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ORIGINAL ARTICLE

Pollen grain size, stigma depth, and style length: the relationships revisited

Robert William Cruden

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Abstract I examine data and review information in the literature to test hypotheses proposed by Delpino and Darwin to explain the source of nutrients utilized by pollen tubes. In 1867, Delpino, in his discussion of distyly, suggested that the positive relationship between pollen grain size and style length was based on the pollen grains containing sufficient nutrients to sustain the growth of their pollen tubes through their respective styles. Darwin (The different forms of flowers on plants of the same species, 2nd edn. J. Murray, London, 1884) rejected Delpino's suggestion based on his examination of distylous species whose morphs produced pollen grains whose sizes were not proportionate to the lengths of their respective styles. Darwin then proposed that pollen tubes first grow autotrophically, i.e., through the stigma, then heterotrophically in the style. This should result in a positive relationship between pollen grain size and stigma depth, if pollen tubes grow autotrophically through the stigma. I examined 15 species in Fabaceae and 20 species in Proteaceae to test the two hypotheses. Pollen grain size was correlated with stigma depth among the Fabaceae, i.e., consistent with Darwin's hypothesis, and was not correlated with style length in either family, i.e., inconsistent with Delpino's proposal. Comparisons of related species, in general, were consistent with Darwin's hypothesis. In addition, information in the literature provided no evidence that pollen tubes obtain resources on or in the stigma, i.e., pollen tube growth from the stigmatic surface to the style was autotrophic. In contrast, pollen tubes obtain an array of resources from the transmission tissue, thus there is little reason for pollen grains to contain those resources. In addition, I suggest that positive correlations between pollen grain size and style/pistil length may be a result of both being correlated with stigma depth.

Keywords Fabaceae · Flower size · Pollen grain size · Stigma depth · Style length · Proteaceae

R. W. Cruden (✉)
Department of Biology, University of Iowa,
Iowa City, IA 52240, USA
e-mail: robert-cruden@uiowa.edu

Present Address:
R. W. Cruden
550 Woodhill Dr., Saline, MI 48176, USA

Introduction

By the end of the nineteenth century nearly all the elements included in recent discussions of the relationship between pollen grain size and style length had been outlined. In 1830, in a letter to Charles-François Brisseau de Mirbel, French botanist and father of cytology, Giovanni Amici, the Italian astronomer, botanist, microscopist and inventor of lenses, made two important observations. First, he related that he had observed in a number of species that pollen tubes grew intact through the style (Amici 1830). Second and most relevant to this discussion, he suggested that pollen grains did not contain sufficient material to account for their growth through very long styles and proposed that they received nourishment from the conducting tissue (Amici 1830). Subsequently, Delpino, who was apparently unaware of Amici's proposal, suggested that the nutrients in the pollen grains of distylous species were proportional to the length of the styles through which their pollen tubes grew (Delpino 1867, p. 286). Darwin (1884, p. 250) rejected Delpino's proposition based on his examination of distylous species, whose floral morphs had

styles that differed in length but produced pollen grains that were equivalent in size, pollen grains that were not proportional in size to the lengths of their respective styles, etc., "These cases seem to prove that the differences in size between pollen grains in the two forms is not determined by the length of the pistil, down which the tubes have to grow." Darwin (p. 251) then reconciled the two arguments; "The tubes are at first developed from matter contained within the grains,…[and] botanists believe that they afterwards draw nourishment from the conducting tissue of the pistil".

Studies during the past 30 years are consistent with the hypotheses outlined above and, although richer in knowledge, we seem but little the wiser. First, a functional relationship between pollen grain size and style length is still subject to the criticism offered by Darwin. Even though a number of studies reported a correlation between pollen grain size and style or pistil length (e.g., Aguilar et al. 2002; Baker and Baker 1979, 1982; Bigazzi and Selvi 2000; Kirk 1993; López et al. 1999, 2006; Ortega Olivencia et al. 1997; Plitmann and Levin 1983; Roulston et al. 2000; Torres 2000; Yang and Guo 2004; also see Sarkissian and Harder 2001) others showed no relationship between the two. The latter included comparisons of homostylous species (e.g., Cruden and Lyon 1985 and references therein, Cruden and Miller-Ward 1981, Knudsen and Olesen 1993, López et al. 2006, also see Small 1988) as well as the morphs of distylous species (Barrett et al. 2000; Casper 1983; Darwin 1884; Feinsinger and Busby 1987; Ganders 1979 and references therein, also see Hong and Han 2002).

Second, several lines of evidence support Darwin's (1884) argument that pollen tube growth is initially supported by resources in the pollen grain and growth through the style is supported by nutrients in the style. First, autotrophic growth on and through the stigma is necessary because dry stigmas provide no resources to pollen tubes and there is no evidence that the stigmatic exudates of species with wet stigmas, which are primarily involved in pollen grain adhesion, hydration and germination, include nutrients or other resources that are taken up and utilized by pollen tubes (e.g., Edlund et al. 2004; Graaf et al. 2001; Herrero and Hormaza 1996; but see Ciampolini et al. 1983). Second, there is considerable evidence supporting Amici's (1830) proposal that pollen tubes obtain nutrients from the style (e.g., Campbell and Ascher 1975; Cheung 1996; Gawlik 1984; González et al. 1996; Herrero and Hormaza 1996; Lind et al. 1996; Schmidt-Adam and Murray 2002; Wu et al. 1995).

Based on the repeated observation that pollen tubes obtain resources from the stylar transmission tissue (see above), the distance between the stigmatic surface and resources in the stylar transmission tissue should provide an estimate of the distance pollen tubes grow autotrophically. It follows that pollen grains have to contain sufficient resources to support the growth of their pollen tubes across that distance, which should be reflected in a positive correlation between pollen grain size and stigma depth (see Cruden and Lyon 1985).

My re-examination of the relationships between pollen grain size and both stigma depth and style length was prompted by two considerations. First, I tested the hypothesis that pollen grain size and stigma depth are positively correlated because it was based on one study (Cruden and Lyon 1985) and a few additional observations (Cruden 2000). Second, I examined the relationship between pollen grain size and style length, i.e., Delpino's hypothesis, because a functional relationship is still attributed to positive correlations between the two traits (e.g., Ortega Olivencia et al. 1997; Torres 2000; Yang and Guo 2004; also see Plitmann and Levin 1983; Roulston et al. 2000) even though the two traits are not always correlated (Cruden and Lyon 1985) and there is considerable evidence that pollen grains obtain resources from the transmission tissue (see above). In addition, Delpino's and Darwin's hypotheses needed to be considered in the context of stigmatic and stylar structure and function. I examine relationships between flower size and other floral traits because other studies involving legumes showed flower size was positively correlated with a number of floral traits, including pollen grain size (e.g., Galloni et al. 2007; López et al. 1999; Ortega Olivencia et al. 1997; also see Small 1988). However, if floral traits evolve independently of one another (Cruden 2000) related species might display an array of phenotypes involving floral traits and the relationships among them.

To test the two hypotheses I examine relationships involving pollen grain size, stigma depth, style length, and flower size among 15 species in Fabaceae and those involving pollen grain size, style length, and flower size among 20 species in Proteaceae. I consider Delpino's and Darwin's hypotheses with respect to my results and stigmatic and stylar structure and function.

Materials and methods

The plants

All but two of the species examined in this study were indigenous to eastern New South Wales, Australia (see Appendix 1 for collection localities). Voucher specimens collected in 2006 (06-) were deposited in the Janet Cosh Herbarium at the University of Wollongong, NSW and my determinations were checked by Belinda Pellow, Curator of the Herbarium. Specimens collected in 2008 (08-) were

deposited in the University of Michigan Herbarium (MICH), Ann Arbor, MI, USA.

Methods

In 2006, flowers were examined using a Leica MZ75 dissection microscope and the flower parts measured at different magnifications. Keel length was measured with a mm ruler, the distance from the stigma to the lowest ovule and style length of large flowers were measured at ×6.3 and those of small flowers at ×20, and stigma depth at ×50. Pollen grains were examined using an Olympus CH2 microscope and their size measured at ×400. In 2008, additional measurements of pollen grains were made using an Olympus BHA microscope at ×480. The measurements were made with ocular micrometers and converted to mm, etc. For most species measurements of flower parts were made on one flower from each of ten plants (but see tables).

Pollen grains of the legumes were obtained and treated in several ways. One, an anther was removed from both the upper and lower whorls of anthers, the anther sacs separated and one from each anther was placed in a large drop of 60% glycerol on a slide. Pollen grains were removed from the anther sacs, which were then removed, a cover slip applied and the diameters of ten widely, spaced pollen grains were measured. Even though I used a high concentration of glycerol the pollen grains expanded, thus measurements over estimate the size of the pollen grains in the anther. Two, for 13 species, I scattered pollen from an open flower onto a slide by striking the filaments with an insect pin. I then measured the lengths and diameters of the pollen grains and used them to determine the volumes of the ellipsoidal pollen grains. Three, because their pollen grains changed shape immediately following dispersal the pollen gains of two species were dispersed into or onto a drop of immersion oil and a cover slip applied. This minimized their exposure to the air (see below).

The pollen grains of the Proteaceae were handled in two ways. Those of some species were deposited on a dry slide by vibrating the presenter and then measured or they were removed from the presenter and placed into a drop of 95% ethanol on a slide, washed with successive drops of 95% ethanol, and a drop of 60% glycerol added. The size of the grains was equivalent in the two treatments. Further, because the pollen grains were more or less dorso-ventrally flattened and triangular in optical cross-section I used the cross-sectional area as a measure of pollen grain size. Pollen grains of Proteaceae were tested for starch by staining with IKI.

I used keel length as a measure of flower size in the legumes rather than the length of the standard (see López et al. 1999; Small 1988) because the blade of the standard was variable with respect to height and width, e.g., it was much wider than high in *Bossiaea* and *Dillwynia* and higher than wide in *Phyllota* and some *Pultenaea*. Likewise, wing length might serve as a predictor of flower size. In the species considered here there was a strong correlation between keel length and wing length whether all the species were analyzed ($r = 0.950$, $P \ll 0.001$) or just those in Mirbelieae ($r = 0.927$, $P < 0.001$) and in comparisons involving flower size the results were equivalent whether I used keel length or wing length. Flower size in Proteaceae was based on tepal length, which was obtained from Harden (1991), Olde and Marriott (1995), or preserved material. The median length was used in the analyses.

I used the diameters of pollen grains hydrated in 60% glycerol (see above) as a measure of pollen grain size in the analyses of the legumes. The method is simple, easily replicated, and allowed me to compare pollen grain size among species because the pollen grains of all the species became spherical or nearly so. I used pollen grains from mature flower buds and assumed that the size of the pollen grains would change very little before the flower opened and that the diameters of hydrated pollen grains were correlated with the volumes of pollen grains in open flowers. The two assumptions were tested and both were supported (see below). Then I used pollen grain volume to examine its relationship with stigma depth and that with style length among all the species to determine if the two measures of pollen grain size produced equivalent results and they did (see below).

I compared the diameters of hydrated pollen grains from mature flower buds and those from open flowers to test my assumption that they were equivalent in size. I compared pollen grains from buds and flowers from a single plant, as pollen grain size differed between plants (Cruden personal observation). I examined all the species except *Bossiaea obcordata*, *Dillwynia floribunda*, and *Pultenaea linophylla*. There was no difference between the two stages in any species (range of *U*s for 11 species: 200–243, $n = 20, 20$, $P > 0.2$; for *Hardenbergia violacea* ($U = 169$, $n = 20, 15$, $P > 0.2$).

I compared the diameters of hydrated pollen grains from mature flower buds with the volumes of dry pollen grains from open flowers (Table 1) to test my assumption that they were correlated. Analysis with the Spearman coefficient of rank correlation showed a strong correlation between them ($r = 0.993$, $P \ll 0.001$). The less than perfect correlation is the result of one, possibly two, ties. First, the diameters of the pollen grains of *Bossiaea obcordata* and *Daviesia alata* were equivalent but the volumes of the pollen grains of *D. alata* were larger. Second, the volumes of the pollen grains of *Indigofera australis* were either equivalent in size to those of *Dillwynia retorta*, providing the correlation given above, or if smaller, as was probably the case, the correlation is higher ($r = 0.999$). Also, the diameters of the pollen grains

Table 1 Pollen grain diameter, pollen grain volume, stigma depth, style length, distance from the stigma to the lowest ovule, and keel length in 15 species in Fabaceae

Genus/species	Pollen grain diameter (μm^2)	Pollen grain volume (μm^3)	Stigma depth (mm)	Style length (mm)	Stigma to lowest ovule (mm)	Keel length (mm)
Tribe Bossiaeae						
Bossiaea ensata	$23.78^{a} \pm 0.27$ (9)	$2{,}166^{a} \pm 48$	$0.073^{a} \pm 0.003$ (9)	$2.95^{a} \pm 0.07$	7.05 ± 0.16	$7.9^{a} \pm 0.2$
Bossiaea obcordata	$20.33^{b} \pm 0.27$ (7)	$1{,}341^{b} \pm 23$ (9)	$0.047^{b} \pm 0.004$ (6)	$3.04^{a} \pm 0.17$ (6)	5.69 ± 0.26 (6)	$8.5^{a} \pm 0.3$ (6)
Tribe Mirbelieae						
Gomphilobium grandiflorum	24.28 ± 0.17	$2{,}509 \pm 59$	0.062 ± 0.004 (9)	7.61 ± 0.28	12.24 ± 0.38	16.3 ± 0.3
Sphaerolobium minus	28.14 ± 0.38 (7)	$3{,}374 \pm 62$	0.046 ± 0.002 (8)	3.63 ± 0.11 (8)	4.87 ± 0.10 (8)	4.9 ± 0.2 (8)
Daviesia alata	20.00 ± 0.20	$1{,}720 \pm 13$	0.019 ± 0.001	1.63 ± 0.03	3.28 ± 0.06	5.0 ± 0.1
Mirbelia rubiifolia	31.90 ± 0.28	$6{,}160 \pm 143$	0.086 ± 0.003	0.43 ± 0.03	2.13 ± 0.09	3.5 ± 0.1
Phyllota humifusa	24.08 ± 1.01	$2{,}368 \pm 68$	0.050 ± 0.004	7.09 ± 0.12	7.68 ± 0.12	7.1 ± 0.1
Aotus ericoides	24.60 ± 0.29	$2{,}641 \pm 61$	0.040 ± 0.000	5.44 ± 0.11	6.08 ± 0.11	8.6 ± 0.2
Pultenaea tuberculata	$29.83^{a} \pm 0.30$	$4{,}237^{a} \pm 71$	$0.049^{a} \pm 0.002$	$10.23^{a} \pm 0.27$	11.60 ± 0.26	$11.2^{a} \pm 0.2$
Pultenaea linophylla	$28.22^{b} \pm 0.20$	4,109 (2)	$0.044^{ab} \pm 0.003$ (9)	$7.63^{b} \pm 0.14$	8.29 ± 0.14	$8.0^{b} \pm 0.1$
Pultenaea villosa	$23.23^{c} \pm 0.24$	$2{,}305^{b} \pm 42$	$0.037^{b} \pm 0.000$	$5.96^{c} \pm 0.12$	6.84 ± 0.12	$7.2^{c} \pm 0.1$
Dillwynia floribunda	$37.74^{a} \pm 0.32$	$1{,}002^{a} \pm 343$ (8)	$0.160^{a} \pm 0.004$	$3.29^{a} \pm 0.06$	4.07 ± 0.08	$6.1^{a} \pm 0.2$
Dillwynia retorta	$33.78^{b} \pm 0.49$ (5)	$8{,}412^{b} \pm 259$	$0.157^{a} \pm 0.004$ (8)	$2.21^{b} \pm 0.07$	3.03 ± 0.07	$5.8^{a} \pm 0.2$
Tribe Indigofereae						
Indigofera australis	36.5 ± 0.32	$10{,}362 \pm 322$ (4)	0.159 ± 0.006	2.06 ± 0.03	5.89 ± 0.11	8.7 ± 0.2
Tribe Phaseoleae						
Hardenbergia violacea	35.08 ± 0.44	$9{,}541 \pm 340$ (9)	0.189 ± 0.005	1.58 ± 0.21	4.05 ± 0.06	5.1 ± 0.1

Pollen grain diameter was based on hydrated pollen grains and pollen grain volume was based on dry pollen grains from dehisced anthers (but see "Materials and methods"). Systematic arrangement follows that of Crisp and Weston (1995) and Doyle et al. (1997). Derived taxa are at the bottom of the table. Numbers in parentheses indicate the number of plants sampled if other than ten. Means ± a standard error are given. In comparisons of species in the same genus different superscripts following the means indicate they are significantly different and the same superscript indicates the means are equivalent

of *B. ensata* and *Pultenaea villosa* were equivalent in size and although the pollen grains of *P. villosa* appear to be a little larger their volumes were equivalent ($U = 76$, $n = 10, 10$, $P > 0.05$). In addition, I examined the relationship of pollen grain volume with stigma depth and that with style length among all the species. Although the correlations ($r = 0.782$ and $r = -0.195$, respectively) differed a little from those reported in the results ($r = 0.757$ and $r = -0.204$, respectively) they were effectively the same, i.e., the two measures of pollen grain size produced equivalent results.

In contrast to the pollen grains of most of the species, the pollen grains from two species began to elongate seconds after dispersal. To obtain measurements of the pollen grains of these species at the moment of release I held flowers such that their pollen was released into a drop of immersion oil on a microscope slide. The pollen grains of *H. violacea* were more or less spherical and I measured their diameters. The pollen grains used in this test were obtained from location 16 in 2008 and were equivalent in size to those obtained from location 10 in 2006. The pollen grains of *I. australis* were triangular in outline when viewed from above and bulged in the middle when viewed from the side. In essence, they were triangular boxes whose tops and bottoms bulged. I measured the cross-sectional height of the pollen grain above the base of bulge to provide an estimate of the height as if the surface were flat. I then calculated the volumes of the pollen grains assuming they were triangular prisms. Because the edges of these pollen grains were rounded the mean given in Table 1 probably over estimates their volume.

The sampling of the Fabaceae and Proteaceae reflects different objectives. I emphasized the sampling of genera in Fabaceae relative to species to reduce the possible impact of having a large number of species in one genus in the sample. Thus, most of the genera (8 of 11) were represented by a single species. Most of the genera (8 of 11) and species (11 of 15) were in the Tribe Mirbelieae (Table 1). I sampled the Proteaceae so as to include most of the genera that were common locally (*Persoonia* excluded) and, when possible, to sample 2 or 3 species in each genus. With the exception of the two species in

Table 2 Pollen grain size, style length and tepal length in various Proteaceae

Subfamily/genus/species	Pollen grain size ($\mu m^2 \pm SE$)	Style length (mm ± SE)	Tepal length (mm)
Subfamily Symphionematoideae			
Symphionema montanum	$282^a \pm 6$	$2.3^a \pm 0.1$	4–5
Symphionema paludosum	$1{,}088^b \pm 20$	$2.4^a \pm 0.0$	4–5
Subfamily Proteoideae			
Tribe Conospermeae			
Conospermum taxifolium	$1{,}892^a \pm 25$	$3.3^a \pm 0.1$	6–7
Conospermum longifolium	$2{,}053^b \pm 41$	$3.9^b \pm 0.1$	c.8
Conospermum tenuifolium	$2{,}966^c \pm 45$	$3.9^b \pm 0.0$	c.4
Tribe Petrophileae			
Petrophile pedunculata	$393^a \pm 6$	$7.5^a \pm 0.1$	8–10
Petrophile pulchella	$803^b \pm 15$	$8.1^a \pm 0.2$	10–14
Petrophile sessilis	$813^b \pm 17$	$6.1^b \pm 0.1$	10–14
Tribe Leucadendreae			
Subtribe Isopogoninae			
Isopogon anemonifolius	$2{,}021^a \pm 42$	$15.5^a \pm 0.4$	10–12
Isopogon anethifolius	$390^b \pm 8.5$	$15.6^a \pm 0.2$	10–15
Subtribe Adenanthinae			
Adenanthos detmoldi	1,058 ± 2	46.0 ± 0.6	20–22
Subfamily Grevilleoideae			
Tribe Roupaleae			
Lambertia formosa	1,154 ± 21	44.1 ± 0.7	30–40
Tribe Embothrieae			
Subtribe Lomatiinae			
Lomatia fraserii	$461^a \pm 8$	$5.5^a \pm 0.1$	7–9
Lomatia silaifolia	$518^b \pm 9$	$6.3^b \pm 0.1$	c.15
Subtribe Embothriinae			
Telopea speciosissima	1,341 ± 29	20.4 ± 0.4	22–24
Subtribe Hakeinae			
Grevillea buxifolia	$2{,}196^a \pm 8$	$22.0^a \pm 0.4$	19–21
Grevillea laurifolia	$1{,}864^b \pm 56$	$20.1^b \pm 0.3$	13–25
Grevillea sphacelata	$1{,}894^b \pm 16$	$8.1^c \pm 0.1$	9–12
Hakea laevipes	$2{,}263^a \pm 45$	$3.7^a \pm 0.1$	c.4
Hakea teretifolia	$5{,}133^b \pm 135$	$11.2^b \pm 0.2$	4–6

Pollen grain size is the area of the optical cross-section of the more or less dorso-ventrally flattened, triangular shaped pollen grains. With the exception of *Telopea speciosissima* one flower from each of ten plants was sampled and for *Telopea* the sample sizes were eight and seven for pollen grain size and style length, respectively. Systematic arrangement follows that of Weston and Barker (2006) except for the species, which are arranged alphabetically. Derived taxa are at the base of table. Means ± a standard error are given. In comparisons of species in the same genus different superscripts following the means indicate they are significantly different and the same superscript indicates the means are equivalent

Symphionema, the species in Proteaceae were more or less evenly divided among two subfamilies and nine genera (Table 2). Three genera were represented by a single species, three by two species, and three by three species.

Statistics

I used the Spearman coefficient of rank correlation to examine relationships among traits, the Mann–Whitney *U*-test to examine differences in traits between species, and the STP method (Sokal and Rohlf 1969, p. 395) to test differences in traits among species. A sequential Bonferroni method (Rice 1989) was used to determine significance, i.e., $\alpha \leq 0.05$, for multiple tests using the Spearman coefficient of rank correlation.

The legume data were examined for the possible influence of relatedness using the phyletic analyses of Mirbelieae by Crisp and Weston (1995) and Fabaceae by Doyle et al. (1997). The genera were ordered as presented in Crisp and Weston (1995) and Doyle et al. (1997) (see Table 1) and tested for possible correlations between genus and style length, stigma depth, and pollen grain size. To test whether subfamily might influence the possible relationships I examined all of the genera and those in Mirbelieae separately.

The data from the Proteaceae were examined somewhat differently. To test for possible effects of relatedness I used the Kruskal-Wallis test with tied ranks (Zar 1999) in conjunction with "nonparametric multiple comparisons with unequal sample sizes" (Zar 1999, p. 225) to

test for possible differences in style length and pollen grain size among subfamilies and tribes. The possible influence of phyletic differences among tribes and also genera were examined with the Spearman coefficient of rank correlation. The subfamilies, tribes and genera were ordered as outlined by Weston and Barker (2006) (see Table 2).

Results

Fabaceae

I used pollen grain diameter as a measure of pollen grain size to test the relationship of pollen grain size with stigma depth and style length (Table 1). Among all the species and only those in Mirbelieae there were significant correlations between pollen grain diameter and stigma depth ($r = 0.757$, $n = 15$, $P < 0.002$ and $r = 0.772$, $n = 11$, $P < 0.01$, respectively). In contrast, pollen grain diameter and style length were not correlated among all the species ($r = -0.204$, $P > 0.5$) or those in Mirbelieae ($r = -0.173$, $P > 0.5$). In addition, it has been suggested that a relationship between pollen grain size and the distance from the stigma to the lowest ovule is the appropriate distance to measure. The two traits were not correlated among all the species ($r = -0.275$, $n = 15$, $P > 0.2$) or just those in Mirbelieae ($r = -0.318$, $n = 11$, $P > 0.2$).

Flower size

The data provide mixed evidence for positive relationships between flower size and other floral traits. There were positive correlations between keel length and both style length and distance from the stigma to the lowest ovule among all the species ($n = 15$) ($r = 0.604$, $P = 0.02$ and $r = 0.789$, $P < 0.001$, respectively) but no correlation between keel length and either stigma depth ($r = -0.093$, $P > 0.5$) or pollen grain diameter ($r = -0.089$, $P > 0.5$). The correlation between keel length and style length was not significant when multiple tests ($n = 4$) were taken into consideration. Likewise, among the Mirbelieae ($n = 11$) there were positive correlations between keel length and both style length and the distance from the stigma to the lowest ovule ($r = 0.846$, $P < 0.002$ and $r = 0.873$, $P < 0.001$, respectively) but no correlation between keel length and either stigma depth ($r = -0.146$, $P > 0.5$) or pollen grain diameter ($r = -0.182$, $P > 0.5$).

The results described above were independent of phyletic relationships. There was no relationship between genus (fixed effect) and style length among all the genera ($r = -0.246$, $n = 11$, $P > 0.2$) or just those in Mirbelieae ($r = 0.071$, $n = 8$, $P > 0.5$). Likewise, there was no relationship between genus and stigma depth among all the genera ($r = 0.452$, $n = 11$, $P > 0.1$) or those in Mirbelieae ($r = 0.119$, $n = 8$, $P > 0.5$) and no relationship between genus and keel length among all the genera ($r = 0.064$, $P > 0.5$) or those in Mirbelieae ($r = 0.119$, $P > 0.5$). However, there was a weak correlation between genus and pollen grain diameter among all genera ($r = 0.718$, $n = 11$, $P < 0.02$) but not among the genera in Mirbelieae ($r = 0.405$, $n = 8$, $P > 0.2$). The correlation between the two among all genera was not significant when multiple tests ($n = 4$) were considered.

Interspecific comparisons

Data from three genera show considerable variation in floral traits and the relationships among them (Table 1). In the two species of *Bossiaea* both keel length and style length were equivalent ($U = 45.5$, $n = 6, 10$, $P > 0.05$ and $U = 30.5$, $n = 6, 10$, $P > 0.1$, respectively) but *B. ensata* produced larger pollen grains ($U = 63$, $n = 7, 9$, $P < 0.001$) and had deeper stigmas ($U = 54$, $n = 9, 6$, $P < 0.001$) than *B. obcordata*. In the two species of *Dillwynia* keel length and stigma depth were equivalent ($U = 65.5$ and 62.5, respectively, $P > 0.2$) (Table 1), the styles of *D. floribunda* were longer ($U = 100$, $n = 10, 10$, $P < 0.001$) and it produced larger pollen grains ($U = 50$, $n = 5, 10$, $P < 0.001$) than *D. retorta* (Table 1).

The differences in floral traits among the species in *Pultenaea* were positively associated, in large part, with flower size (Table 1). The keels of *Pultenaea tuberculata* were longer than those of both *P. villosa* and *P. linophylla* ($U_s = 100 > U_{0.01[3,10]} = 89$) and those of *P. linophylla* were longer than those of *P. villosa* ($U_s = 95.5$, $P < 0.01$). Likewise, the styles of *P. tuberculata* were longer than those of *P. linophylla* ($U_s = 100$, $P < 0.01$) and *P. villosa* ($U_s = 100$, $P < 0.01$) and those of *P. linophylla* were longer than those of *P. villosa* ($U_s = 100$, $P < 0.01$). Also, the pollen grains of *P. tuberculata* were larger than those of both *P. linophylla* ($U_s = 92.5$, $P < 0.01$) and *P. villosa* ($U_s = 100$, $P < 0.01$) and those of *P. linophylla* were larger than those of *P. villosa* ($U_s = 100$, $P < 0.01$). Finally, the stigmas of *P. tuberculata* were deeper than those of *P. villosa* ($U_s = 90$, $P < 0.01$) but those of *P. linophylla* were intermediate in depth to those of *P. tuberculata* ($U_s = 69.5$, $P > 0.05$) and *P. villosa* ($U_s = 80$, $P > 0.05$).

Proteaceae

There was no relationship between pollen grain size and style length among the genera ($r = 0.127$, $n = 10$, $P > 0.5$) or species ($r = 0.093$, $n = 20$, $P > 0.5$) in Proteaceae (Table 2). Likewise, there was no relationship

between the two among genera ($r = 0.313$, $n = 7$, $P > 0.5$) or species ($r = 0.114$, $n = 16$, $P > 0.5$) when the bird-pollinated species *Adenanthos detmoldii*, *Grevillea laurifolia*, *Lambertia formosa*, and *Telopea speciosissima* were excluded from the analysis. The relationship between pollen grain size and stigma depth was not examined because some or most of the stigmatic tissue of the species in Proteaceae considered here was included within the presenter (Cruden personal observation, also see Clifford and Sedgley 1993; Herscovitch and Martin 1989), thus it was not possible to distinguish where the stigmatic tissue ended and the stylar transmission tissue began.

Flower size

There were weak positive correlations between tepal length and style length among the subtribes ($r = 0.750$, $n = 9$, $P < 0.05$) and genera ($r = 0.718$, $n = 10$, $P < 0.05$) and a strong correlation among species ($r = 0.651$, $n = 20$, $P < 0.005$). The first two tests were not significant when multiple tests ($n = 2$) were considered. There was no relationship between tepal length and pollen grain size among the subtribes ($r = -0.017$, $P > 0.5$), genera ($r = -0.242$, $P > 0.5$), or species ($r = 0.408$, $P > 0.05$).

There was little or no influence of phylogeny or taxon on these results. The Kruskal–Wallis test with tied ranks (Zar 1999) showed no differences in pollen grain size, i.e., the cross-sectional area of the pollen grain, among subfamilies or tribes ($H_c = 0.805$, $X^2_{0.05,2}* = 5.991$, $P > 0.5$ and $H_c = 8.018$, $X^2_{0.05,5}* = 11.070$, $P > 0.1$, respectively). Likewise, there was no correlation between pollen grain size and either tribe or subtribe ($r = 0.300$, $n = 6$, $P > 0.5$ and $r = 0.300$, $n = 9$, $P > 0.2$, respectively). In contrast, there was a weak but significant difference among subfamilies ($H_c = 6.391$, $P < 0.05$) with respect to style length that was attributable to the short styles of *Symphionema* (Sympionematoideae) relative to those of species in Grevilleoideae ($Q = 2.4517$, $P < 0.05$). The differences in style length between Symphionematoideae and Proteoideae and Proteoideae and Grevilleoideae were not significant ($Q = 1.8876$, $P > 0.2$ and $Q = 0.9359$, $P > 0.5$, respectively). However, correlation analyses showed no relationship between tribe and style length whether all the tribes were included ($r = 0.829$, $n = 6$, $P = 0.1$) or Symphionematoideae was excluded ($r = 0.700$, $n = 5$, $P > 0.1$). Also, style length was not correlated with either subtribe or genus whether *Symphionema* was included in the analysis ($r = 0.517$, $n = 9$, $P > 0.1$ and $r = 0.430$, $n = 10$, $P > 0.2$, respectively) or excluded from it ($r = 0.310$, $n = 8$, $P = 0.5$ and $r = 0.233$, $n = 9$, $P > 0.5$). Finally, tepal length was not correlated with either subtribe ($r = 0.400$, $P > 0.2$) or genus ($r = 0.142$, $P > 0.5$).

Interspecific comparisons

Comparisons of congenerics in Proteaceae displayed a number of relationships among tepal length, style length and pollen grain size (Table 2). The styles of *Hakea laevipes* were much shorter ($U = 100$, $P < 0.001$) and its pollen grains smaller ($U = 100$, $P < 0.001$) than those of *H. teretifolia* and both were positively associated with tepal length (Table 2). Likewise, the styles of *Lomatia fraserii* were shorter ($U = 95$, $n = 10$, 10, $P < 0.001$) and its pollen grains somewhat smaller ($U = 28$, $n = 3$, 10, $P < 0.025$) than those of *L. silaifolia* and it had smaller flowers (Table 2). In contrast, the styles of the two species in *Isopogon* were equivalent in length ($U = 54$, $P > 0.1$) as were their tepals (Table 2) and *I. anemonifolius* produced much larger pollen grains than *I. anethifolius* ($U = 100$, $P < 0.001$). Also, the styles of the two species in *Symphionema* were equivalent in length ($U = 51.5$, $n = 8$, 10, $P > 0.1$) and *S. paludosum* produced larger pollen grains than *S. montanum* ($U = 100$, $P < 0.001$).

Comparisons of species in other genera revealed a variety of relationships. The styles of *Petrophile sessilis* were shorter than those of *P. pedunculata* and *P. pulchella* (for each $U_s = 100 > U_{0.01[3,10]} = 89$) and the styles of the latter were equivalent in length ($U_s = 75$, $< U_{0.05[3,10]} = 81$). The pollen grains of *P. pedunculata* were smaller than those of *P. pulchella* ($U_s = 81$, $P = 0.05$) and *P. sessilis* ($U_s = 100$, $P < 0.01$) and the latter two species had equivalent sized pollen grains ($U_s = 56.5$, $P \gg 0.1$). In *Grevillea*, the pollen grains of *G. buxifolia* (bee-pollinated) were larger than those *G. laurifolia* (bird-pollinated) and *G. sphacelata* (bee-pollinated) ($U_s = 95$ and $U_s = 100$, $P < 0.01$, respectively) and those of *G. laurifolia* and *G. sphacelata* were equivalent in size ($U_s = 56$, $P > 0.1$) (Table 2). The styles of *G. buxifolia* were longer than those of both *G. laurifolia* and *G. sphacelata* ($U_s = 95$ and $U_s = 100$, $P < 0.01$, respectively) and those of *G. laurifolia* were longer than those of *G. sphacelata* ($U_s = 100$, $P < 0.01$). Finally, in *Conospermum* (Table 2) the styles of *C. taxifolium* were shorter than those *C. tenuifolium* ($U_s = 92.5$, $P < 0.01$) and *C. longifolium* ($U_s = 100$, $P < 0.01$), which were equivalent in length ($U_s = 50.5$, $P \gg 0.05$). The pollen grains of *C. taxifolium* were smaller than those of *C. longifolium* ($U_s = 87$, $P < 0.05$) and *C. tenuifolium* ($U_s = 100$, $P < 0.01$) and those of *C. longifolium* were smaller than those and *C. tenuifolium* ($U_s = 100$, $P < 0.01$).

I tested pollen grains of all the Proteaceae for starch, except *Symphionema paludosum* and *H. laevipes*, and all tested positive (also see Herscovitch and Martin 1989).

Discussion

In 1867, Delpino proposed that pollen grains contained sufficient resources to support the growth of their pollen tubes through the style (Delpino 1867, p. 286). Darwin rejected this idea and suggested that pollen tubes first grew autotrophically, i.e., through the stigma, and then heterotrophically through the style (Darwin 1884, p. 251). Despite evidence to the contrary Delpino's argument is still used to explain positive relationships between pollen grain size and style length (e.g., Ortega Olivencia et al. 1997; Torres 2000; Yang and Guo 2004). Below I briefly consider the relationships involving pollen grain size with stigma depth and style length, examine variation in floral traits among related species, and describe two sets of phenotypes that suggest the quite different phenotypes that related species might exhibit. Then I consider Delpino's proposition and suggest an alternative explanation for positive relationships between pollen grain size and style length. I examine Darwin's proposition by reviewing stigmatic and stylar structure and function, which show that pollen tubes are unlikely to acquire resources on or in the stigma but acquire a variety of resources in the style. I also consider variation in pistil traits, which dictate the distance pollen tubes have to grow autotrophically, and pollen grain traits that may reflect that distance.

The results presented above mirror those reported earlier by Cruden and Lyon (1985). There was a strong, positive correlation between pollen grain size and stigma depth among the species in Fabaceae whether all the species were considered or just those in Mirbelieae. In contrast, pollen grain size and style length were not correlated either among the legumes or 20 species in Proteaceae and among the legumes there was no correlation between pollen grain size and the distance from the stigma to the lowest ovule. Previously, we reported positive correlations between pollen grain size and stigma depth and no correlations between pollen grain size and style length among species in *Solanum* (Solanaceae) (6 spp.), Apiaceae (6 spp.), Brassicaceae (8 spp.), and 10 unrelated facultatively xenogamous species (Cruden and Lyon 1985). Also, my colleagues and I found no correlation between pollen grain size and style length among 19 unrelated species (Cruden and Miller-Ward 1981) or 14 species in *Erythrina* (Caesalpinaceae) (Cruden and Lyon 1985).

Comparisons of related species showed considerable variation in relationships among floral traits and provided mixed support for the two hypotheses. In two of the three comparisons involving species in Fabaceae pollen grain size was positively associated with stigma depth, i.e., consistent with Darwin's argument. In *Bossiaea* stigma depth and pollen grain size differed significantly between species and were positively associated but neither was associated with either style or keel length, which were equivalent in length. In *Pultenaea* pollen grain size was positively associated with stigma depth and also with style length and keel length. In *Dillwynia* pollen grain size was positively associated with both style length and keel length and stigma depths were equivalent.

Likewise, related species in Proteaceae exhibited considerable variation in the relationships among pollen grain size, style length, and flower size. In half of the comparisons (7/13) there was no relationship between pollen grain size and style length, thus inconsistent with Delpino's argument, in four of the comparisons there were positive associations among all three traits and in only two comparisons were the data clearly consistent with Delpino's argument. The seven comparisons include three different relationships between pollen grain size and style length as well as exhibiting different relationships with tepal length. In four comparisons the pollen grains of one species were larger than those of a second species but their styles were equivalent length. The tepals of the former were either longer (*Petrophile pulchella* vs. *P. pedunculata*), equivalent in length (species in *Isopogon* and *Symphionema*) or shorter (*Conospermum tenuifolium* vs. *C. longifolium*) than those of the second species. In two comparisons pollen grain size of the two species was equivalent but the styles of one species were longer. The tepals of the latter species were either longer (*Grevillea laurifolia* vs. *G. sphacelata*) or equivalent in length (*P. pulchella* vs. *P. sessilis*) to those of the second species. The third phenotype was illustrated by *P. pedunculata* whose pollen grains were smaller, its styles longer and tepals shorter than those of *P. sessilis*. In four comparisons all three traits were positively associated. These included comparisons of the species in *Hakea* and *Lomatia* as well as *G. buxifolia* with *G. sphacelata* and *C. longifolium* with *C. taxifolium*. Two comparisons were consistent with Delpino's argument. The pollen grains of *C. tenuifolium* and *G. buxifolia* were larger and their styles longer than those of *C. taxifolium* and *G. sphacelata*, respectively. The tepals of *C. tenuifolium* were shorter than those of *C. taxifolium* and those of *G. buxifolia* and *G. laurifolia* were equivalent in length.

The variation in relationships among the floral traits described above provides a striking contrast with the positive relationships among flower size, style length, pollen grain number and amount of pollen in various European legumes (e.g., Galloni et al. 2007; López et al. 1999; Ortega Olivencia et al. 1997; Small 1988). In addition to showing that flower size need not be correlated with other floral traits, my data illustrate some of the numerous combinations of traits and relationships exhibited by related species. Such variation is consistent with the assumption that the traits may evolve independently (Cruden 2000) and that related species should exhibit a

considerable number of phenotypes. Below I describe two sets of phenotypes to suggest the quite different phenotypes that might be exhibited by related species.

In a number of comparisons of related species flower size and style length were equivalent and, presumably, so were pollinator size and the position of the pollen bearing area on the pollinator (PBA) but there were striking differences in pollen traits. In *Isopogon* and *Symphionema* the tepals, styles and pollen presentation areas were equivalent in length and the positions of the PBA were equivalent. However, *I. anemonifolius* and *S. paludosum* produced fewer and larger pollen grains and less pollen than *I. anethifolius* and *S. montanum*, respectively (this study, Cruden 1997, personal observation). The production of fewer pollen grains and less pollen were associated with larger stigma areas (Cruden 1997), i.e., more efficient pollination (Cruden 1997, 2000). Similar relationships were observed between species in *Bossiaea* (Cruden personal observation) and *Cryptantha* (Casper 1983).

The second set of phenotypes is exhibited by species whose floral traits were positively correlated and differences in flower size might be indicative of functional relationships between various traits and pollinator size. In *Pultenaea* (this study, Cruden personal observation) and various European legumes (e.g., López et al. 1999; Ortega Olivencia et al. 1997) there were positive correlations among flower size, style length, pollen grain number, the amount of pollen and/or nectar volume (e.g., Galloni et al. 2007; López et al. 1999; Ortega Olivencia et al. 1997) as well as flower size with pollinator size (Herrera 2001). In addition, larger flowers frequently have larger anthers that form larger pollen presentation areas, which generate larger PBAs, thus it seems reasonable to assume that PBAs may be correlated with flower size and pollinator size. In essence, the positive relationship between flower size and pollinator size may be accompanied by positive relationships with a number of traits.

Delpino's hypothesis

The only support for Delpino's (1867) proposition that pollen grains contain sufficient resources to support the growth of their pollen tubes through a style are the numerous observations of correlations between pollen grain size and style length (e.g., Aguilar et al. 2002; Baker and Baker 1979, 1982; Bigazzi and Selvi 2000; López et al. 1999, 2006; Plitmann and Levin 1983; Roulston et al. 2000; Torres 2000; Yang and Guo 2004). It is explicit in Delpino's hypothesis and variants thereof that pollen grain size and style length are correlated but it is not evident how such models might accommodate taxa in which the two traits are not correlated, as was the case in various distylous species (Barrett et al. 2000; Casper 1983; Darwin 1884, pp. 250–252; Feinsinger and Busby 1987; Ganders 1979 and refs. therein, also see Hong and Han 2002) and among related homostylous species (this study; Cruden and Lyon 1985 and references therein, Knudsen and Olesen 1993; López et al. 2006; also see Small 1988). In most of the distylous species pollen grain size of the two morphs was equivalent but the styles or pistils of the long-styled plants were considerably longer than those of the short-styled plants. In contrast, pollen grain size differed between the two morphs of *Suteria* (Rubiaceae) and their styles were equivalent in length (Darwin 1884).

Equivalent relationships were observed between related homostylous species. The pollen grains of *Petrophile pulchella* and *P. sessilis* were equivalent in size but their styles differed in length as did those of *Grevillea laurifolia* and *G. sphacelata* (Table 2). The relationship was reversed in *Bossiaea*, (Fabaceae), *Isopogon*, *Symphionema*, *Conospermum*, and *Petrophile* (Proteaceae). The pollen grains of species in these genera differed in size but their styles or pistils were equivalent in length (Table 2). The same relationship was reported in Sonneratiaceae (Germeraad et al. 1968). Likewise, the styles of *Crinum erubescens* Solander and *Hymenocallis acutifolia* (Herb.) Sweet (Amaryllidaceae) were equivalent in length, i.e., 22–30 cm long, but the pollen grains of the former were half the size and its stigmas shallower than those of *H. acutifolia* (Cruden personal observation). Also, in sets of related taxa some had small pollen grains and long styles relative to others that had much larger pollen grains and shorter styles, e.g., *Adenanthos* and *Lambertia* vis-a-vis *Conospermum* and *Hakea* (this study) and species in *Trigonella* vis-a-vis *Medicago* (Small 1988). In only 3 of 16 comparisons, i.e., those involving species in *Dillwynia*, *Conospermum*, and *Grevillea*, was pollen grain size positively associated with style length and not positively associated with either stigma depth and/or flower size.

The positive relationship between pollen grain size and style length is explained if both are correlated with one or more other traits, for example, both pollen grain size and stigma depth were correlated with style length in *Polygonum* (Cruden and Lyon 1985) and *Pultenaea* (this study). In general, larger pollen grains are associated with deeper stigmas (this study, Cruden 2000; Cruden and Lyon 1985) and longer styles frequently have deeper stigmas, e.g., in many distylous species. In such plants positive correlations between pollen grain size and style length are probably inevitable. Variations on this theme might explain many of the correlations observed between pollen grain size and style length (see above).

Darwin's hypothesis

The available data are consistent with Darwin's (1884) observation that pollen tubes initially grow autotrophically, i.e., through the stigma, then heterotrophically through the style. The positive correlations between pollen grain size and stigma depth (this study, Cruden and Lyon 1985) are consistent with pollen grains containing sufficient resources to support the growth of their pollen tubes through the stigma and differences in the constituents of stigmatic exudates and the ECM are consistent with such growth being autotrophic (see below). Autotrophic growth through the stigma is obligatory in species with dry stigmas (e.g., Bigazzi and Selvi 2000) and there is no evidence that pollen tubes obtain nutrients from the stigmatic exudates of species with wet stigmas (e.g., Edlund et al. 2004; Graaf et al. 2001; Herrero and Hormaza 1996; Swanson et al. 2004) although there was some speculation to the contrary (Ciampolini et al. 1983). Further, in many species the stigmatic tissues below the stigmatic surface are anatomically and cytologically different from the transmission tissue in the style and have no secretory function (e.g., Bigazzi and Selvi 2000; Cresti et al. 1982; Herrero and Dickinson 1979).

The constituents of stigmatic exudates are generally different from those in the ECM (e.g. Ciampolini et al. 1981, 1995; Ghosh and Shivanna 1984; Herrero and Dickinson 1979; Heslop-Harrison and Heslop-Harrison 1982; Miki-Hirosige et al. 1987; Schmidt-Adam and Murray 2002; Webb and Williams 1988) and have different functions. Stigmatic exudates include molecules that are involved in pollen grain attachment, hydration, and germination, and may serve to protect the stigma from drying (e.g., Ciampolini et al. 1981; Edlund et al. 2004; Lord and Webster 1979; Swanson et al. 2004; Tilton and Horner 1980) whereas molecules in the ECM provide resources to growing pollen tubes (see above), control the direction they grow (e.g., Lush et al. 2000; Cheung et al. 2000; also see Swanson et al. 2004), influence their rates of growth and/or play a role in sexual selection (e.g., Herrero and Hormaza 1996; Hormaza and Herrero 1996; also see Swanson et al. 2004), and are involved in gametophytic incompatibility reactions (de Nettancourt 1977; Richards 1986).

In a few species the "stigmatic" exudate was produced by the stylar transmission tissue rather then the stigma, e.g., species in Amaryllidaceae (Ciampolini et al. 1990; Shivanna and Sastri 1981), Apiaceae (Weber 1994; Weber and Frosch 1995) and Commelinaceae (Owens et al. 1984). The styles, which were either hollow (Ciampolini et al. 1990; Owens et al. 1984; Shivanna and Sastri 1981), half-solid (Weber and Frosch 1995) or solid (Weber 1994), were continuous with the stigmatic surface. In the species with hollow styles the stigmatic and stylar exudates may have been similar but the staining of the exudate produced by *Smyrnium* (Apiaceae) suggested that the exudate that reached the stigmatic surface was different from that in the transmission tissue (Weber 1994). Also, Clarke et al. (1979) suggested that equivalent molecules may have different functions on the stigma vis-a-vis in the transmission tissue.

In contrast to the stigmas, the extra cellular matrix (ECM) of the stylar transmission tissues of many, if not most, species provide resources to the pollen tubes and others are synthesized as pollen grains germinate and/or in the pollen tubes (see below). A variety of molecules were taken up from the ECM and utilized by pollen tubes, including sugars (Wu et al. 1995), polysaccharides (Labarca and Loewus 1972), nucleic acids (Campbell and Ascher 1975), amino acids (Gawlik 1984), and proteins (Cheung 1996; Lind et al. 1996). Other studies reported that resources, which disappeared from styles with compatible pollen tubes, were present in the styles of unpollinated controls (e.g., González et al. 1996) and those with incompatible pollen tubes (Herrero and Dickinson 1979). Also, the disappearance of starch or other molecules from the transmission tissue and/or ECM concomitant with the passage of compatible pollen tubes (e.g., Cruden and Lyon 1985; Hopping and Jerram 1979; Schmidt-Adam and Murray 2002; also see Cresti et al. 1982) is consistent with their being utilized by those pollen tubes.

Further, differences in the growth rates of pollen tubes in the stigma and style are consistent with the pollen grains obtaining resources from ECM. In *Lilium*, *Petunia* and *Gasteria* the rate of growth through the stigma was 1/10–1/2 that in the style (Herrero and Dickinson 1980; Janson et al. 1994; Lubliner et al. 2003; Willemse 1996) and in *Petunia* the change in growth rate was correlated with the transition zone between the stigma and style (Herrero and Dickinson 1980; Lubliner et al. 2003).

A few workers have speculated that the relationship between pollen grain size and style length reflects a resource found only in the pollen grains, e.g., a mRNA (Plitmann and Levin 1983) or a protein (Roulston et al. 2000). The observation that mRNAs in pollen tubes are long lived is consistent with the hypothesis. However, there is evidence of transcription of mRNAs in pollen tubes (Mascarenhas 1993; Weterings et al. 1992, 1995; also see Haskell and Rogers 1985; Süss and Tupý 1982; Tupý et al. 1977) and though this may not falsify the hypothesis it raises doubts about its general applicability. Also, recent studies, which show that in vitro assays give less reliable results then in vivo assays (Hiscock et al. 1995; also see Lord 2001), cast doubt on earlier studies that failed to detect transcription or translation in pollen tubes.

Roulston et al. (2000) showed that the amount of protein in pollen grains was positively correlated with both pollen

grain size and style length and speculated that a protein might be a limiting factor. This argument may explain the relationship among species whose pollen grains contain sufficient protein to grow through the style (Mascarenhas 1993) but not in species whose pollen grains failed to germinate if translation was inhibited or translation occurred during pollen grain germination and/or in the pollen tubes (Hulzink et al. 2002; Mascarenhas 1993 and references therein, Taylor and Helper 1997). For example, Hiscock et al. (1995) identified nearly 40 proteins in pollen grains germinating in vivo that were not present in ungerminated pollen grains and had not been detected with in vitro assays. In addition, several workers reported the uptake of proteins from the ECM and their utilization by pollen tubes (Cheung 1996; Lind et al. 1996). In effect, mature pollen grains need not contain all the proteins required by their pollen tubes.

Variation in pistil and pollen traits

Most recent discussions of the relationship between pollen grain size and style and/or pistil length have ignored the substantial variation in the structure and function exhibited by angiosperm pistils. Stigmas, which are generally classified as either wet or dry, are morphologically diverse, ranging from a fringe of papillae at the tip of the style, e.g., species in Commelinaceae (Owens et al. 1984), to quite long lobes with complex anatomies, e.g., species in Onagraceae (Dickinson and Lawson 1975). The different kinds of stigmas may be associated with styles that are hollow, half-solid, or solid. In addition, the stylar transmission tissue may be continuous with the stigmatic surface, contiguous with it, or separated from it by non-secretory tissue.

Likewise there is considerable variation in pollen grain size and storage products that may serve as a source of energy for the autotrophic growth of pollen tubes. Differences in pollen grain size are well documented and numbers of studies distinguished between starch containing, i.e., starchy, and starchless pollen grains (e.g., Baker and Baker 1979; Franchi et al. 1996; Grayum 1985). Starchless pollen grains were frequently small and tested positive for lipids (Baker and Baker 1979; Grayum 1985; also see Roulston and Buchmann 2000). The earlier studies generated the idea that if pollen grains did not store starch they stored lipid, which would provide sufficient energy to support the growth of pollen tubes through relatively long styles. This conclusion reflected, at least in part, the universal acceptance and application of the Delpino model. However, recent studies showed that pollen grains may store sugars or a mix of sugars and starch (Speranza et al. 1997), starch and lipids (Wang et al. 2004) and the pollen grains of legumes may contain sugars, which is suggested by their swelling when exposed to water (this study), as well as lipids (Baker and Baker 1979). The variation in energy sources for pollen tube growth makes considerable sense in the context of the variation in the distance pollen tubes grow autotrophically.

In addition, storage products may have functions other than providing a source of energy to growing pollen tubes. Sugars in the cytoplasm may play a role in protecting pollen grains from desiccation (Pacini 1996; also see Roulston and Buchmann 2000) and lipids may be utilized in membrane synthesis. In a recent study, Rodríguez-Garcia et al. (2003) reported that lipid in the pollen grains of *Olea europea* L. was mobilized and moved into the pollen tube shortly after germination and disappeared as the pollen tube elongated. This is consistent with incorporation into cell membranes as well as serving as a source of energy. Finally, pollen grain size may reflect selective pressures other than those associated with providing resources for pollen tube growth (Cruden 2000). For example, it takes fewer large pollen grains to produce a pollen population effect relative to small pollen grains. This is due, at least in part, to the amounts of compounds sequestered in the exine that facilitate the growth of pollen tubes through the stigmatic surface and, in general, they should be proportional to pollen grain size. Thus, relatively large pollen grains might contain relatively small amounts of storage products and be associated with short distances between the stigmatic surface and the stylar ECM.

Stigma-pollen relationships

The differences in the structure and function of stigmas and styles coupled with differences in pollen grain traits suggest that plants should exhibit considerable variation in relationships between them. First, the pollen grains of plants whose pollen grains are large and styles quite short might contain sufficient resources to grow the length of the style autotrophically. In such taxa there should be a correlation between pollen grain size and style length and/or the distance from the stigma to the lowest ovule and their ECMs might lack the nutrients and other resources utilized by pollen tubes.

Second, in species whose stylar transmission tissues are continuous with the stigmatic surface the pollen tubes have only to grow across the stigmatic surface to reach resources in the stylar transmission tissue (e.g., Ciampolini et al. 1990; Ghorbel and Nabli 1998; Owens et al. 1984; Shivanna and Sastri 1981: Slater and Calder 1990; Weber 1994; Weber and Frosch 1995). The pollen grains are expected to be small and/or store sugar as a source of energy. Given the short distances involved, differences in pollen grain size might be quite subtle and difficult to detect.

Third, in a number of species the stylar transmission tissue was contiguous with the stigmatic surface (e.g., Ciampolini et al. 1995; Heslop-Harrison et al. 1981; Owens et al. 1984; Ghosh and Shivanna 1984; Tilton and Horner 1980; Weber 1994; Welk et al. 1965). In such species the stigmatic surface is composed of one to a few layers of secretory cells, thus pollen tubes have to grow through them to reach the stylar transmission tissue and their pollen grains are expected to be smaller and/or store sugar. If stigma depth varies among related species a positive relationship between pollen grain size and stigma depth is expected.

Fourth, in many species the stigmatic surfaces and stylar transmission tissues are separated by non-secretory tissue (e.g., Bigazzi and Selvi 2000; Ciampolini et al. 1983; Cresti et al. 1982, 1986; Ghosh and Shivanna 1982; Herrero and Dickinson 1979; Herscovitch and Martin 1989; Jensen and Fisher 1969; Kenrick and Knox 1981; Olson 1991; Shivanna and Owens 1989; Williams et al. 1982), which may be a mm or more in depth. Pollen grain size should vary considerably and smaller pollen grains might store lipid and larger pollen grains starch and/or sugar. Because the distance between the stigmatic surface and the stylar transmission tissue is variable one might expect a positive relationship between pollen grain size and stigma depth, especially among related species, as was the case among species in *Polygonum*, *Solanum* (Cruden and Lyon 1985) and Mirbelieae (this study).

In addition, species in a number of families have long stigma lobes, e.g., in Amaryllidaceae, Cactaceae and Onagraceae, or long style branches, e.g., in Iridaceae, Xyridaceae, and Malvaceae. Many species in these families have large or oversize pollen grains and those of species in Onagraceae and Malvaceae stored starch. Even so, it seems unlikely that such pollen grains contain sufficient resources to grow through quite long stigma lobes and style branches, e.g., 10 mm in *Oenothera* (Dickinson and Lawson 1975) and 7 mm in *Gladiolus* (Ameele 1982). The large, starch containing pollen grains of *Oenothera* may reflect their very thick stigma lobes. Likewise, the stigmas of many Malvaceae are quite deep. In these species there should be a positive relationship between pollen grain size and distance to the transmission tissue in the stigma lobes or style branches.

Finally, Delpino's explanation for the relationship between pollen grain size and style length was attractive because it was simple, easily understood, and made intuitive sense. The biological reality, as outlined above, is quite different. The variation in structure and function observed in pistils and pollen grains was paralleled by considerable variation in other floral traits as well as the relationships among them. The distance that pollen tubes grow autotrophically varies considerably and that distance should be reflected in pollen grain size and the energy source for the autotrophic growth of the pollen tubes. This may be reflected in the positive relationships observed between stigma depth and pollen grain size among related genera and species (this study, Cruden and Lyon 1985).

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Appendix 1: Sources of material included in this study

Species	Collection number	Location
Fabaceae		
Aotus ericoides (Vent.) G. Don	06-1	1
Bossiaea ensata Sieber ex DC.	06-7	5
Bossiaea obcordata (Vent.) Druce	06-12	3
Daviesia alata Sm.	06-15	9
Dillwynia floribunda Sm.	06-2	1
Dillwynia retorta (J. C. Wendl.) Druce	06-36	14
Gomphilobium grandiflorum Sm.	06-11, 08-12	5
Hardenbergia violacea (Schneev.) Stearn	06-13, 08-17	10/16
Indigofera australis Willd.	06-16, 08-15	12
Mirbelia rubiifolia (Andrews) G. Don.	06-3	2
Phyllota humifusa Benth.	06-9, 08-13	5
Pultenaea linophylla Schrad.	06-4	1
Pultenaea tuberculata Pers.	06-10, 08-14	5
Pultenaea villosa Willd.	06-19	13
Sphaerolobium minus Labill.	06-5	5
Proteaceae		
Adenanthos detmoldi F. Muell.	952559	WA, Scott River.

continued

Species	Collection number	Location
Conospermum longifolium Sm.	06-37	6
Conospermum taxifolium Sm.	06-8	5
Conospermum tenuifolium R. Br.	06-20	13
Grevillea buxifolia (Sm.) R. Br.	06-27	7
Grevillea laurifolia Sieber ex Spreng.	06-34	15
Grevillea sphacelata R. Br.	08-20	7
Hakea laevipes Gand.	06-39	8
Hakea teretifolia (Salisb.) Britton	06-23	4
Isopogon anemonifolius Knight	06-30	5
Isopogon anethifolius (Salisb.) Knight	06-26	6
Lambertia formosa Sm.	06-28	5
Lomatia fraserii R. Br.	860533	Victoria, Bendoc.
Lomatia silaifolia (Sm.) R. Br.		6
Petrophile pedunculata R. Br.	06-41	11
Petrophile pulchella (Schrad.) R. Br.	06-40	6
Petrophile sessilis Sieber ex Schult. and Schult. f.	06-38	6
Symphionema montanum R. Br.	06-35	15
Symphionema paludosum R. Br.	06-6	5
Telopea speciosissima (Sm.) R. Br.		11

Locations are in eastern New South Wales to the south and west of Sydney. The species name is followed by my collection number and the number of the collection locality. Collections made in 2006 are indicate by 06- and those collected in 2008 by 08-. Material of two species was obtained from the Mt. Annan Botanic Garden, Mt. Annan, NSW. The species name is followed by the garden's accession number and locality

Northwest of Wollongong:

1. Intersection of Mt. Kiera Rd. and Harry Graham Rd. (road to Mt. Kembla).
2. Picton Road, 30–50 m east of Mt. Kiera Rd.
3. Cordeaux Dam Road, ca. 0.7 km south of Picton Road (Rt. 88).

Route 60 northeast of Wollongong:

4. Route 60, between Sublime Point and Darkes Forest Road, opposite Bomerang Golf Course.
5. Darkes Forest Road, ca. 200 m north and south of track into the Dharawal State Recreation Area (near end of paved road).
6. Woronora Dam Road, ca. 4.7 km N Route 60.
7. Woronora Dam Road, SE of track leading to Sarahs Knob.

Route 9, southeast of Robertson:

8. Route 9, ca. half way between Carrington Falls Road and Barren Grounds Nature Preserve.
9. Carrington Falls Road, ca. 0.8 km from Route 9.
10. Carrington Falls Road, ca. 1.4 km from Route 9.
11. Carrington Falls Road, 0.5–1.0 km south of Kangaroo River.

Belmore Falls Road, south of Robertson:

12. Ca. 6.8 km from Route 48 in Robertson (6.1 km from South Street).
13. Ca. 7.6 km from Route 48 in Robertson (6.9 km from South Street).

Blue Mountains:

14. Wentworth Falls, below car park.
15. Blackheath. Fairfax track, in woods at Govett's Leap

Road to Wombeyan Caves:

16. Ca. 15 km NW of the tunnel and ca. 31 km NW of Greenhills Rd.

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