.

2 QUANTITATIVE GENETICS OF POLLEN GRAIN SIZE

2.1 Introduction

Strong gametophytic selection due to pollen competition occurs when many microgametophytes from several donors vie for a limited number of ovules. If this competition leads to nonrandom fertilization with respect to pollen genotype (see Marshall and Ellstrand, 1986; Marshall and Folsom, 1992; Snow, 1986, 1994; Snow and Spira, 1996), selection based on pollen competition should lead to fixation of alleles for the best-competing phenotype, and thus to loss of genetic variation (e.g. Walsh and Charlesworth, 1992). The opportunity for such selection can be substantial because stigmas often receive an order of magnitude more pollen grains than the number of available ovules in natural populations (Snow, 1994; but see Burd, 1995) and animal pollination typically delivers pollen from several donors (reviewed by Harder and Barrett, 1995).

Pollen characteristics that could provide pollen donors with consistent mating advantage across maternal plants include average grain size. That pollen size influences male reproductive success is reasonable, given that it varies positively with pistil characteristics (e.g., Baker and Baker, 1979; Plitmann and Levin, 1983; Cruden and Lyon, 1985; Williams and Rouse, 1990; Dulberger, 1992; Kirk, 1993; Vonhof and Harder, 1995), pollen-tube growth rate (van Breukelen, 1982; Lord and Eckard, 1984; Perez and Moore, 1985; Gore et al., 1990; Williams and Rouse, 1990; Manicacci and Barrett, 1995), and post-fertilization siring ability (Cruzan, 1990; Lau and Stephenson, 1993, 1994).

Previous studies of pollen size identified a range of control by additive genes. Of the ten floral traits studied in *Spergularia marina*, pollen grain volume had the lowest genetic

coefficient of variation (sensu Houle, 1992), which suggests low potential for response to selection on this trait (Delesalle and Mazer, 1995). In addition, in two populations of *Mimulus guttatus*, narrow-sense heritabilities for pollen diameter based on sire regression did not differ significantly from zero (Fenster and Carr, 1997). These results suggest that the inter-individual variation in pollen size reported in phenotypic studies (Prentice et al., 1984; Stanton and Preston, 1986; Devlin, 1989; McKone and Webb, 1988; McKone, 1989, 1990; Thomson, McKenna, and Cruzan, 1989; Cruzan, 1990; Young and Stanton, 1990a, b; Nakamura and Wheeler, 1992; Mazer and Hultgård, 1993; Vonhof and Harder, 1995; Pfahler, Pereira, and Barnett, 1996) largely reflects the influence of environmental factors. However, in *Raphanus sativus*, Mazer and Schick (1991; see also Mazer,1992) found significant additive genetic variance for pollen grain volume (but see Young et al., 1994) and artificial selection effectively caused divergence in pollen diameter in *Phaseolus vulgaris* L. (Montes-R and White, 1996). Results from these studies suggest that phenotypic variation in pollen size is, to some degree, genetically determined and heritable. Although natural selection should fix alleles in fitness related traits (Fisher, 1930; Falconer, 1989), various circumstances may maintain heritable variation in pollen size including mutation-selection balance (Lande, 1976a), and/or various genotype × environment interactions (e.g., Haldane and Jayakar, 1963; Via and Lande, 1987; Gillespie and Turelli, 1989).

The potential for rapid evolutionary modification of pollen size also depends on genetic associations with other traits. Due to the highly integrated nature of floral development, traits that individually influence reproductive success likely exhibit genetic correlations (e.g., Cheverud, 1984, Shore and Barrett, 1990; Conner and Via, 1993; Fenster

and Carr, 1997). For example, in *Raphanus sativus*, pollen production and petal size are positively genetically correlated and therefore, cannot evolve independently (Stanton and Young, 1994). Such correlations could slow evolution towards new, optimal reproductive phenotypes (Antonovics, 1976; Lande, 1979; Meagher, 1992) or be maintained by selection as adaptive trait combinations (Stanton and Young, 1994). For example, if pollinators preferentially visit plants with large flowers and large pollen (as in *Raphanus sativus*, Young and Stanton, 1990b; Stanton et al., 1991), and pollen export increases with visitation, then both traits should experience positive directional selection. However, a negative genetic correlation between these traits would impede the evolution of an effective floral phenotype.

In this chapter, I evaluate the potential for and constraints on the evolution of pollen size. In particular, I present results from an artificial selection experiment that tested for heritable variation in pollen grain size and correlated effects of selection on other floral traits. If variation in pollen size influences the male reproductive success of plants in a population, then the extent to which natural selection can change the pattern of phenotypic variation in subsequent generations will depend on the heritability and degree to which selection acts independently on pollen size.

2.2 Methods

2.2.1 Study species, selection protocol, and data collection

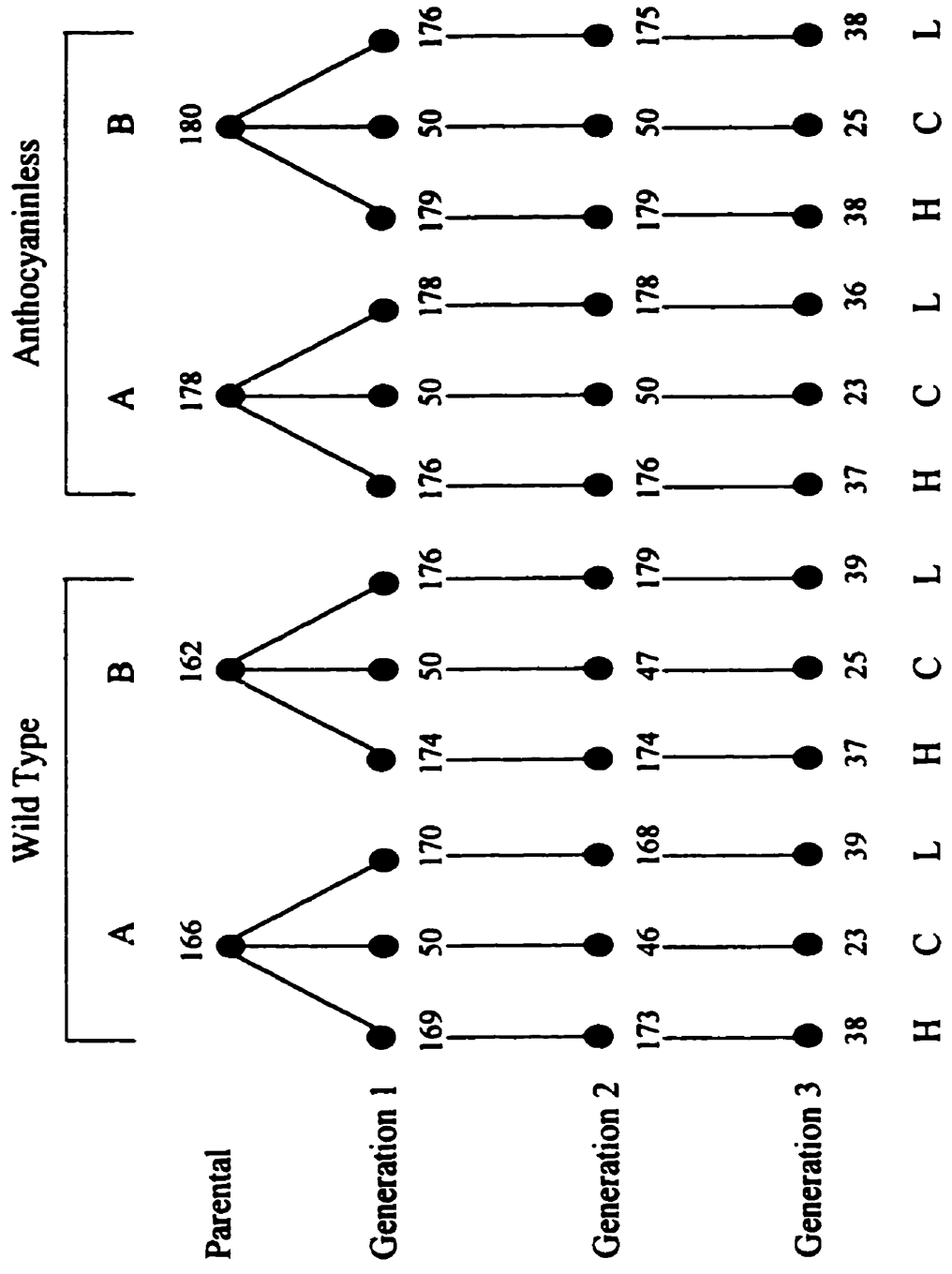
To investigate the heritable basis of pollen-size variation and the correlated effects of selection on pollen size on other traits, I applied divergent selection on pollen diameter in *Brassica rapa* L. (syn. *campestris*, Brassicaceae). *Brassica rapa* is a self-incompatible

annual or biennial mustard native to Eurasia and now widely naturalized in North America. In this study, I used a rapid-cycling stock of *B. rapa* previously developed by several cycles of selection for early flowering, rapid seed maturation, absence of seed dormancy, small plant size and high female fertility (Williams and Hill, 1986). Flowering of rapid-cycling *B. rapa* commences approximately 13 days after planting, and continues for up to 7 days. During this period, 3 or 4 flower buds open daily on inflorescences which produce 20 to 25 flowers. Plants were grown singly in 4 × 4 × 5.5cm potting units in standard greenhouse soil with 0.2 g of fertilizer (Osmocote 14:14:14 controlled release fertilizer) and maintained in a laboratory (23-26.5°C) under cool-white fluorescent bulbs (24 h/day).

Plants used in the study were derived from two morphologically distinct base populations (Wisconsin Fast Plants, University of Wisconsin, Madison, WI, USA): wild-type and anthocyaninless. Wild-type plants produce anthocyanin, which colors the base of stems and leaf petioles purple. In anthocyaninless plants, a recessive allele at the "anthocyanin" locus prevents production of anthocyanin, resulting in completely green stems and leaves. These differences in anthocyanin production were not relevant for this study, but proved useful in a subsequent study of the effects of pollen size on gametophytic competition (Chapter 3).

For each population, the experimental design involved two selection lines (high and low) and a randomly selected control-line, each replicated twice (Fig. 2.1). Individuals in the parental generation were grown from seed obtained from the Carolina Biological Supply Company (Burlington, North Carolina, USA). Replicate lines of each population were established from different seed lots. I staggered the planting of high-, low- and control-line

Fig. 2.1. **Pedigree of genetic lines created during three generations of divergent selection on pollen diameter, with a randomly selected control (H=high-, L=low- and C=control-lines). Each population included two replicates (A and B). The numbers of individuals measured for pollen size in each line are indicated throughout.**


Wild Type
Anthocyaninless
A
B
A
B
Parental
166
162
178
180
Generation 1
169
50
170
174
176
50
176
176
50
178
179
50
176
Generation 2
173
46
168
174
179
47
176
176
50
178
179
50
175
Generation 3
38
23
39
37
39
25
37
23
36
38
25
38
H
C
L
H
L
C
H
C
L
H
C
L

plants within a replicate by approximately two weeks to overcome the constraints imposed by the short flowering period of the plants (maximum 7 days) relative to the time required for data collection and pollinations.

Pollen was measured and counted for plants in each generation with a Particle Data, Elzone 180XY electronic particle analyzer. From each plant, I collected all anthers from the 4^{th}, 5^{th} or 6^{th} (occasionally 7^{th}) flower after dehiscence (see Fig. 2.1 for population sizes). I first suspended each pollen sample in 1.5ml of 70% ethanol and then added 25ml of 0.63% NaCl solution and sonicated for 5 min. Anthers were examined under a dissecting microscope to ensure that all pollen had been removed prior to analysis. The particle analyzer then processed each pollen sample by drawing a 1-ml subsample through a 190-μm diameter aperture. As particles pass through the aperture, they change the resistance to an electric current flowing between electrodes on either side of the aperture. The analyzer counts these resistance changes and measures their amplitude. The amplitude changes are converted into particle diameters and displayed as a frequency histogram of counted particles by 128 logarithmic diameter classes. Total pollen volume per flower was calculated by converting diameter to volume and summing the product of the number of particles in each size class and the average size for that class. Two 1-ml subsamples were analyzed and the average pollen diameter (μm), pollen volume per flower (μm^3), and pollen number were calculated for each pollen sample.

After quantifying pollen size in the four parental populations, I chose the 20 individuals with the largest and 20 individuals with the smallest mean pollen diameter as the parents for the "high" and "low" selection lines. Ten pollen donors were assigned randomly

to each parental plant within the high and low selection lines and single-donor crosses performed; therefore, each parental plant received 10 pollinations and donated pollen for pollinations on 10 other plants.

From the remaining individuals in the parental populations, I chose 50 randomly to generate the control-line. The 50 plants in the control-line were paired randomly with one other individual from the group and mated; two flowers were pollinated for each combination to ensure at least one fully seeded pod. During all pollinations, newly dehisced anthers were brushed across the stigma to cover it with pollen.

I imposed selection for three generations. During offspring generations 1 and 2, the 20 individuals with the largest and 20 individuals with the smallest mean pollen diameters were chosen to propagate the high- and low-lines, respectively. All control-line plants assayed for pollen diameter in each generation were mated randomly with one other individual. For offspring generation 1, I randomly assigned parental pairs as long as they did not share a parent from the previous generation (i.e. no half-sib crosses). In offspring generation 2, only plants with a coefficient of relatedness less than or equal to 0.0625 were paired and mated. This breeding design was adopted to minimize accumulation of genetic load.

To determine the genetic association between pollen diameter and other floral traits, I collected the 5^{th}, 6^{th} or 7^{th} (occasionally 8^{th}) flower from all individuals in offspring generations 1 and 2 (high- and low-lines only) and stored them in separate microtubes in 70% ethanol. I subsequently measured petal length, maximum petal width, filament length, and style length at 6X and ovule number at 25X under a dissecting microscope for 40 to 50

randomly chosen flowers per replicate. In addition, I used counts of pollen number and pollen volume per flower obtained from the automated particle analyzer to test for a tradeoff between pollen size and total pollen production.

2.2.2. Data analyses

The realized narrow-sense heritability of pollen diameter was estimated from the regression of selection response on cumulative selection differential (Falconer, 1989). I calculated the response after each generation of selection in three ways and therefore obtained three estimates of realized heritability. The mean response in each generation (R) was calculated alternatively as:

$$R_{1a} = \overline{X}_H - \overline{X}_C \qquad (1a)$$

$$R_{1b} = \overline{X}_L - \overline{X}_C \qquad (1b)$$

$$R_2 = \overline{X}_H - \overline{X}_L \qquad (2)$$

where $\overline{X}_H$, $\overline{X}_L$, and $\overline{X}_C$ are the mean pollen diameters for the high-, low-, and control-lines, respectively. Measurement of the selection response as the difference between selected and control-line means (R_{1a} and R_{1b}) or as the divergence between the high- and low-line means (R_2) eliminates common environmental effects, although the latter method estimates heritability more precisely (Hill, 1972). Selection differentials were calculated for each generation as:

$$\sum_{i=1}(\mu_{s,i} - \mu)p_i \qquad (3)$$

(Falconer, 1989), where $\mu_{s,i}$ is the pollen diameter of each individual selected for breeding, μ is the mean pollen diameter in the entire population, and p_i is the proportion of individuals measured in the next generation that were the offspring of plant i. To calculate heritability based on the divergence between the high- and low-lines, I summed the selection differentials for each line (absolute values) to obtain the total selection applied. Variances for realized heritabilities were estimated according to the formulas given by Ross (1997, p. 140-142).

To assess the direct response of pollen diameter to selection and the correlated responses of pollen number and pollen volume, I compared trait means in each population (anthocyaninless and wild-type) with a general linear model that considered replicate (A or B), selection line (high, low, control), generation, and their interactions as the independent variables. Pollen number and pollen volume were square-root transformed prior to analyses to meet the assumptions of the model. To determine the extent of correlated response to selection for the remaining floral traits, I used the same general linear model, but considered only offspring generations 1 and 2 in the high- and low-lines. (I discuss how I distinguished correlated responses to selection from non-genetic causes of variation in the Results). I analyzed the two populations separately because their different selection histories could have resulted in different magnitudes and patterns of genetic variation.

I further examined the relation between pollen size and number to test the expectation that pollen production (n) should vary inversely with investment per pollen grain (v = volume) from division of a fixed expenditure of resources on male gametes (R), such

that:

$$n \propto R / v \qquad (4)$$

(Vonhof and Harder, 1995). Because I measured a linear dimension (d = pollen diameter), whereas investment involves a cubic dimension (v), the relevant relation becomes

$$n \propto R / d^3. \qquad (5)$$

I tested this expectation by regression analysis after log transforming both sides of eq. 5

$$\ln(n) = \ln(R) - 3\ln(d). \qquad (6)$$

The full general linear model for this analysis also included population (anthocyaninless and wild-type), replicate nested within population, and their interactions. Final model selection involved backward elimination (α=0.05) of nonsignificant effects. I used a single-sample t-test to test the hypothesis that the partial regression coefficient for ln(pollen diameter) did not differ significantly from –3.

2.3 Results

2.3.1 Direct responses to selection

Directional selection for large and small pollen diameter caused divergence between lines; the distributions of pollen size for plants in the high- and low-lines overlapped less and less with each subsequent generation (see Fig. 2.2). However, the replicates for each population did not respond consistently (see generation×line×replicate effect, Table 2.1). In particular, anthocyaninless replicate A and wild-type replicate B populations responded relatively symmetrically, whereas the high-line of anthocyaninless replicate B and the low line of wild-type replicate A responded more than the corresponding lines subject to

Fig. 2.2. Changes in the distributions of pollen diameter during three generations of artificial selection for the anthocyaninless replicate B population. Histograms for the three offspring generations represent the high- (——), low- (- - - -), and control- (·····) lines. Vertical lines represent the means of each distribution.

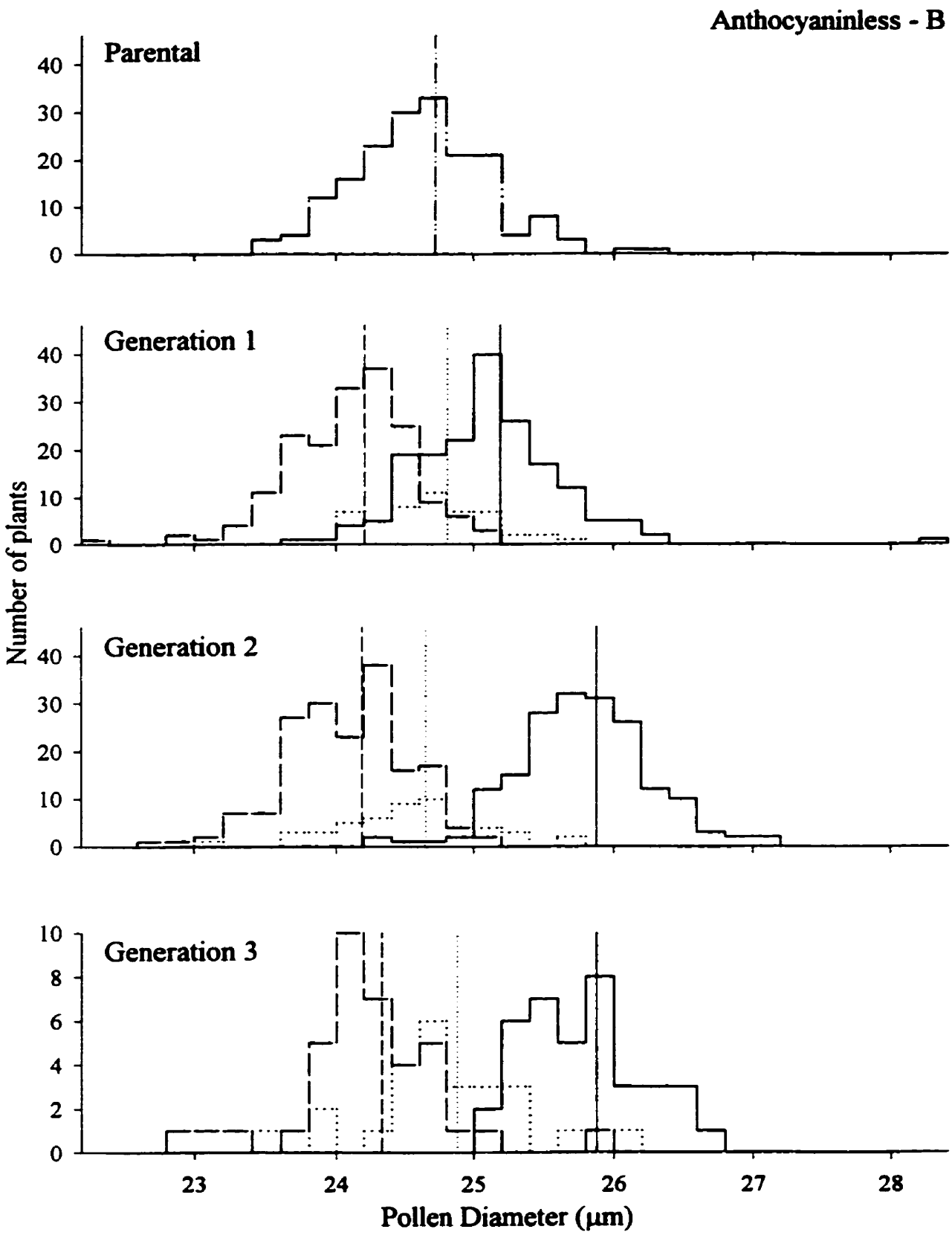

Anthocyaninless - B
Parental
Generation 1
Generation 2
Generation 3
Number of plants
Pollen Diameter (μm)
0
10
20
30
40
2
4
6
8
23
24
25
26
27
28

Table 2.1. ANOVA evaluating the direct response to selection on pollen diameter in each population.

Source	Anthocyaninless	Wild Type
Generation	$F_{3, 2152}=34.32^{***}$	$F_{3, 2085}=35.57^{***}$
Line	$F_{2, 2152}=1108.70^{***}$	$F_{2, 2085}=842.76^{***}$
Replicate	$F_{1, 2152}=29.41^{***}$	$F_{1, 2085}=56.83^{***}$
Gen×Line	$F_{4, 2152}=38.39^{***}$	$F_{4, 2085}=97.92^{***}$
Gen×Rep	$F_{3, 2152}=8.53^{***}$	$F_{3, 2085}=53.74^{***}$
Line×Rep	$F_{2, 2152}=1.49$	$F_{2, 2085}=9.40^{***}$
Gen×Line×Rep	$F_{4, 2152}=15.01^{***}$	$F_{4, 2085}=2.88^{*}$

$^{*}P<0.05$, $^{***}P<0.0001$

selection in the opposing directions (Figs. 2.3a and 2.4a). Differences in the magnitude of response for large versus small pollen were reflected in the average response per generation in the high- and low-lines (Table 2.2). The most asymmetrical response occurred in the wild-type replicate A population: mean pollen diameter (±SE) increased by 0.11 (± 0.06) μm per generation in the high-line, whereas it decreased by 0.46 (± 0.10) μm per generation in the low-line (Table 2.2). Responses were evaluated as deviations from control given that control-line means increased significantly in the anthocyaninless and wild-type replicate A populations. Although control-lines had smaller populations than high- and low-lines, I rejected drift as a possible explanation for these increases in pollen diameter using Lande's (1976b) approach. Changes in control-line means likely reflect altered environmental conditions or inadvertent selection.

After the third round of selection, individuals in the high-, low-, and control-lines had significantly different mean pollen diameters across all replicates (Figs. 2.3a and 2.4a). By offspring generation 3, plants in the low-lines produced pollen grains that averaged 0.55 to 1.26 μm smaller than those produced by plants in the parental generation. Conversely, pollen from high-lines were 0.41 to 1.00 μm larger (Figs. 2.3b and 2.4b). By the third offspring generation, pollen grains from high-line plants averaged 1.55 to 2.04 μm larger in diameter than pollen from low-line plants.

Differences in the magnitude of response were reflected in the estimates of realized heritability (±SE), which ranged from 0.13 ±0.04 to 0.61 ±0.08 (Table 2.3, also see Figs. 2.3b-c and 2.4b-c). Not surprisingly, these extremes in heritability involved high- and low-line plants in the wild-type replicate A population, which responded asymmetrically to

Fig. 2.3. (a) Direct responses of pollen diameter (± 95% CI) to three generations of artificial selection for anthocyaninless plants in the high- (H ——), low- (L - - - -), and control- (C ·····) lines. Means with confidence intervals that do not overlap differ significantly. (b) Deviations of each selection line from the control-line (H-C and L-C, see equations 1a and 1b) relative to the cumulative selection differential. (c) Differences between the high and low selection lines (H-L, see equation 2) relative to the cumulative selection differential (absolute value).

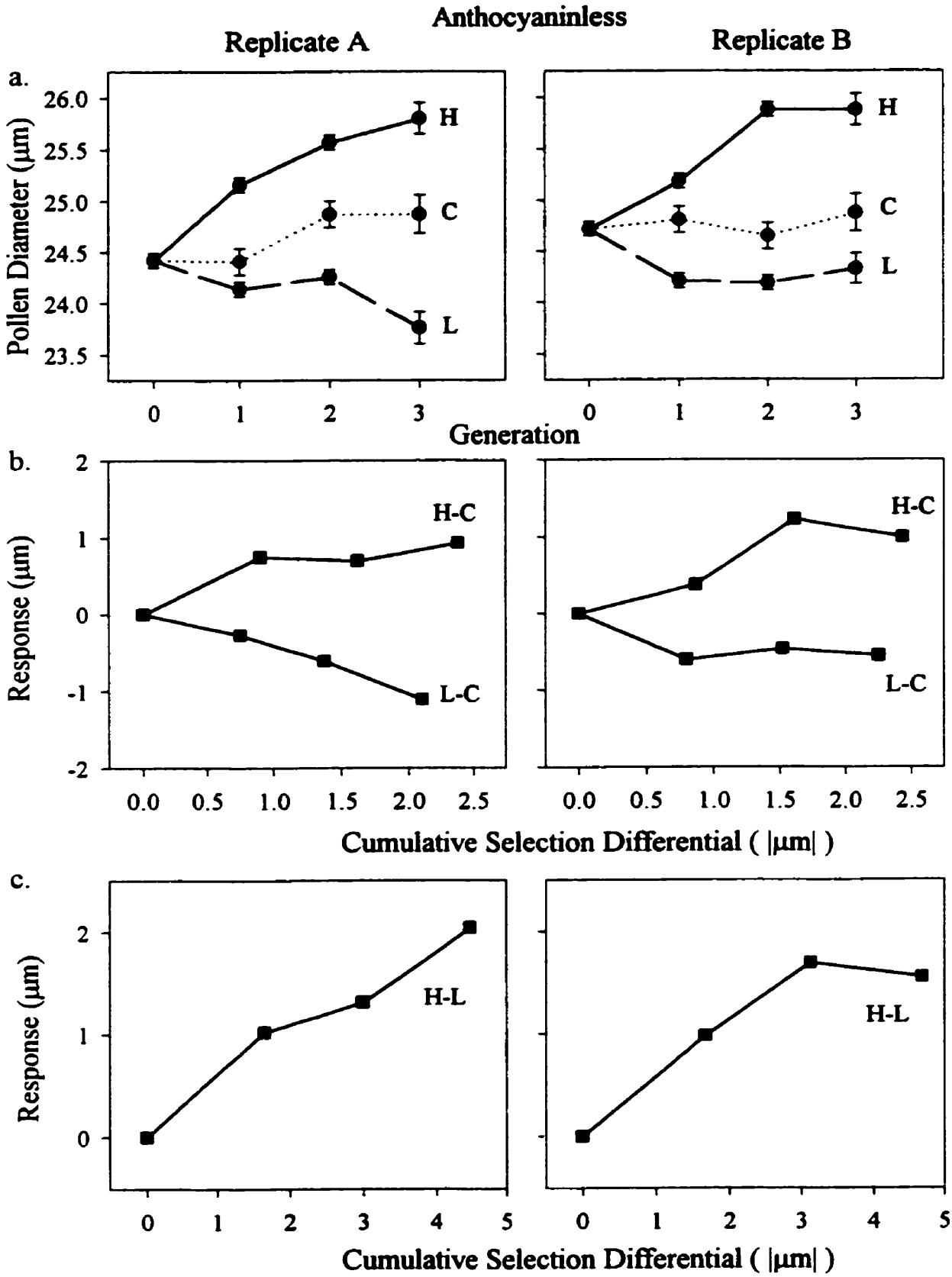

Anthocyaninless
Replicate A
Replicate B
a.
Pollen Diameter (µm)
26.0
25.5
25.0
24.5
24.0
23.5
H
C
L
0
1
2
3
Generation
b.
Response (µm)
2
1
0
-1
-2
H-C
L-C
0.0
0.5
1.0
1.5
2.0
2.5
Cumulative Selection Differential (|µm|)
c.
Response (µm)
2
1
0
H-L
0
1
2
3
4
5
Cumulative Selection Differential (|µm|)

Fig. 2.4. (a) Direct response of pollen diameter (± 95% CI) to three generations of artificial selection for wild-type plants in the high- (H ——), low- (L - - - -), and control- (C ·····) lines. Means with confidence intervals that do not overlap differ significantly. (b) Deviations of each selection line from the control-line (H-C and L-C, see equations 1a and 1b) relative to the cumulative selection differential. (c) Differences between the high and low selection lines (H-L, see equation 2) relative to the cumulative selection differential (absolute value).

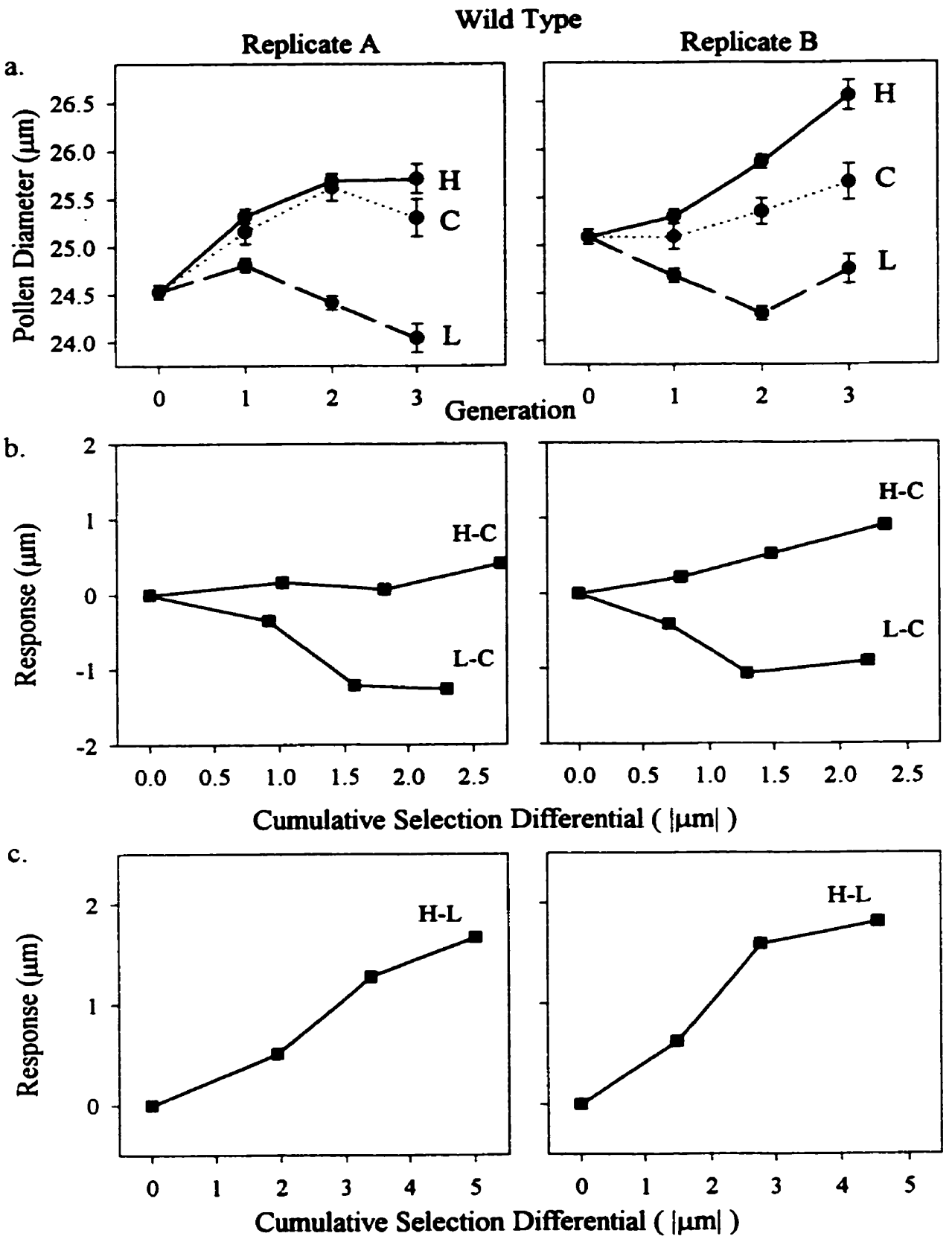

Wild Type
Replicate A
Replicate B
a.
Pollen Diameter (µm)
26.5
26.0
25.5
25.0
24.5
24.0
H
C
L
0
1
2
3
Generation
b.
Response (µm)
2
1
0
-1
-2
H-C
L-C
0.0
0.5
1.0
1.5
2.0
2.5
Cumulative Selection Differential (|µm|)
c.
Response (µm)
2
1
0
H-L
0
1
2
3
4
5
Cumulative Selection Differential (|µm|)

Table 2.2. The average response per generation (μm ±SE) to divergent selection on pollen diameter.

	Anthocyaninless		Wild Type	
	A	B	A	B
High	0.27 ± 0.11	0.39 ± 0.15	0.11 ± 0.06	0.30 ± 0.03
Low	-0.37 ± 0.04	-0.15 ± 0.11	-0.46 ± 0.10	-0.34 ± 0.12

Table 2.3. **Estimates of realized heritability (±SE) of pollen diameter from regressions of selection response on cumulative selection differential. Separate heritabilities were calculated for the high and low lines (equations 1a and 1b) as well as a single estimate based on the divergence between lines (equation 2).**

	Anthocyaninless		Wild Type	
	A	**B**	**A**	**B**
High	0.36 ± 0.06	0.48 ± 0.07	0.13 ± 0.04	0.39 ± 0.06
Low	0.53 ± 0.07	0.21 ± 0.05	0.61 ± 0.08	0.45 ± 0.07
Divergence	0.44 ± 0.06	0.35 ± 0.06	0.35 ± 0.05	0.42 ± 0.06

selection. Single estimates of heritability based on the divergence between high- and low-lines did not vary substantially among the four replicate populations (mean h^2=0.39, see Table 2.3).

2.3.2 Correlated responses to selection

Given the bidirectional experimental design, I distinguished correlated responses to selection by the floral traits from non-genetic causes of variation in two ways. First, a significant "generation×line" effect, whereby the high and low selection lines responded in opposite directions indicated a genetic association with pollen diameter. Alternatively, I interpreted a significant "line" effect as indirect evidence for a genetic correlation between pollen diameter and floral traits measured in offspring generations 1 and 2. In this case, significant and consistent differences in trait means between all replicates of the high- and low-lines implied a correlated response to selection during the first round of selection.

Selection on pollen diameter induced correlated responses in pollen number, petal length, petal width, style length and ovule number. The correlated responses for all these traits, except pollen number, indicated positive genetic associations with pollen size. Total pollen volume (Table 2.4) and filament length (Table 2.5) did not change systematically in response to selection; changes in trait means likely represent the effects of altered environmental conditions.

Pollen number changed in response to selection on mean pollen diameter, although the correlated response was not consistent between replicates of each population (see gen×line×rep effect, Table 2.4). A tradeoff between pollen size and number was evident in

Table 2.4. ANOVA evaluating the correlated responses of pollen number and volume of pollen produced (μm^3) to selection on pollen diameter.

	Anthocyaninless		Wild-Type	
Source	Pollen number	Pollen volume	Pollen number	Pollen volume
Generation	$F_{3, 2121}=25.38^{***}$	$F_{3, 2121}=24.40^{***}$	$F_{3, 2065}=28.73^{***}$	$F_{3, 2065}=23.28^{***}$
Line	$F_{2, 2121}=24.56^{***}$	$F_{2, 2121}=32.58^{***}$	$F_{2, 2065}=81.09^{***}$	$F_{2, 2065}=3.84^{*}$
Replicate	$F_{1, 2121}=15.28^{***}$	$F_{1, 2121}=29.52^{***}$	$F_{1, 2065}=6.15^{*}$	$F_{1, 2065}=22.74^{***}$
Gen×Line	$F_{4, 2121}=7.94^{***}$	$F_{4, 2121}=12.70^{***}$	$F_{4, 2065}=10.81^{***}$	$F_{4, 2065}=5.99^{***}$
Gen×Rep	$F_{3, 2121}=4.34^{*}$	$F_{3, 2121}=5.35^{*}$	$F_{3, 2065}=6.53^{**}$	$F_{3, 2065}=9.10^{***}$
Line×Rep	$F_{2, 2121}=14.00^{***}$	$F_{2, 2121}=13.11^{***}$	$F_{2, 2065}=2.97$	$F_{2, 2065}=4.88^{*}$
Gen×Line×Rep	$F_{4, 2121}=4.27^{*}$	$F_{4, 2121}=6.50^{***}$	$F_{4, 2065}=2.76^{*}$	$F_{4, 2065}=1.84$

$^{*}P<0.05$, $^{***}P<0.0001$

Table 2.5. ANOVA evaluating the correlated responses of five floral traits to selection on pollen diameter.

Source	Petal length	Petal width	Style length	Filament length	Ovule number
Anthocyaninless					
Generation	$F_{1,343}=6.90^{*}$	$F_{1,343}=3.06$	$F_{1,338}=23.71^{***}$	$F_{1,343}=4.90^{*}$	$F_{1,342}=54.81^{***}$
Line	$F_{1,343}=37.48^{***}$	$F_{1,343}=31.89^{***}$	$F_{1,338}=29.20^{***}$	$F_{1,343}=1.30$	$F_{1,342}=14.98^{***}$
Replicate	$F_{1,343}=15.95^{***}$	$F_{1,343}=8.31^{*}$	$F_{1,338}=32.97^{***}$	$F_{1,343}=25.72^{***}$	$F_{1,342}=30.92^{***}$
Gen×Line	$F_{1,343}=18.74^{***}$	$F_{1,343}=15.71^{***}$	$F_{1,338}=23.38^{***}$	$F_{1,343}=4.45^{*}$	$F_{1,342}=1.10$
Gen×Rep	$F_{1,343}=2.31$	$F_{1,343}=0.96$	$F_{1,338}=29.61^{***}$	$F_{1,343}=6.12^{*}$	$F_{1,342}=12.64^{**}$
Line×Rep	$F_{1,343}=3.48$	$F_{1,343}=28.91^{***}$	$F_{1,338}=0.97$	$F_{1,343}=9.19^{*}$	$F_{1,342}=3.49$
Gen×Line×Rep	$F_{1,343}=0.00$	$F_{1,343}=0.66$	$F_{1,338}=6.61^{*}$	$F_{1,343}=2.47$	$F_{1,342}=0.04$

...continued

Table 2.5. Continued.

Source	Petal length	Petal width	Style length	Filament length	Ovule number
Wild Type					
Generation	$F_{1,323}=5.55^{*}$	$F_{1,323}=80.29^{***}$	$F_{1,322}=0.57$	$F_{1,323}=0.21$	$F_{1,323}=41.49^{***}$
Line	$F_{1,323}=40.71^{***}$	$F_{1,323}=35.95^{***}$	$F_{1,322}=8.33^{*}$	$F_{1,323}=5.08^{*}$	$F_{1,323}=34.14^{***}$
Replicate	$F_{1,323}=33.42^{***}$	$F_{1,323}=8.29^{*}$	$F_{1,322}=17.97^{***}$	$F_{1,323}=20.65^{***}$	$F_{1,323}=3.40$
Gen×Line	$F_{1,323}=0.57$	$F_{1,323}=12.82^{**}$	$F_{1,322}=1.10$	$F_{1,323}=1.01$	$F_{1,323}=1.51$
Gen×Rep	$F_{1,323}=0.00$	$F_{1,323}=0.15$	$F_{1,322}=4.98^{*}$	$F_{1,323}=0.20$	$F_{1,323}=1.73$
Line×Rep	$F_{1,323}=35.69^{***}$	$F_{1,323}=57.69^{***}$	$F_{1,322}=0.00$	$F_{1,323}=3.32$	$F_{1,323}=2.53$
Gen×Line×Rep	$F_{1,323}=22.35^{***}$	$F_{1,323}=7.51^{*}$	$F_{1,322}=0.33$	$F_{1,323}=2.82$	$F_{1,323}=0.48$

$^{*}P<0.05$, $^{**}P<0.001$, $^{***}P<0.0001$

the anthocyaninless replicate A population and both wild-type replicates (Fig. 2.5). By offspring generation 2, low-line plants produced significantly more pollen grains than high-line plants. However, in general, pollen number did not differ significantly between the high- and control-lines. The apparent size-number tradeoff in the low-lines is consistent with the greater response to selection for smaller pollen grains in these replicates (see Table 2.2). Overall, mean pollen diameter significantly affected pollen grain number ($F_{1,35}$=10.04, P<0.01), irrespective of population ($F_{1,35}$=2.41, P>0.10) or replicate ($F_{2,35}$=1.73, P>0.10). The partial regression coefficient for ln(pollen diameter), which equaled –2.36 ±0.74 (± SE), was statistically indistinguishable from –3 (two-tailed single-sample t-test: t_{35}=0.86, P>0.05), suggesting that the pollen size-number tradeoff arose simply from the division of a fixed expenditure of resources to male gametes (see Fig. 2.6). Although pollen number diverged between the high- and low-lines in the anthocyaninless replicate A population and both wild-type replicates, total pollen volume did not (Fig. 2.7, see Table 2.4 for statistical details), affirming that artificial selection on pollen diameter did not change total resource allocation to pollen.

The consistent decrease in pollen number (and pollen volume) between the parental and first offspring generations likely resulted from a change in my perception of the end points of the frequency histogram of pollen grains displayed by the counter. Particles to the left and right of the main particle distribution represent contaminant particles which I deleted from the frequency histogram prior to analysis. In the parental generation, I was likely more conservative when deleting these contaminant particles, resulting in larger estimates of pollen number.

Fig. 2.5. Correlated responses of mean pollen number (± 95% CI) in the high selection (——), low selection (- - - -), and control (·····) lines. Means with confidence intervals that do not overlap differ significantly.

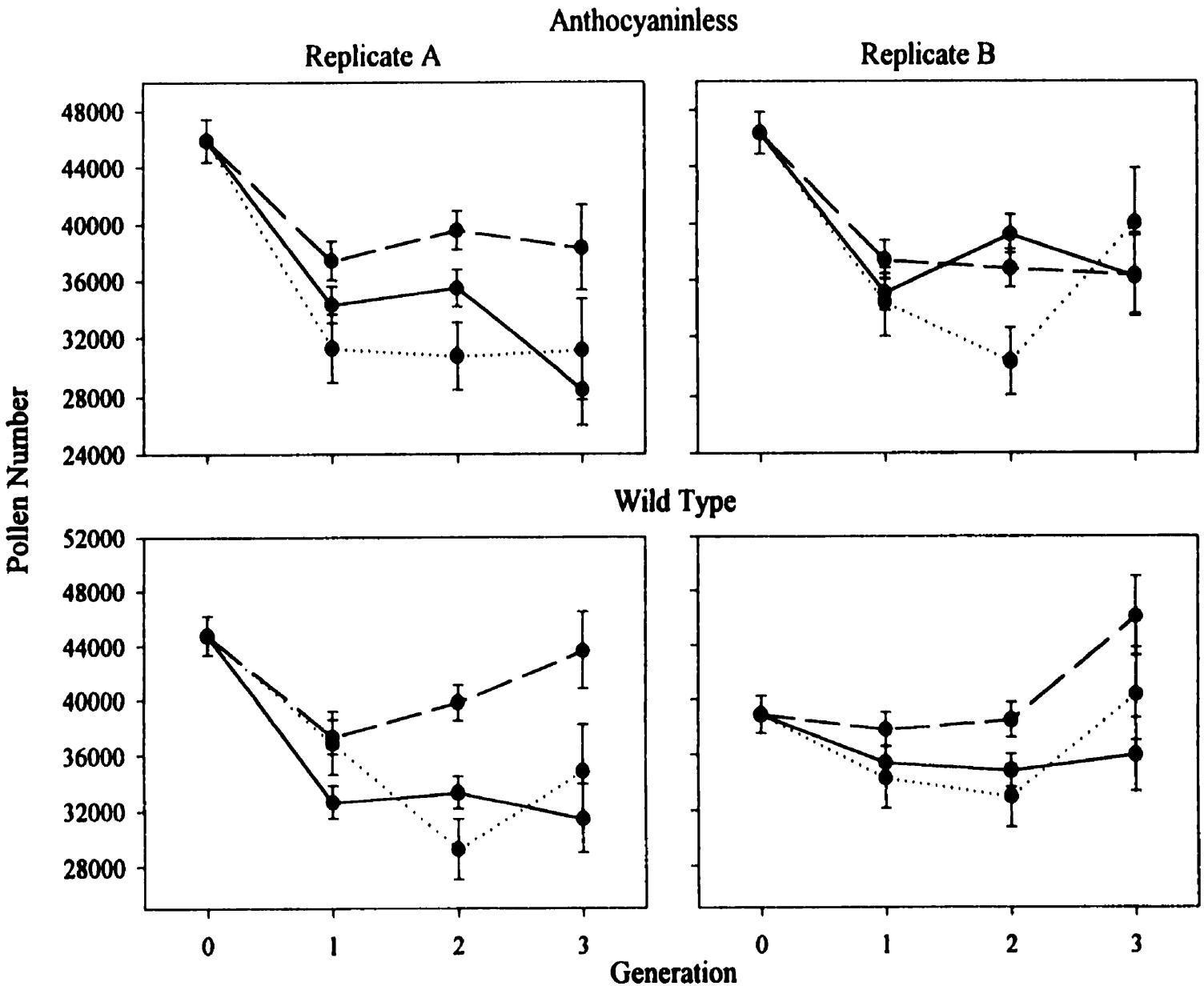

Anthocyaninless
Replicate A
Replicate B
Wild Type
Pollen Number
48000
44000
40000
36000
32000
28000
24000
52000
48000
44000
40000
36000
32000
28000
0
1
2
3
Generation

Fig. 2.6. The relation between mean pollen diameter (d) and the mean number of pollen grains per flower (n) for the 40 genetic lines in Fig. 2.1. The dashed line represents the predicted number of pollen grains based on $\ln(n) = 18.08 - 2.36 \ln(d)$. The influence of population and replicate (nested within population) have been controlled by adding the residuals from the entire general linear model for each genetic line to the predicted values for the observed pollen diameter.

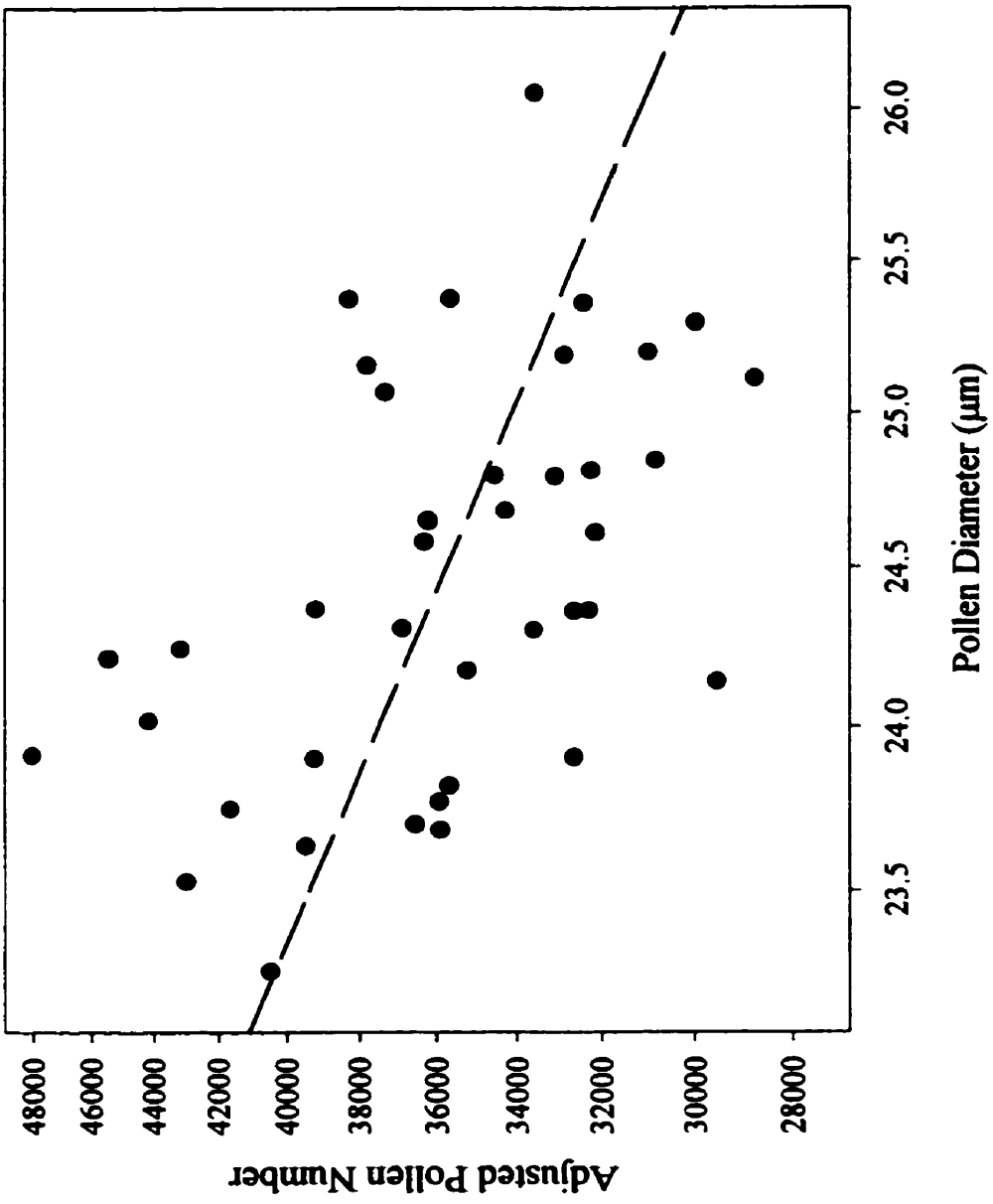
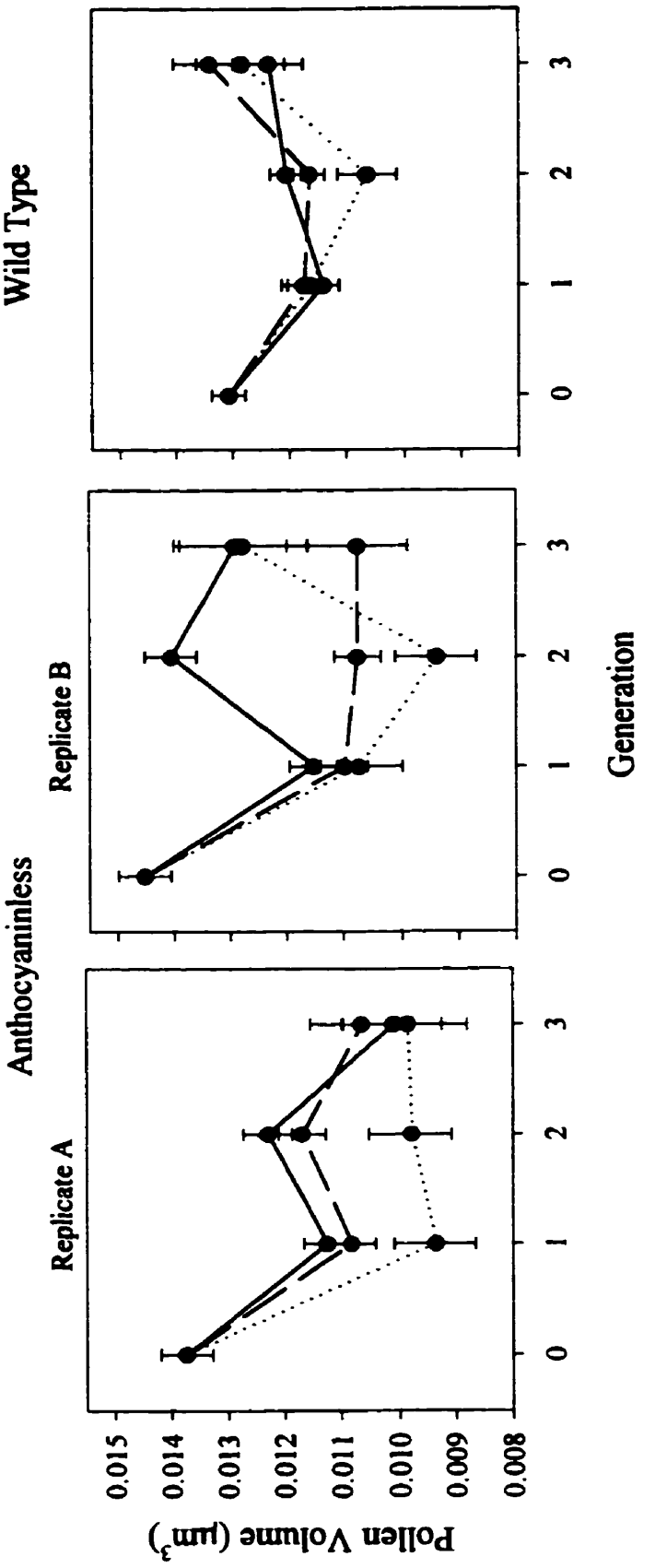

Adjusted Pollen Number
48000
46000
44000
42000
40000
38000
36000
34000
32000
30000
28000
23.5
24.0
24.5
25.0
25.5
26.0
Pollen Diameter (µm)

Fig. 2.7. Correlated responses of mean pollen volume per flower (± 95% CI) in the high selection (——), low selection (- - - -), and control (·····) lines. Means with confidence intervals that do not overlap differ significantly.

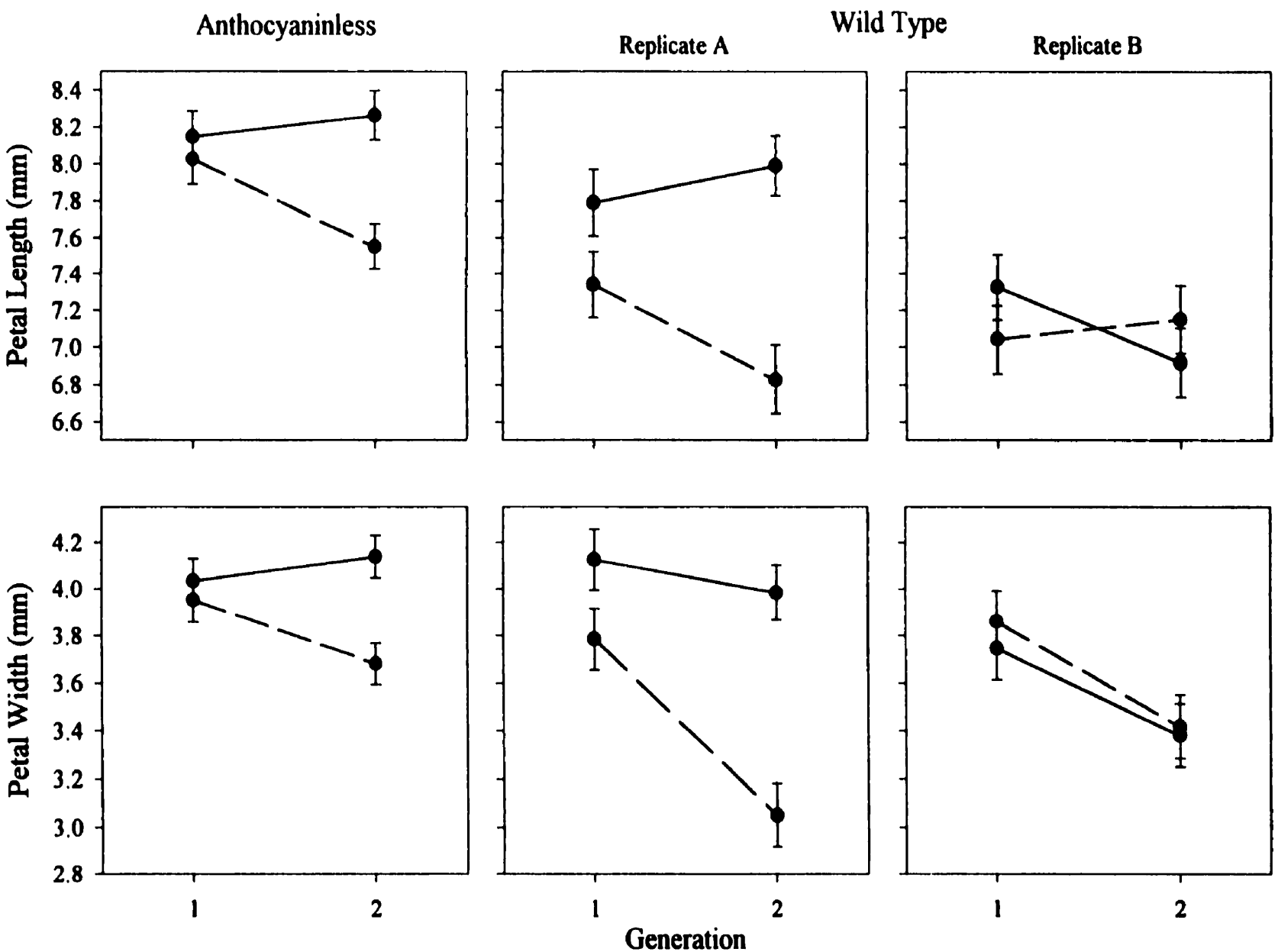

Anthocyaninless
Wild Type
Replicate A
Replicate B
Pollen Volume (μm³)
0.015
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0
1
2
3
Generation

Measurements of petal length, petal width, style length and ovule number involved only individuals in the high- and low-lines in offspring generations 1 and 2. Without control-line measures, responses must be interpreted cautiously. If these floral traits changed systematically in the control line, then changes in trait means between generations in the high- and low-lines likely resulted from changes in environmental conditions. To indicate a genetic association with pollen diameter, replicates must have responded consistently between generations. Given that plants from different replicates were not grown simultaneously, it seems unlikely that environmental factors induced consistent responses in floral traits.

Selection on pollen diameter induced correlated responses in petal length and width. In the anthocyaninless population, the replicates responded consistently (Table 2.5), with significant decreases in petal length and width in the low-lines (Fig. 2.8). By offspring generation 2, high-line plants produced significantly larger petals than low-line plants. In the wild-type population, petal length and width did not change consistently between replicates in response to selection (significant generation×line×replicate interaction, Table 2.5). However, except for petal length in wild-type replicate B, both petal measures decreased consistently in the low-lines (Fig. 2.8). That selection for small pollen diameter decreased petal length and width in 7 of the 8 low-line replicates suggests positive genetic correlations between flower size and pollen grain size.

Selection for small pollen significantly decreased average style length in both anthocyaninless low-line replicates. Even though the direction of response differed between high-line replicates (significant generation×line×replicate effect, Table 2.5), styles were

Fig. 2.8. Correlated responses of mean petal length and width (± 95% CI) in the high selection (——) and low selection (- - - -) lines. Means with confidence intervals that do not overlap differ significantly.


Anthocyaninless
Wild Type
Replicate A
Replicate B
Petal Length (mm)
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
Petal Width (mm)
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
1
2
Generation

consistently shorter in low- versus high-lines by the second offspring generation (Fig. 2.9). These results suggest a positive genetic association between style length and pollen grain size in the anthocyaninless population. Although style lengths of wild-type plants did not respond to the second generation of selection (non-significant generation×line effect, Table 2.5), the significant 'line' effect provides indirect evidence for a genetic association with pollen diameter. In both wild-type replicates, high-line plants produced significantly longer styles by the second offspring generation (Fig. 2.9), suggesting a correlated response to selection during the first round of selection. Results for both wild-type and anthocyaninless plants suggest a positive genetic association between pollen size and style length.

Ovule number also responded indirectly to selection on pollen diameter (significant 'line' effect, Table 2.5). By offspring generation 2, high-line plants produced significantly more ovules than low-line plants in both populations (Fig. 2.10). This consistent difference among all replicates indicates a positive correlated response in ovule number during the first generation of selection.

2.4 Discussion

Genetic variation in pollen diameter allowed significant divergence between the high and low selection lines. In general, additive genetic effects accounted for over 30% of the observed phenotypic variation in pollen grain size in *B. rapa*. Such heritable genetic variation has important implications for gametophytic selection in natural populations, specifically the potential for pollen-size evolution. If pollen size affects mating success, then natural selection can effect evolutionary change favoring pollen sizes that promote

Fig. 2.9. Correlated changes of mean style length (± 95% CI) in the anthocyaninless high (——) and low (- - - -) selection lines, and mean style length for wild-type high- and low-line plants.

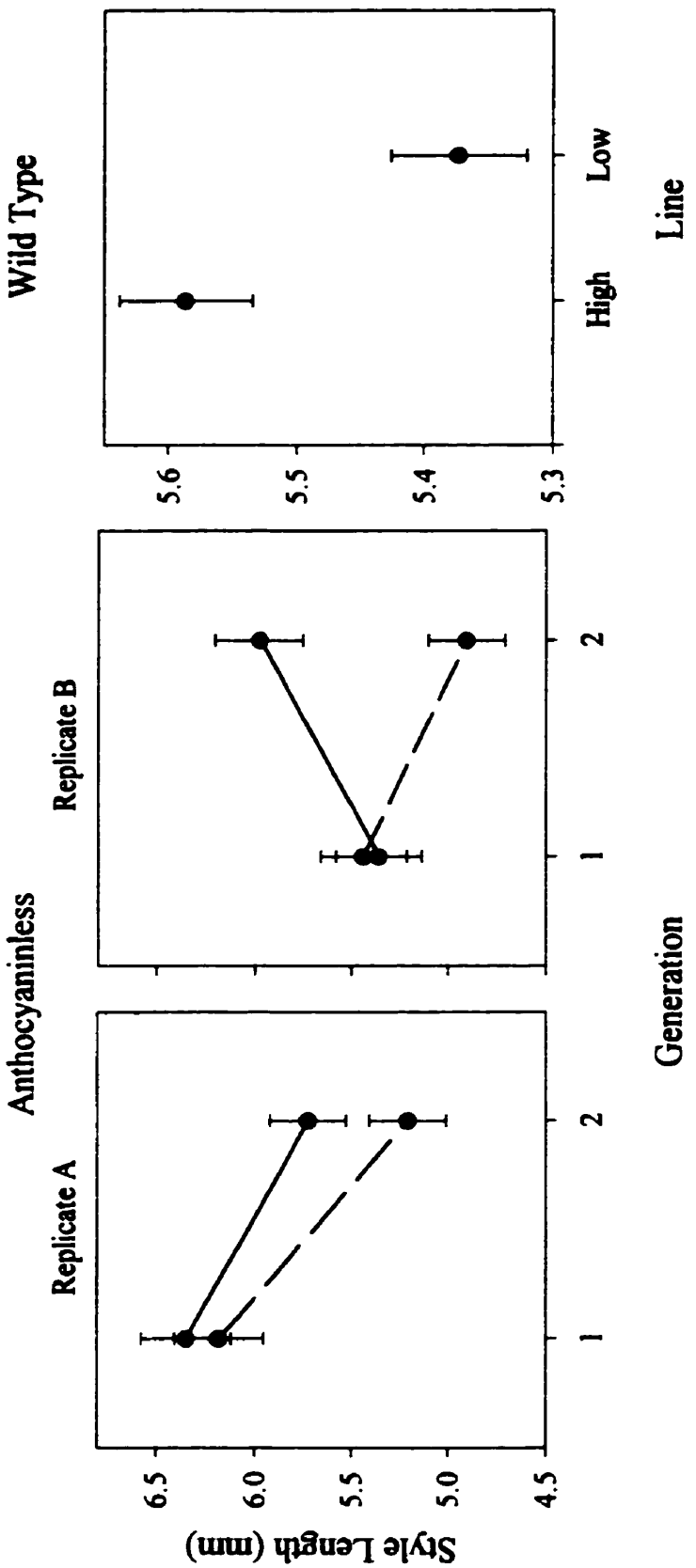
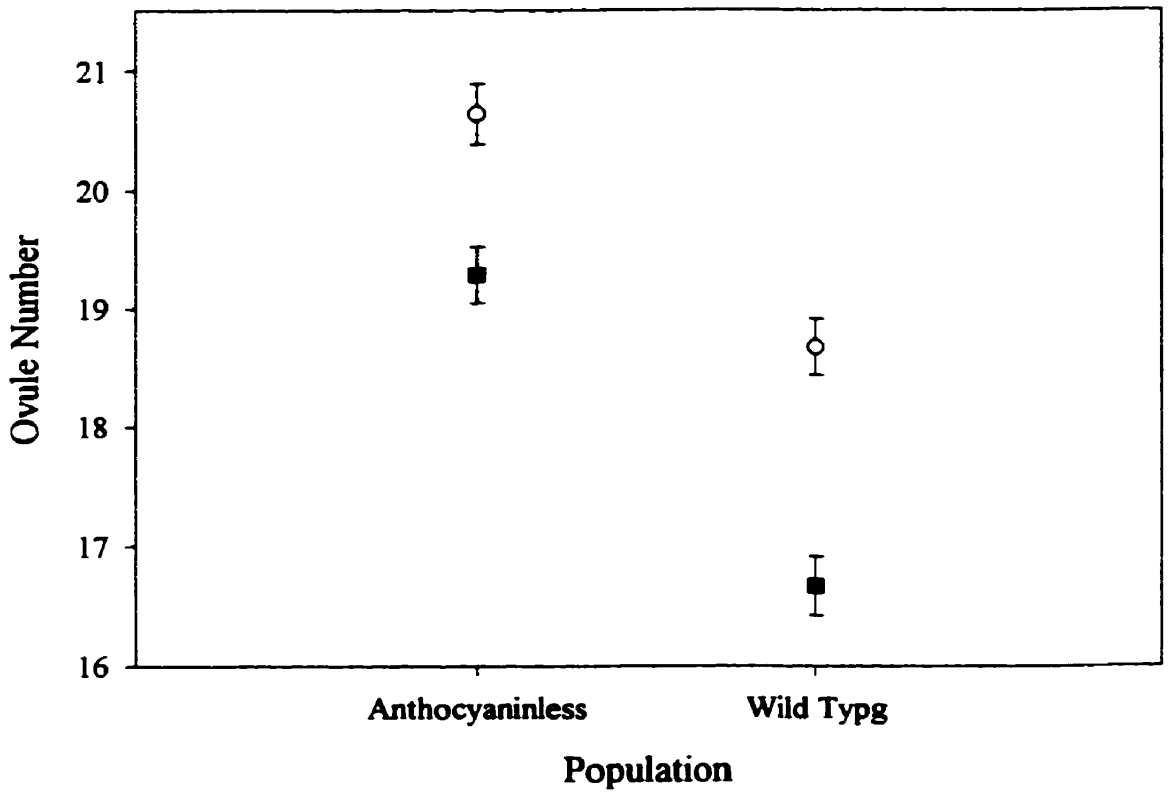

Anthocyaninless
Replicate A
Replicate B
Wild Type
Style Length (mm)
6.5
6.0
5.5
5.0
4.5
1
2
Generation
5.6
5.5
5.4
5.3
High
Low
Line

Fig. 2.10. **Mean ovule number (± 95% CI) in the anthocyaninless and wild-type high- (O) and low-line (■) plants.**


21
20
19
18
17
16
Ovule Number
Anthocyaninless
Wild Typg
Population

pollination and/or fertilization. However, if pollen size associates closely with fitness, then the maintenance of significant additive genetic variation is surprising. Although mutation-selection balance in large populations constitutes an important source of genetic variation (Lande, 1976a), the magnitude of the variance maintained by mutation is unlikely to be great (Charlesworth, 1987).

Genetic variation in pollen diameter may persist because selection varies spatially and/or temporally (Haldane and Jayakar, 1963). Changes in pollinator type and abundance (see Galen, 1989; Schemske and Horvitz, 1989; O'Neil and Schmitt, 1993; Robertson, Diaz and Macnair, 1994) and/or changes in population structure may mediate changes in the intensity and/or direction of selection (but see Harder, 1998). For example, suppose plants in a sparse population attract few pollinators and suffer insufficient pollination, whereas plants in a dense population receive large pollen loads from multiple donors. The first environment places a premium on more (smaller) pollen grains, whereas the second environment may favor larger (fewer) grains, if pollen size confers an advantage during post-pollination processes. If the optimal pollen size varies with these different pollination conditions, then the direction and magnitude of selection will differ. With extensive pollen and/or seed dispersal, both populations would maintain genetic variation in pollen size.

Despite significant heritable variation for pollen diameter, the correlated responses by other floral characters suggest that pollen size cannot evolve independently. Genetic correlations between traits may be due to linkage disequilibrium, pleiotropy, or a combination of these factors (Falconer, 1989). The former mechanism causes transient associations between traits and the correlation structure should vary among populations.

Strong generation×line×replicate interactions (see Tables 2.4 and 2.5) signify linkage disequilibrium as the likely proximate cause of the observed genetic correlations. Correlations caused by pleiotropy are of greater evolutionary consequence as they persist and so can greatly alter the rate and direction of evolution. In addition, pleiotropic correlations should be similar among different populations. Regardless of the genetic mechanism, genetic correlations between floral traits may be maintained by selection as adaptive trait combinations if they result in effective reproductive phenotypes (Stanton and Young, 1994).

The only negative genetic correlation detected in this study involved pollen size and number, which has also been found in other studies (Mazer and Hultgård, 1993; Stanton and Young, 1994; but see Fenster and Carr, 1997). Given that total allocation to pollen did not change, the inverse relation arose simply from the division of a fixed expenditure of resources among pollen grains within individual flowers. 'More, smaller' and 'fewer, larger' are alternative partitions of the same total investment into male gametophytes. The optimal compromise struck between these alternatives should vary depending on the prevailing pollination and fertilization environment (Harder, 1998).

The remaining genetic correlations between pollen grain size and flower size, style length, and ovule number were positive. Such associations support current models of the genetic control of flower development, with individual genes influencing the phenotypic expression of many floral organs (reviewed in Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). For example, in *Arabidopsis thaliana* and *Antirrhinum majus*, genes that direct stamen development also influence petal and carpel development. In these two

distantly related species, genes controlling floral phenotype are homologous at the DNA level, suggesting that the basic processes of floral development are evolutionarily old. Therefore, positive genetic associations among floral traits should be common (also see Hill and Lord, 1989 and references therein).

The positive genetic correlation between pollen size and flower size in *B. rapa* (as in *Mimulus guttatus*, Fenster and Carr, 1997) may govern intrapopulation gender specialization among hermaphroditic plants (see Bawa and Webb, 1983; Devlin and Stephenson, 1987; Thomson, 1989) when flower size predominantly influences male function (e.g., Campbell, 1989; Galen, 1989). For example, in *Raphanus sativus*, flowers with large petals receive more pollinator visits, and rates of pollen removal increase with visitation, whereas individuals with larger flowers do not produce more seeds (Young and Stanton, 1990b; Stanton et al., 1991). Therefore, selection should favor concordant investment in pollinator attraction (e.g. petals) and pollen (see Stanton and Galloway, 1990) because increasing investment in petals without increasing pollen investment is likely to be wasteful (Stanton and Young, 1994). Hence, natural selection may maintain the positive genetic correlation between pollen size and flower size in animal-pollinated species as an effective sex allocation strategy.

The positive genetic correlation between pollen size and style length in *B. rapa* provides a genetic explanation for the corresponding interspecific pattern found in a wide variety of angiosperms (Lee, 1978; Baker and Baker, 1983; Plitmann and Levin, 1983; Elisens, 1986; Williams and Rouse, 1990; Kirk, 1993; Olivencia et al., 1997; Harder, 1998). Positive assortative mating within species may maintain this correlation. If plants producing

large pollen have relatively greater siring success when competing in long styles, then genes for large pollen will become associated with genes for long styles. This association will foster a positive genetic correlation between the traits through linkage disequilibrium. In contrast, with disassortative mating, the correlation between pollen size and style length becomes negative. For example, in distylous species, short-styled flowers typically produce larger pollen than long-styled flowers because 'legitimate' fertilization of long-styled flowers requires longer pollen tubes (Darwin, 1884; but see Ganders, 1979). Therefore, mating patterns may maintain associations between pollen size and pistil characteristics in the same individual. This provides the opportunity for runaway sexual selection which involves genetic coupling between a trait and preference for that trait (Fisher, 1958; O'Donald, 1980; Lande, 1981; Kirkpatrick, 1982). If long styles signify maternal preference for large pollen, then females with long styles will produce offspring with large pollen, setting up a genetic covariance. Therefore, selection for large pollen causes indirect selection for long styles because the genes encoding them are in linkage disequilibrium.

The genetic association between pollen size and ovule number is particularly interesting in the context of sex allocation. Sex allocation theory proposes that given fixed reproductive resources, male and female investments by hermaphrodites should vary inversely (Charlesworth and Charlesworth, 1981; Charnov, 1982). Plants that invest less in pollen (for e.g., via size while holding number constant) should have more resources available for ovule production, and vice versa. As expected, pollen size and ovule number exhibited a negative genetic correlation in *Primula scotica* (Mazer and Hultgård, 1993). In contrast, in *B. rapa*, these traits correlated positively. However, resource expenditure on

pollen did not change, so that plants with large pollen produced fewer grains (see Fig. 2.7). Similarly, if ovule number varies inversely with ovule size, then size-number responses in pollen and ovules should preclude a trade-off between sexual functions. In addition, measurements of pollen and ovule allocation by individual flowers provide little information on the trade-off between sexual functions for entire plants (Mazer, 1992).

The extensive variation in pollen size among species (Wodehouse, 1935; Muller, 1979) and limited variation within species (e.g. Vonhof and Harder, 1995) suggests strong selection on pollen size. The functional advantage of pollen size presumably depends on each species' particular pollination and fertilization conditions (Harder, 1998). Although strong selection generally diminishes genetic variation, artificial disruptive selection on pollen size in lab-grown populations of *B. rapa* caused divergence between lines. This response suggests that natural selection on pollen grain size in wild populations could effect evolutionary change in this trait; however, the relevance of heritability measures from laboratory populations for natural populations remains unclear. The range of genotypes observed in the rapid-cycling stock of *B. rapa* may underestimate the range of genetic variation available in natural populations, with higher additive genetic variation in the field. Alternatively, growth of plants under controlled laboratory conditions likely reduced environmental variation, making additive genetic variation easier to detect. In fact, the four parental populations had substantially lower coefficients of variation for pollen diameter (anthocyaninless - replicate A: 1.95%, replicate B: 1.99%; wild-type - replicate A: 2.41%, replicate B: 1.79%) than wild *Raphanus sativus* populations at three densities (high: 8.54%, medium: 9.80%; low: 10.49%; Mazer, 1992) and many papilionaceous legumes (Vonhof

and Harder, 1995). For these reasons, Mitchell-Olds and Rutledge (1986) emphasized the need to measure heritability and genetic correlations under natural conditions. Nonetheless, the contrasting pattern of pollen-size variation among and within species suggests that evolution has effectively caused changes in pollen size in the past. However, pollen size could resist rapid evolutionary modification in *B. rapa* if the observed genetic associations reflect strong pleiotropy and the fitness consequences of the correlated traits constrain their independent evolution.

3 THE INFLUENCE OF POLLEN SIZE ON POST-POLLINATION COMPETITIVE ABILITY

3.1 Introduction

Male reproductive success depends on a plant's ability to disperse pollen and fertilize ovules. Floral characters that increase successful transfer of pollen to conspecific stigmas may therefore enhance male fitness and be selected through male function. Not surprisingly, characteristics of floral displays that influence pollen export have been the focus of detailed study (e.g., Willson and Bertin, 1979; Bell, 1985; Stanton, Snow and Handel, 1986; Cruzan, Neal and Willson, 1988; Thomson, 1988; Galen and Stanton, 1989; Harder and Thomson, 1989; Young and Stanton, 1990b). However, delivery of pollen to stigmas does not ensure male reproductive success. Pollen grains that reach a conspecific stigma must germinate and produce pollen tubes that penetrate the style to the ovary to release their sperm into an embryo sac. Pollen grains that germinate first and/or have faster growing pollen tubes should fertilize more ovules, and thereby transmit more genes to the next generation. Hence, given the incipient mating pattern established by pollen dispersal, post-pollination processes ultimately determine male fitness.

Competition for ovules among pollen donors occurs when stigmas receive large, mixed-donor pollen loads (reviewed in Snow, 1994). Under these conditions, only donors with the best-competing pollen phenotypes will succeed in the race for fertilization of limited ovules. Willson and Burley (1983) argued that plants should invest minimally per pollen grain when pollen typically compete little, but pollen should be investment rich when competition is high. Differences in relative investment in pollen by donors may vary with some morphological characters of pollen.

Pollen size may represent relative investment in pollen and in turn influence competitive and fertilization abilities. Pollen accumulates diverse products during development, including RNAs, proteins, enzymes, ribosomes, starch, lipids, and phosphorus storage compounds which may affect germination, pollen-tube growth and fertilization ability (Cruzan, 1990; Lau and Stephenson, 1993, 1994; Aizen and Raffaele, 1996). In addition, interspecific studies show that pollen-tube growth rate varies positively with pollen size (van Breukelen, 1982; Perez and Moore, 1985; Gore et al., 1990; Williams and Rouse, 1990). Intraspecific studies relating pollen size to rates of tube elongation have uncovered the same relation, but have generally been restricted to heterostylous (Glover and Barrett, 1983; Anderson and Barrett, 1986; Manicacci and Barrett, 1995) and cleistogamous species (Lord and Eckard, 1984), in which different floral morphs differ in pollen size. However, these studies have little relevance to male mating success in natural populations because pollen grains from different morphs do not compete for fertilization because of incompatibility (heterostyly) and lack of opportunity (cleistogamy). Whether intraspecific pollen size variation influences the relative competitive ability of males in species with monomorphic flowers remains largely unknown.

The influence of pollen size on male reproductive success has been addressed experimentally only twice. Lau and Stephenson (1994) found that *Cucurbita pepo* plants grown in high phosphorus soils produced significantly larger pollen grains with higher phosphate concentration that sired significantly more seeds than pollen produced by plants grown in low phosphorus soils (see also Lau and Stephenson, 1993). A second study by Cruzan (1990) evaluated the effects of pollen size on male reproductive success of *Erythronium grandiflorum*. In this species, the second flowers of two-flowered plants

produce smaller pollen than first flowers. In vivo, pollen from the two flower positions germinated with equal frequency and their pollen tubes grew at similar rates, suggesting that differences in the internal resource content of pollen was not associated with the size of pollen and, hence, did not reflect pollen competitive ability. However, flowers on an *E. grandiflorum* inflorescence open sequentially, with approximately 24 h between anthesis of each flower (and hence dehiscence of anthers). To accommodate this time lag, Cruzan refrigerated pollen from first flowers until second flowers opened. Hence, any effects of pollen size on competitive ability could have been compromised by differences in pollen age and/or storage. Hence, Cruzan's failure to find siring differences between pollen from first and second flowers need not indicate that pollen size does not alter gametophytic competition. Furthermore, Cruzan's (1990) and Lau and Stephenson's (1993, 1994) experiments did not assess the consequences of genetic differences in pollen size, which are most relevant in the context of intrasexual selection.

In this chapter, I test the prediction that large pollen sires more seeds than small pollen in a competitive environment, using pollen from plants that differ genetically in their pollen sizes. With this experiment, I will ascertain the importance of post-pollination processes in the evolution of pollen size.

3.2 Methods

3.2.1 Study species and experimental design

I tested the influence of pollen size on post-fertilization siring ability in a laboratory experiment using *Brassica rapa* L. (syn. *campestris*, Brassicaceae). *Brassica rapa* is a self-incompatible annual or biennial mustard native to Eurasia and now widely naturalized in

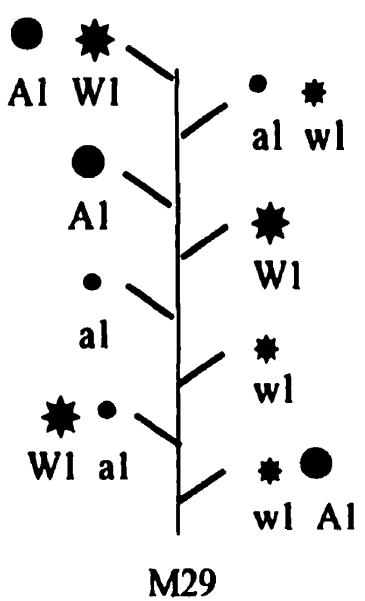
North America. In this study, I used genetic lines of *B. rapa* from two populations (wild-type and anthocyaninless) developed by three generations of divergent selection on pollen size (see Chapter 2). Wild-type plants produce anthocyanin which colors the base of stems and leaf petioles purple; anthocyaninless plants lack anthocyanin pigment, resulting in completely green stems and leaves. Expression of anthocyanin is controlled by two alleles at a single locus (e.g, *A*, *a*), with *A* dominant to *a*. Wild-type plants used in the study were homozygous dominant (*AA*); anthocyaninless plants were homozygous recessive (*aa*). When individual flowers on anthocyaninless plants (*aa*) receive pollen from two homozygous pollen donors (*AA* and *aa*), the progeny of wild-type donors typically produce anthocyanin (*Aa*), whereas those from anthocyaninless donors do not (*aa*). I used the Mendelian inheritance of anthocyanin production as a genetic marker to test the influence of pollen size on siring success. Plants were grown and maintained as described in Chapter 2.

I used anthocyaninless individuals as pollen recipients and wild-type and anthocyaninless plants as pollen donors in experimental hand pollinations. Ninety maternal plants were chosen from plants with no known history of direct selection on pollen size (control-line offspring generations 2 and 3, see Figs. 2.1 and 3.1). Pollen donors were chosen from 50 plants from each of four genetic lines that had experienced three generations of artificial selection on pollen diameter: wild-type large, wild-type small, anthocyaninless large, and anthocyaninless small (Fig. 3.1). From plants in the pool of potential pollen donors, I collected the four long anthers from the 3rd or 4th flower and measured pollen diameter with a Particle Data, Elzone 180XY electronic particle analyzer (see Chapter 2). I chose the 10 (or 11) individuals with the largest or smallest grain

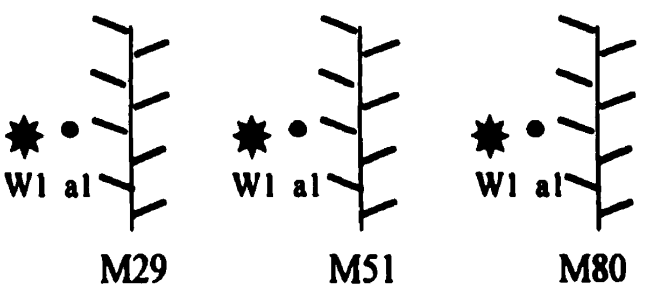
Fig. 3.1. **Example of the design for testing the effects of pollen-size on paternity in *Brassica rapa*. Pollen donors and maternal plants were chosen from genetic lines generated by divergent selection on pollen size (e.g. AB3H: anthocyaninless, replicate B, offspring generation 3, high line; see Fig. 2.1). Each maternal plant received 8 pollination treatments, replicated on two levels (I and II).**

	Population	Genotype	Pollen Size	Genetic Line	Code
Pollen donors	Anthocyaninless	*aa*	large ●	AA3H, AB3H	A1-A10
			small •	AA3L, AB3L	a1-a10
	Wild Type	*AA*	large ✸	WA3H, WB3H	W1-W10
			small ✸	WA3L, WB3L	w1-w10
Maternal plants	Anthocyaninless	*aa*	control	AA2C, AA3C AB2C, AB3C	M1-M90

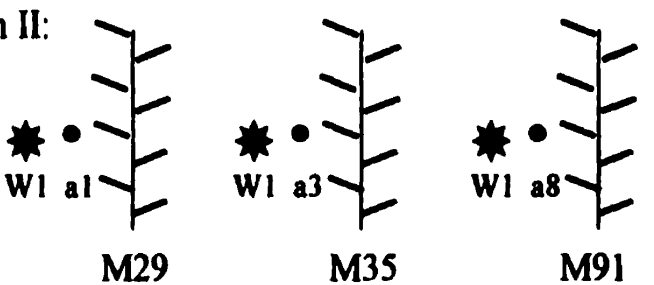
Pollination treatments:



Replication I:



Replication II:



diameters, depending on the pollen-size class, as the pollen donors for the experiment [hereafter referred to as W1-W10 (wild-type large), w1-w10 (wild-type small), A1-A11 (anthocyaninless large), and a1-a11 (anthocyaninless small)].

Each maternal plant received eight pollinations involving four pollen donors (Fig. 3.1) assigned randomly along the inflorescence. To test the competitive ability of large versus small pollen, I pollinated two flowers on each maternal plant with the following mixtures: i) equal numbers of large wild-type and small anthocyaninless pollen (*W/a*), and ii) equal numbers of small wild-type and large anthocyaninless pollen (*w/A*). To confirm that wild and anthocyaninless pollen of the same size class did not differ in competitive ability, I also pollinated two additional flowers with equal numbers of iii) large wild-type and large anthocyaninless pollen (*W/A*); and iv) small wild-type and small anthocyaninless pollen (*w/a*) using the same donors involved in the size-competition crosses. Finally, I assessed the fertilization potential of each pollen type in the absence of between-donor pollen competition with four single-donor crosses (*W*, *w*, *A*, *a*). These pollinations were replicated on two levels. First, pollen mixtures from each pair of donors (e.g. W1/a1) were applied to stigmas on three maternal plants (see Replication I, Fig. 3.1). Second, each large-pollen donor (e.g. W1) competed against different small-pollen donors from the other population (e.g. a1, a3, and a8) on three maternal plants, and vice versa (see Replication II, Fig. 3.1).

For all pollinations, I collected dehisced long-level anthers from donors and placed them in microtubes where the pollen was mixed using 60 lb. monofilament fishing line. The pollen-covered fishing line was then brushed across the stigma, covering it densely with pollen to ensure competition among pollen tubes for ovules. Before preparing the

two-donor pollen mixtures (*W/a*, *w/A*, *W/A*, and *w/a*), I counted pollen production per anther with the automated particle analyzer. I then adjusted the relative number of anthers collected from each plant to equalize the number of pollen grains from each individual. Between pollinations, I cut the tip of the fishing line, rinsed it in water and wiped it dry to avoid pollen contamination.

3.2.2 Seed collection and paternity assignments

I collected 550 pods from the maternal plants 25 days after the last pollination (of the 720 pollinations performed, 170 failed to develop seed). While harvesting seeds, I recorded the fate of all the ovules in each side of a pod under a dissecting microscope as either i) unfertilized, ii) fertilized but subsequently aborted, or iii) fertilized and developed into a mature seed. Following seed collection, I germinated seeds on petri plates with moistened filter paper and used the presence or absence of anthocyanin in seedlings to determine paternity.

I assessed the competitive ability of pollen from a given donor in terms of the proportion of seeds sired. Differences in relative siring success by different donors may partly reflect dissimilar pollen performance (i.e. speed of germination and/or pollen tube growth rate); however, post-fertilization processes such as differential seed abortion (e.g., Casper, 1988; Marshall, 1988) can also affect siring success. To differentiate between these two mechanisms, I noted the position of unfertilized ovules, aborted ovules and seeds within the pod relative to the base of the style. Preliminary studies had suggested that ovules located at the base of the style are fertilized first, whereas the ovules at the peduncular end of the ovary are fertilized last by the slowest growing pollen tubes. I

therefore predicted that large pollen grains in *W/a* and *w/A* crosses would fertilize relatively more ovules near the stylar end of the ovary if relative siring success primarily reflects differences in pollen performance. If relative siring success does not vary with seed position, then post-fertilization processes likely influence paternity of large- and small-pollen donors.

Germination of seeds from wild-type, single-donor crosses revealed a broad range of anthocyanin expression, with some wild-type progeny resembling anthocyaninless plants. Absence of anthocyanin resulted from particular maternal plant × wild-type donor combinations. Therefore, to avoid overestimation of paternity by anthocyaninless donors in two-donor crosses, I restricted analyses of seed siring to maternal plants for which the single-donor pollination by the wild-type donor resulted in anthocyanin expression in ≥80% of seedlings. This condition reduced sample sizes for seed-siring data involving two-donors (*W/a*: n=30; *w/A*: n=24; *W/A*: n=27; *w/a*: n=29), with concurrent loss of effective replication across maternal plants.

3.2.3 Data analysis

To confirm that putative large- and small-pollen donors produced pollen of different size, I compared mean pollen diameter among the four genetic lines (wild-type large, wild-type small, anthocyaninless large, and anthocyaninless small) by two-factor ANOVA. The analysis considered population (wild-type or anthocyaninless), pollen-size class (large or small) and their interaction as the independent variables using untransformed data.

Analysis of the outcome of the experimental pollinations considered three binomial dependent variables: whether an ovule was fertilized (fertilization frequency), whether a fertilized ovule failed to develop (abortion frequency), and whether an ovule developed into a seed (seedset). Given the binomial nature of these variables, I analyzed their variation with generalized linear models (McCullagh and Nelder, 1989) that incorporated a logit link function ($\ln [p / (1 - p)]$, where p is the probability of a 'success') and a binomial error distribution (GENMOD procedure of SAS, release 7.0, SAS Institute Inc., 1998).

I divided the pollination treatments into three groups: single-donor pollinations (*W*, *w*, *A*, *a*), same-size competitive crosses (*W/A*, *w/a*), and different-size competitive crosses (*W/a*, *w/A*), each of which I analyzed separately. The analyses for single-donor treatments considered population (wild-type or anthocyaninless), pollen size (large or small), and their interaction as independent effects (n=82 maternal plants, maximum of 4 pods/maternal plant). For the same-size competitive crosses, I tested for differences between hand pollinations involving large or small pollen ['cross size', large (*W/A*) or small (*w/a*): n=73 maternal plants, maximum of 2 pods/maternal plant]. The analyses for the competitive crosses involving different-sized pollen considered the morph of the large pollen donor ['morph': wild-type (*W/a*) or anthocyaninless (*w/A*)] as the independent variable (n=73 maternal plants, maximum of 2 pods/maternal plant). All these analyses also included ovule position from the stylar end of the ovary as a covariate (only ovule positions 1-11 were considered due to the paucity of data for ovules farther from the style). I also tested for possible interactions between the covariate and main effects and removed these interactions when they were not statistically significant (α=0.05). Finally, the

analyses recognized that pods on individual maternal plants involve repeated measurements of the same subject. To account for the lack of independence that can accompany such repeated measures, the GENMOD procedure characterized the covariance structure of the dependent variables within plants by restricted maximum likelihood.

Fertilization frequency, abortion frequency and seedset did not vary significantly with any of the main effects in the analyses of single-donor, same-size competitive, and different-size competitive crosses (see Table 3.1). I therefore pooled the data from all crosses for an overall analysis which tested the effect of 'cross type' (single-donor, same-size competitive, and different-size competitive) on fertilization, seedset, and abortion (n=87 maternal plants, maximum of 8 pods/maternal plant). [Hereafter, 'two-donor crosses' refers to both same-size competitive crosses (*W/A*, *w/a*), and different-size competitive crosses (*W/a*, *w/A*)].

To test whether wild-type and anthocyaninless pollen of the same size class compete equally, I analyzed the proportion of wild-type seeds in pods pollinated with *W/A* and *w/a* pollen mixtures in a generalized linear model with 'cross size' [large (*W/A*) or small (*w/a*)] as the main effect, ovule position as a covariate, and their interaction. This analysis considered only seeds occupying the first eight ovule positions in the ovary as very few seeds were harvested from more distant positions.

To determine whether pollen size influences post-pollination siring ability, I considered the proportion of seeds sired by pollen from large versus small donors in different-size competitive crosses (i.e. proportion of seeds sired by wild-type and anthocyaninless donors in *W/a* and *w/A* treatments, respectively). This analysis considered the morph of the large donor [wild-type (*W/a*) or anthocyaninless (*w/A*)], ovule position

Table 3.1. Generalized linear models of the proportions of ovules fertilized, fertilized ovules aborted, and ovules that set seed in single- and two-donor crosses. For crosses involving donors with same-sized pollen, 'cross size' refers to whether the treatment involved large (*W/A*) or small (*w/a*) pollen. For pollinations involving donors of different size, 'morph' refers to the population of the large donor [wild-type (*W/a*) or anthocyaninless (*w/A*)]. For instances in which ovule position significantly affected a dependent variable, the partial regression coefficient (±SE) is provided in parentheses below the corresponding likelihood ratio (*G*). Interactions between the covariate and main effects were non-significant and were removed from the models.

Source	Fertilization	Abortion	Seedset
Single-donor			
Population	G_1=0.02	G_1=1.38	G_1=0.39
Size class	G_1=1.98	G_1=1.32	G_1=2.59
Population×Size class	G_1=0.06	G_1=1.56	G_1=0.24
Ovule position	G_1=38.27***	G_1=0.02	G_1=32.47***
	(-0.22 ±0.02)		(-0.16 ±0.02)
Same-size competitive			
Cross size	G_1=0.52	G_1=0.09	G_1=0.16
Ovule position	G_1=33.98***	G_1=0.50	G_1=26.65***
	(-0.24 ±0.03)		(-0.16 ±0.03)
Different-size competitive			
Morph	G_1=1.49	G_1=0.00	G_1=0.94
Ovule position	G_1=39.24***	G_1=2.49	G_1=26.64***
	(-0.26 ±0.03)		(-0.16 ±0.02)

$^{***}P<0.0001$

(1-8) as a covariate, and their interaction. I log-transformed ovule position prior to the analysis to straighten the relation between the logit of the dependent variable and ovule position.

3.3 Results

Mean pollen grain diameter differed significantly between wild-type and anthocyaninless large- and small-pollen donors (population: $F_{1,38}$=13.96, P<0.001; pollen size: $F_{1,38}$=1530.05, P<0.001; pop.×size: $F_{1,38}$=5.63, P<0.05). Pollen donors from large-pollen lines produced significantly larger pollen (range: 26.54-27.51μm) than those from small-pollen lines (range: 23.28-24.42μm). Wild-type large and anthocyaninless large lines produced pollen of equivalent size, whereas plants from the wild-type small line produced significantly larger pollen than the anthocyaninless small pollen line (Fig. 3.2).

Fertilization frequency, abortion frequency and seedset did not vary significantly with any of the main effects in the analyses of single-donor, same-size competitive, and different-size competitive crosses (Table 3.1). In non-competitive situations, donors with small pollen fertilized and sired as many seeds as donors with large pollen. Neither the size class of pollen in same-sized competitive crosses [large (*W/A*) or small (*w/a*)], nor the morph of the large donor in *W/a* or *w/A* pollen mixtures influenced the outcome of pollinations (Table 3.1).

Single- and two-donor crosses did not differ significantly in fertilization frequency, abortion frequency or seedset (Table 3.2). However, fertilization and seedset decreased significantly with ovule position. The proportion of ovules fertilized decreased from 0.76

Fig. 3.2. Mean pollen diameter (±95% CI) for anthocyaninless large (●), anthocyaninless small (•), wild-type large (■), and wild-type small (▪) pollen donors. Means with confidence intervals that do not overlap are significantly different.

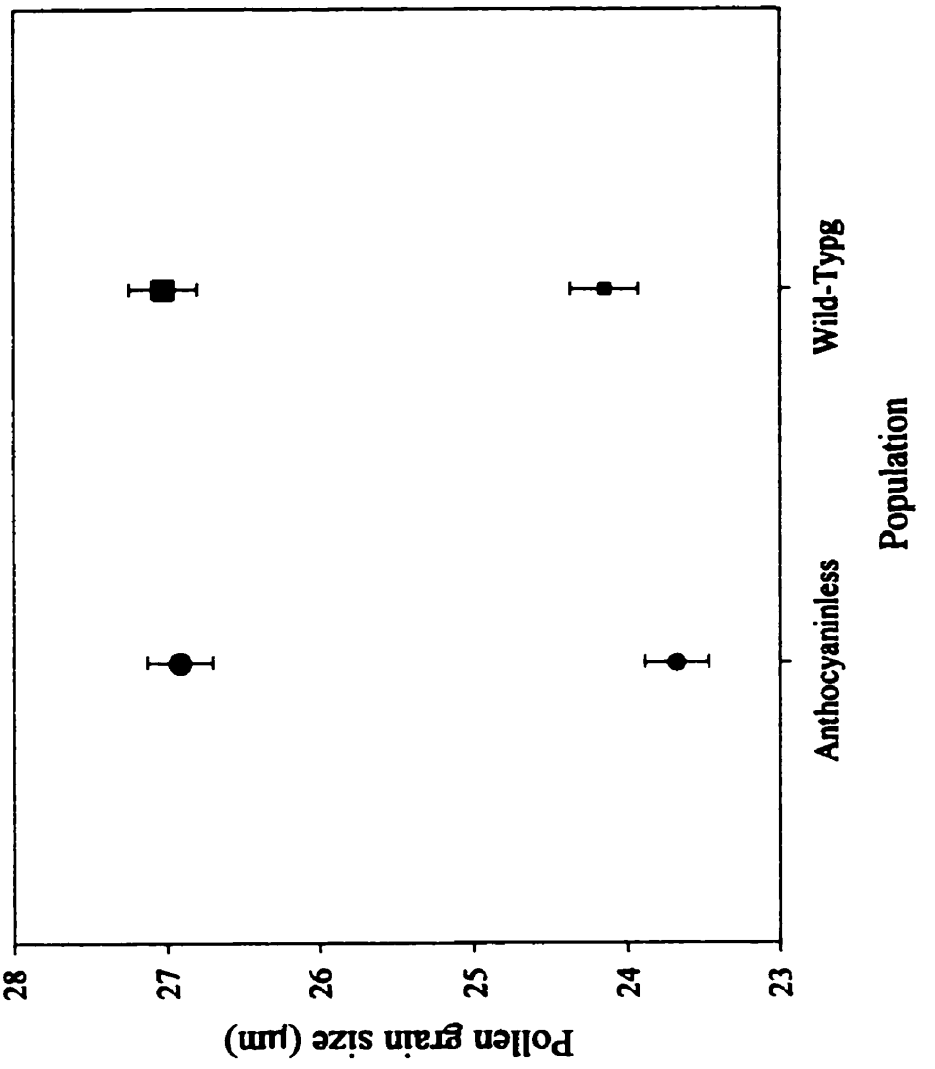

Pollen grain size (µm)
28
27
26
25
24
23
Anthocyaninless
Wild-Typg
Population

Table 3.2. **Generalized linear models of the proportions of ovules fertilized, fertilized ovules aborted, and ovules that set seed. 'Cross type' refers to the three pollination treatments: single-donor (*W*, *w*, *A*, *a*), same-size competitive cross (*W/A*, *w/a*), and different-size competitive cross (*W/a*, *w/A*). For instances in which ovule position significantly affected a dependent variable, the partial regression coefficient (±SE) is provided in parentheses below the corresponding likelihood ratio (*G*). Interactions between the covariate and main effect were non-significant and were removed from the models.**

Source	Fertilization	Abortion	Seedset
Cross type	G_2=1.73	G_2=0.57	G_2=1.70
Ovule position	G_1=43.36***	G_1=1.07	G_1=36.60***
	(-0.23 ±0.02)		(-0.16 ±0.02)

***P<0.0001

at ovule position 1 to 0.33 at ovule position 11 (Fig. 3.3a). Seedset showed a similar trend although abortions reduced the proportions at each ovule position (Fig. 3.3b). The proportion of fertilized ovules aborted did not vary significantly with ovule position (Table 3.2). Approximately one quarter of fertilized ovules (mean ±SE = 0.24 ±0.03) failed to develop.

Same-sized pollen in competitive crosses had equivalent siring ability. The proportion of wild-type seed did not differ between pollinations involving *w/a* versus *W/A* pollen mixtures (cross size: G_1=1.31, P>0.10), and seeds sired by wild-type donors did not occupy particular positions within the pod (ovule position: G_1=1.62, P>0.10; cross size×ovule position: G_1=0.04, P>0.50, removed from model). The competitive abilities of wild and anthocyaninless pollen of the same size class are approximately equal given that the proportion of seeds sired by wild-type pollen fluctuates about the expectation of 0.5 from ovule positions 1 to 8 (Fig. 3.4a). These results illustrate that the anthocyanin marker did not affect the competitive ability of pollen.

Pollen donors with large pollen in *W/a* and *w/A* mixtures had a post-pollination siring advantage (G_1=8.13, P<0.005; see Fig. 3.4b), irrespective of the morph of the large donor (G_1=0.46, P>0.10). Overall, donors with large pollen sired 69.7% (weighted average; lower SE=3.0%, upper SE=2.9%) of the seeds in a pod when competing against donors with small pollen. The proportion of seeds sired by large pollen increased with ovule position (partial regression coefficient ±SE = 0.63 ±0.19). This increase primarily involved the two ovule positions nearest the style.

Fig. 3.3. **The proportions of (a) ovules fertilized (± SE) and (b) ovules developing into seed (± SE) at each ovule position. See Table 3.2 for statistical details.**

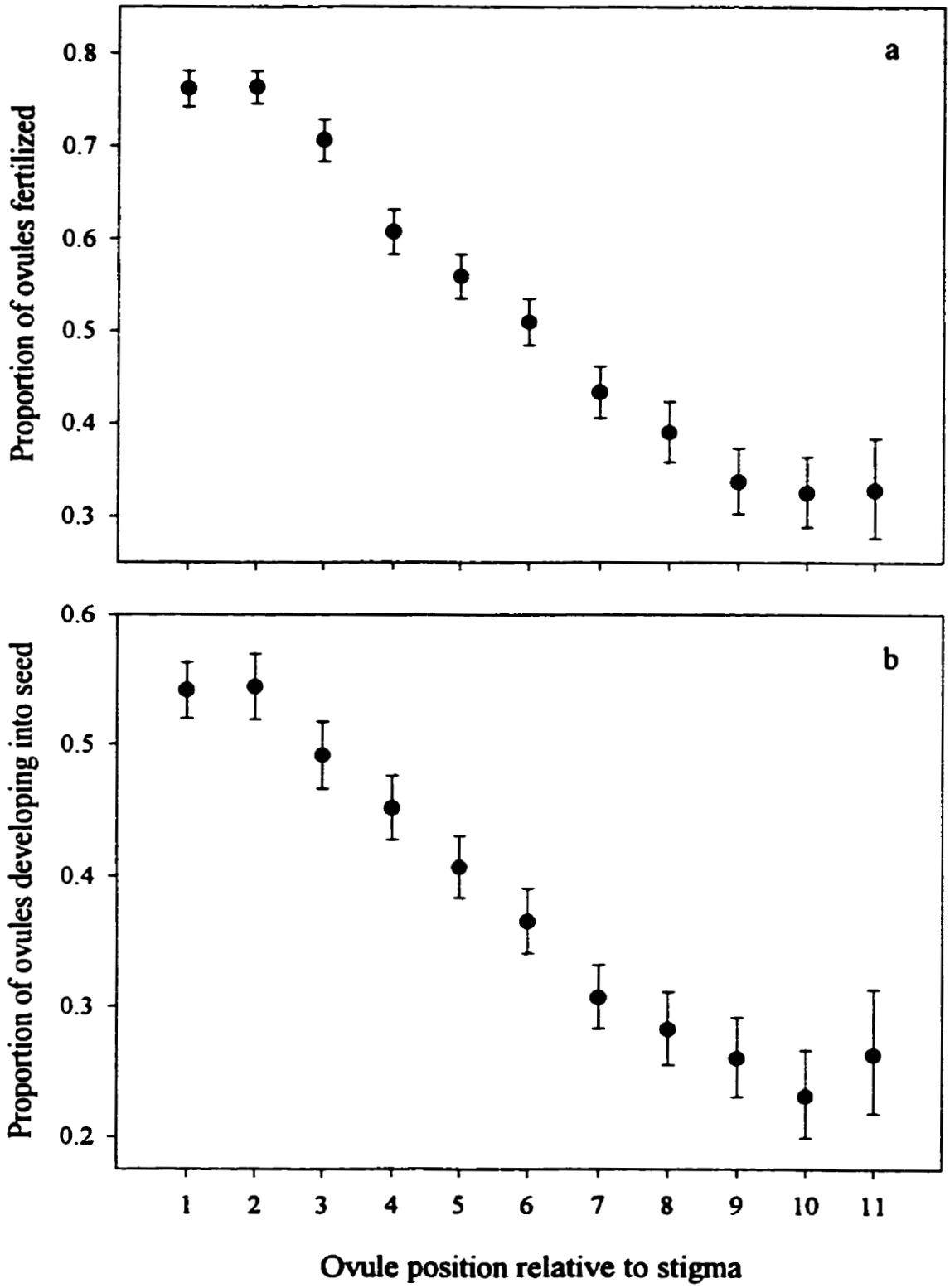
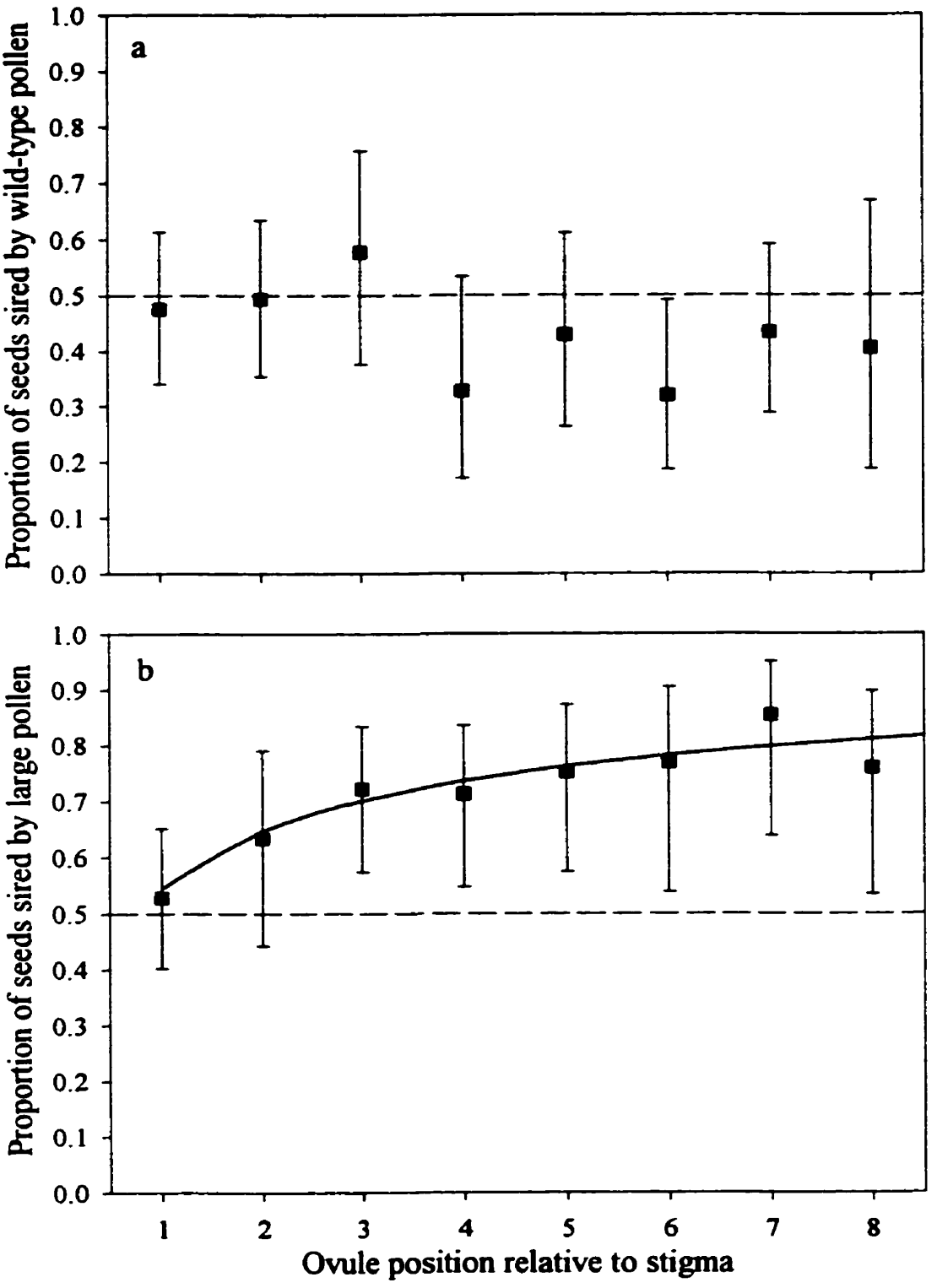

a
b
Proportion of ovules fertilized
Proportion of ovules developing into seed
0.8
0.7
0.6
0.5
0.4
0.3
0.6
0.5
0.4
0.3
0.2
1
2
3
4
5
6
7
8
9
10
11
Ovule position relative to stigma

Fig. 3.4. (a) The proportion of wild-type seed (±95% CI) in pods pollinated with *W/A* and *w/a* pollen mixtures. (b) The proportion of seeds sired by large pollen (±95% CI) in *W/a* and *w/A* crosses. The solid line represents the predicted proportion of seeds sired by the large donor (p) at each ovule position (o) based on $\ln(p/[1-p]) = 0.12 + 0.63 \ln(o)$.


a
b
Proportion of seeds sired by wild-type pollen
Proportion of seeds sired by large pollen
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
5
6
7
8
Ovule position relative to stigma

3.4 Discussion

In *B. rapa*, genetically determined differences in pollen size significantly influence siring success in competitive crosses. Donors with large pollen enjoyed, on average, a 20% siring advantage when competing against donors with small-pollen in the same style. However, in non-competitive single-donor crosses, donors with small pollen sired as many seeds as donors with large pollen. These results suggest that the outcome of pollen competition does not simply reflect intrinsic differences in the fertilization and siring potential of pollen of different size. For example, studies in *Nicotiana* suggest that the amount of RNA present in the pollen grain determines the maximum possible pollen tube length because no new synthesis occurs postpollination (Suss and Tupy, 1976; Tupy, Suss, and Rihova, 1986). If artificial selection for small size had reduced RNA levels to such an extent that maximum tube lengths differed for small and large pollen, then the proportion of ovules fertilized would have been greater for the latter in single-donor crosses. The lack of inherent differences in fertilization and seedset between single donors with large and small pollen suggests that deviation from an equal siring ratio in competitive crosses results from differences in competitive ability during either pre-fertilization performance (i.e. rates of germination, stigmatic penetration, and pollen-tube growth) or post-fertilization ovule abortion.

Post-fertilization processes may lead to greater maturation of ovules sired by the large-pollen donor, despite equal fertilization of ovules by large- and small-pollen. Pollen parents may influence the probability of an ovule's maturation through the contributions of pollen tube cytoplasm (e.g., starch granules or lipid droplets) to the endosperm tissue, with large pollen contributing more than small pollen (see Russell, 1980; Willson and Burley,

1983; Cruzan, 1990). However, pollen size of the paternal plant did not affect abortions in single-donor crosses (see Table 3.1), suggesting that the quantity of paternal input from large- and small-pollen is sufficient for maturation of ovules. This is not surprising given that pollen grains are small compared to the female gametophyte (<1% by volume in *Erythronium grandiflorum*, Cruzan, 1990), so that paternal contributions are unlikely to be substantial. Nonetheless, failure of embryos sired by small pollen could occur in competitive crosses via active abortion of seeds by resource-limited maternal plants based on paternal input. In this case, seeds sired by small pollen would not be aborted in a non-competitive environment, but would be selectively aborted when some seeds are sired by preferred large pollen. Such mate choice should be exercised equally throughout the ovary. However, small pollen sired as many seeds at the base of the style (ovule position 1) as large pollen, but fewer seeds at other ovule positions in competitive crosses (Fig. 3.4b). Therefore, post-fertilization ovule abortion seems to play a minor role in relative paternity of large- and small-pollen in two-donor crosses.

Differences in pre-fertilization pollen performance may explain the relative siring advantage of large pollen in competitive environments. During the initial autotrophic phase of pollen germination and tube growth, pollen grains use endogenous resources (e.g. carbohydrates, proteins, lipids, minerals, etc.; Stanley, 1971) until the heterotrophic interactions between pollen tubes and the stylar tissue commence (references in Cruden and Lyon, 1985; Vasil, 1987). If large pollen contain more endogenous reserves for autotrophic events, then pollen tubes from large pollen may reach the transmission tissue of the style first, where exogenous resources become available for pollen tube growth processes (e.g., cell wall extension, reviewed in Vasil, 1974). The positive interspecific

correlation between pollen size and stigma depth (≡ the distance a pollen tube grows to reach exogenous resources in the stylar tissue, see Cruden and Lyon, 1985) illustrates the importance of endogenous resource content during the initial stages of pollen tube growth.

Differences in pollen performance may also be mediated by differences in the rate of influx of materials from the style during the heterotrophic phase of pollen-tube growth. Large pollen of *B. rapa* may produce pollen tubes that are wider than those produced by small pollen (see Scribailo and Barrett, 1991), influencing the rate of uptake of stylar secretions. Although the equal fertilization potential of large and small pollen grains in non-competitive crosses suggests that the pollen-size differences involved in this study did not compromise the ability to mobilize resources in the transmission tissue, the rate of mobilization may have been greater for large-pollen donors with wider tubes. Hence, if the siring advantage of large pollen in competitive environments results from higher rates of pollen germination, stigmatic penetration, and pollen-tube growth, then differences in endogenous grain reserves and/or the rate of influx of stylar secretions are likely proximate explanations.

Despite the overall siring advantage of large pollen over small pollen in different-size competitive crosses, the positive relation between relative siring success of large pollen and ovule position (Fig. 3.4b) contradicts the expectation that siring success is a simple consequence of higher rates of germination, stigmatic penetration and pollen tube growth. In *B. rapa*, ovules located at the base of the style are fertilized first, whereas ovules at the peduncular end of the ovary are fertilized last by the slowest growing pollen tubes (see Fig. 3.3a). Because pollen grains that differ in performance sire seeds in different regions of the

ovary, I expected large pollen grains to fertilize ovules at the stylar end preferentially. However, small pollen fertilized as many ovules in this position as large pollen. The first possible explanation for this result proposes that large and small pollen may have similar rates of pollen germination and initial pollen tube growth until pollen tubes reach the first few ovules below the style. After traversing this length, pollen tubes from small pollen may be unable to sustain the same growth rate as those from large pollen, resulting in the siring advantage of large pollen for basal ovules. Kumar and Sarkar (1980) reported such a temporal association between pollen diameter and rate of in vitro growth in *Zea mays*. In particular, they found no correlation between pollen size and pollen tube growth rate ½, 1 and 2 h after inoculation, but inbred lines with large pollen had significantly higher rates of tube elongation after 3 h. Therefore, although size may not confer an initial competitive advantage, large pollen may maintain its maximal pollen-tube growth rate longer.

Alternatively, large pollen may germinate slower but produce faster growing pollen tubes than small pollen. Following arrival on a stigma, a pollen grain must hydrate to initiate germination (pollen tubes emerge at the point of maximum volume). Hydration involves water transfer from the stigma (reviewed in Heslop-Harrison, 1987), so that smaller pollen grains, with their higher surface-to-volume ratio, may rehydrate and germinate faster that larger grains. Such an advantage in germination time may explain why small pollen fertilized as many ovules at the base of the style as large pollen, even if pollen tubes from large pollen grow faster. These faster growing pollen tubes would eventually catch-up and surpass the slower-growing tubes of small pollen, resulting in a siring advantage of large pollen for basal ovules. Which of the two explanations accounts for the observed pattern of relative siring success remains unknown.

If variation in pollen size results in differential mating success among donors in natural populations, then sexual selection on pollen size could effect evolutionary change in this trait. For selection to occur, excess pollen from multiple donors must be deposited on stigmas. Opportunity for selection can be substantial because stigmas often receive an order of magnitude more pollen grains than the number of available ovules in natural populations (Snow, 1994; but see Burd, 1995), and animal pollination typically delivers pollen from several donors (reviewed in Harder and Barrett, 1995). However, the opportunity for intrasexual selection depends on interactions between pollen donors and recipients (see Bertin, 1982, 1990; Charlesworth, Schemske, and Sork, 1987; Marshall and Folsom, 1991; Waser, 1993; Herrero and Harmaza, 1996). Sexual selection is possible only when large-pollen donors sire a disproportionately large number of seeds on a wide range of maternal plants (see Snow and Spira, 1991a, b; Marshall, 1998). Despite limited replication, the observed magnitude of the siring advantage of large pollen indicates that maternal plant × pollen donor interactions were small and did not preclude selective mating due to pollen size.

Most previous studies of the association between pollen characters and mating success have focussed on the fitness consequences of variation in pollen production (reviewed in Snow and Lewis, 1993; Ashman, 1998). All else being equal, plants that produce copious pollen transmit more pollen to receptive stigmas (e.g., Schoen and Stewart, 1986; Stanton et al., 1991). This direct effect of pollen production on male fitness underlies evolutionary models of sex allocation (e.g., Charnov, 1982). However, once pollination occurs, realized siring success depends on the dynamics of fertilization and seed

development. Therefore, given the size-number tradeoff imposed by a fixed investment in pollen, the direct effect of pollen production on male fitness could be negated by reduced post-pollination competitive ability of small pollen.

Given the distinct dispersal and post-dispersal aspects of male mating success, both pre- and post-pollination selection contribute to variation in male fertility. When males do not compete for fertilization of ovules (possible with low pollinator abundance), variation in male fitness may simply reflect the relative representation of pollen genotypes that arrive on stigmas. In this situation, plants producing more (yet smaller) pollen grains should transmit more pollen to receptive stigmas and enjoy a fitness advantage. In contrast, when pollinators are abundant and/or efficient, stigmas will receive large, mixed-pollen loads. In this case, individuals producing larger (yet fewer) pollen grains may have a siring advantage in individual pistils under competitive conditions, but suffer a disadvantage in dispersing pollen to fewer stigmas. Hence, the optimal pollen size represents a compromise between the negative relation between size and pollen export and the positive effect of size on gametophytic competition. The most beneficial compromise for a particular species depends on the prevailing pollination environment and the average stylar environment in the population.

4 CONCLUDING DISCUSSION

Natural selection requires heritable fitness differences among phenotypes. In *B. rapa*, significant divergence in pollen diameter over three generations demonstrated heritable genetic control of the observed phenotypic variation in pollen size. In addition, differences in pollen size among *B. rapa* plants influence post-pollination competitive ability and, in turn, siring success. Therefore, given that pollen size has a heritable basis and indicates competitive ability, natural selection could effect evolution of this trait.

The relation between pollen size and male siring success reported in Chapter 3 suggests that pollen size in *B. rapa* should experience upward directional selection, effectively depleting heritable variation. However, additive genetic effects account for 13 to 61% of the observed variation in pollen diameter, suggesting that selection during the history of the *B. rapa* lines that I examined has not exhausted the heritable genetic component of phenotypic variation. Various genotype × environment interactions could account for this maintenance of genetic variation and retard evolutionary change. For example, strong pollen donor × maternal plant interactions may preclude the possibility of selective mating due to pollen size (discussed in Chapter 3). Alternatively, selection on pollen size may vary due to stochastic changes in pollination ecology.

Genetic variation may persist because selection on pollen size varies spatially or temporally due to variation in pollination intensity (for examples involving floral traits other than pollen size, see Campbell, 1989; Schemske and Horvitz, 1989; Kelly, 1992; Wilson, 1995). With frequent pollinator visits, stigmas will likely receive abundant pollen from diverse donors. Such pollination intensifies male-male competition among pollen

grains for fertilization of limited ovules, resulting in strong selection for large pollen size. In contrast, when pollinators are scarce so that stigmas receive insufficient pollen to result in full seedset, a plant's siring success depends on its ability to get to a stigma rather than post-pollination competitive ability. In this case, plants producing more (yet smaller) pollen grains will enjoy a fitness advantage. Hence, variation in pollinator abundance should change the direction of selection on pollen size, thereby maintaining additive genetic variation in natural plant populations.

The contrast of extensive variation in pollen size among species but limited variation within species suggests that selection has effected pollen-size evolution in response to either pollen dispersal or post-pollination processes. However, pollen transport conditions seem to have played a minor role in the evolution of pollen-size, at least for animal-pollinated species (Harder, 1998). The demonstrated consequences of pollen size for siring success suggest post-pollination processes provide the primary impetus for pollen size evolution. Therefore, interspecific pollen-size variation likely reflects differences in conditions for pollen germination, pollen-tube growth and ovule fertilization. For example, mean pollen grain diameters range from 26.2 to 49.1 μm among *Nicotiana* species (Pandey, 1971). Assuming constant style length, I predict that species at the extremes of this range experience different post-pollination environments. Counts of pollen on open-pollinated stigmas in natural populations should reveal a greater number and diversity of pollen for species with larger grains. Alternatively, differences in the intensity of the race for fertilization of ovules may influence pollen-size evolution. With selection for longer styles (e.g., due to shifts to bird pollination), individuals producing large grains should generally

enjoy a fitness advantage, resulting in concurrent directional selection for large pollen. Indeed, positive associations between pollen size and pistil characteristics occur commonly (e.g., Baker and Baker, 1979; Plitmann and Levin, 1983; Cruden and Lyon, 1985; Williams and Rouse, 1990; Dulberger, 1992; Kirk, 1993; Vonhof and Harder, 1995). Future studies identifying associations between pollen size and post-pollination environments should lend further insight into the adaptive significance of interspecific pollen-size variation.

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