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The relationship between the pollen–ovule ratio and pollen size: another comparative test of a sex allocation hypothesis

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ABSTRACT

Hypothesis: Sex allocation theory predicts that the pollen–ovule ratio should decrease linearly with increasing pollen size among seed plants (Charnov, 1982).

Data examined: We retrieved data for pollen–ovule ratio, pollen size, pollen grain number, and mating system from published literature for 311 angiosperm plant species.

Methods: We used model II regressions on cross-species data as well as on phylogenetically independent contrasts (PIC) to quantify the relationship between the pollen–ovule ratio and pollen size. Partial correlations were applied to test if an association between these two traits arises because of a correlation with a third variable, the number of pollen grains.

Results: A linear negative correlation between the pollen–ovule ratio and pollen size does exist for these plant species, both in phylogenetically corrected and uncorrected data. However, the correlation was not consistently found at the taxon and mating system levels. For virtually all groups investigated, the correlation disappeared when we controlled for the effect of pollen grain number. Thus the correlation between the pollen–ovule ratio and pollen size is spurious.

Conclusions: Considering male function, the pollen–ovule ratio depends on the number of pollen grains produced by a flower but not on the size of the pollen grains. For the 'male part' of Charnov's model, its validity can be called into question.

Keywords: comparative analysis, mating system, pollen–ovule ratio, pollen size, sex allocation.

INTRODUCTION

The ratio of pollen to ovules per flower, the pollen–ovule ratio, shows an enormous interspecific variation, spanning six orders of magnitude. For instance, the cleistogamous species *Draba reptans* (Cruden, 1977) produces an average of only four pollen grains per ovule, while wind-pollinated species (e.g. *Betula pendula*) can produce up to 2 million pollen grains per ovule (Pohl, 1937). Two hypotheses have been put forward to explain this variation. First, Cruden, who made the discovery that pollen ovule–ratios are correlated with the degree

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of outbreeding, suggested that the efficiency of pollination shapes the evolution of pollen–ovule ratios (Cruden, 1977). According to this concept, outbreeding plants have high pollen–ovule ratios to guarantee efficient pollination by pollen vectors such as wind, water, and animals. Selfing species require fewer pollen grains produced per ovule to ensure pollination, as the physical distance from the place of pollen production to the stigma is much shorter and overcoming this distance is less complicated. Second, Charnov claimed that the pollen–ovule ratio is determined by the allocation of resources to male and female sex function. The theory reflects the idea that both pollen (male function) and ovules (female function) are equivalent means towards fitness gains (Charnov, 1982). The proportions of resources invested in male function (r), female function ($1 - r$), pollen grain size (C_1), and ovule size (C_2) are related mathematically to the pollen–ovule ratio by the following equation:

$$\log\left(\frac{P}{O}\right) = \log\left(\frac{r}{1-r}\right) + \log C_2 - \log C_1 \tag{1}$$

With respect to this equation, ovule size is reasonably represented by seed size to include resource investment in maturing seeds (Queller, 1984). Given a constant value for r, which Charnov stated should be realized by an evolutionarily stable strategy r^* within a mating system, the pollen–ovule ratio would decrease linearly with increasing pollen grain size if seed size is held constant. In support of his hypothesis, Charnov (1982) found a significant negative relationship between the pollen–ovule ratio and pollen grain volume with a functional regression slope of −1.42 among 19 bee-pollinated species. The data of this preliminary analysis stemmed from an analysis by Cruden and Miller-Ward (1981), who also predicted decreasing pollen–ovule ratios with increasing pollen grain size. This prediction, however, was embedded in the 'efficiency hypothesis' of Cruden. The authors argued that bigger pollen grains contain more compounds that are necessary for germination on and penetration of the stigmatic surface by the pollen tube. Thus 'fewer large grains should be required per seed than small grains'. Queller (1984) argued that the two theories are not mutually exclusive but are supported by theoretical considerations of the sex allocation model.

There is qualitative evidence in the literature that large pollen–ovule ratios coincide with small pollen grains and vice versa (e.g. Mazer and Hultgard, 1993; Barrett *et al.*, 1996; Affre and Thompson, 1998). Some authors (Mione and Anderson, 1992; Gallardo *et al.*, 1994) have addressed this hypothesized relationship directly and found evidence to support Charnov's model for small, taxonomically restricted data sets. However, the reverse pattern, a positive correlation between the pollen–ovule ratio and pollen size, has been observed (Rodriguez-Riano *et al.*, 1999; Tate and Simpson, 2004), although these results were not discussed in detail in terms of Charnov's model. To clarify the contrasting results and test the generality of Charnov's model, a thorough analysis with a larger number of species from different families and across mating systems is desirable. For this purpose, we compiled data for pollen sizes and pollen–ovule ratios from the literature for 311 species. Some of the data sets collected for this study have already been evaluated in the original papers. Thus, it is necessary to point out that some of the correlations at or below the family level in this paper have already been reported elsewhere. This is true in particular for the pollen size–number trade-off within *Pedicularis* (Yang and Guo, 2004) and the correlations between pollen size, pollen grain number, and pollen–ovule ratio within the Fabaceae (Rodriguez-Riano *et al.*, 1999) and *Tarasa* (Tate and Simpson, 2004). However, this study adds to these previous ones by pooling different data and applying statistical methods that have not been used before.

When testing for the hypothesized negative relationship between the pollen–ovule ratio and pollen size, several important issues that might influence the results need to be addressed. Previous studies have presented evidence for a trade-off between pollen size and pollen number – that is, species that produce big pollen grains tend to make less pollen than species that produce relatively small pollen grains (Vonhof and Harder, 1995; Yang and Guo, 2004; but see Cruden and Miller-Ward, 1981; Lopez *et al.*, 1999; Aguilar *et al.*, 2002; Lopez *et al.*, 2005). Assuming also that a positive relationship exists between the pollen–ovule ratio and pollen grain number per flower, any correlation between two of these three variables might be a spurious correlation resulting from correlations of the remaining variable pairs. Furthermore, sex allocation theory predicts that outcrossing species allocate relatively more resources to male function than selfing species (Charlesworth and Charlesworth, 1981; Charnov, 1982). The $\log(r/1 - r)$ term in equation (1) would thus be different for selfing and outcrossing species. Because this term represents the intercept of the regression line of pollen size regressed on pollen–ovule ratio, we would expect the elevations of the slopes (i.e. the intercepts) to differ among mating systems. Finally, a recent study of the relationship between the pollen–ovule ratio and seed size – another prediction of equation (1) – demonstrated the importance of the taxonomical level at which such analyses are performed (Götzenberger *et al.*, 2006).

Consequently, the objectives of our study were to: (1) ascertain if log(pollen size) decreases proportionally with increasing log(pollen–ovule ratio) as predicted by equation (1); (2) determine whether a correlation between these two traits is direct or arises from correlations with pollen grain number; and (3) establish if the relationship between the pollen–ovule ratio and pollen size differs among taxonomic groups and mating systems with respect to regression slope and elevation. The results of the analysis of the relationship between the pollen–ovule ratio and seed size in Götzenberger *et al.* (2006) supported Charnov's hypothesis; the present study can be seen as testing another aspect of Charnov's model to further promote our understanding of sex allocation in plants, particularly factors that shape the wide variation in pollen–ovule ratios among angiosperm plant species.

METHODS

Data sources and supertree building

We compiled data on pollen–ovule ratios and pollen size opportunistically from published literature. Our data set consists in great part of species for which both traits were provided in the same literature source. For some species, pollen size was measured by one of the co-authors. Most often, volume was used to denote pollen size but in some papers pollen grain diameter was used. Therefore, analyses across all species were calculated on pollen volume data while within-taxon analyses were based on pollen volumes or lengths, depending on what measure was available for the relevant taxon. Wherever possible, data on mating system and pollen number produced per flower were obtained from the same source. We had to combine different mating system categorizations, so that the resulting categories were: (1) outcrossed (species stated as self-incompatible, xenogamous or facultative xenogamous) and (2) selfed (species stated as autogamous or facultative autogamous). Facultative xenogamous and facultative autogamous species are sometimes referred to as mixed mating systems because they are able to self and to outcross. However, facultative xenogamous species predominantly outcross and facultative autogamous species predominantly self, so that we assigned them to the outcrossed and selfed categories, respectively.

To account for the phylogenetic relationship of the species in our analysis (see Statistical analyses), a phylogenetic tree was necessary. Therefore, we built an informal supertree by using the APGII phylogeny (Bremer *et al.*, 2003) to assign the species to orders and families. Within-family and -genera relationships were extracted from published phylogenies and grafted onto the family branches (see references at http://evolutionary-ecology.com/data/2142app1.pdf). Except for one genus (*Ononis*), these phylogenies were based on molecular markers. Because of the different molecular markers (ITS, rbcL, chloroplast DNA, etc.) used to infer phylogenetic relationships for different parts of the tree, it was not possible to assign equivalent branch lengths for the whole tree. We used two different trees for further statistical analyses: one tree with all branch lengths set equal, whereas the branch lengths of the second tree were adjusted with the bladj function in the Phylocom software (Webb *et al.*, 2005). This program assigns branch lengths according to the node ages of orders and families given in Wikstrom *et al.* (2001) to the internal nodes of the phylogenetic tree. Then all other branch lengths are defined by setting the nodes evenly between dated nodes and between undated nodes and terminals. Terminals represent the extant species with age 0. The resulting phylogenetic tree and accompanying data can be viewed at http://evolutionary-ecology.com/data/2142app2.pdf.

Statistical analyses

In comparative analyses, species cannot be regarded as independent data points because they share common ancestors (Felsenstein, 1985; Harvey and Pagel, 1991). Therefore, we used the method of phylogenetically independent contrasts (PIC) to incorporate phylogeny in our analyses. Based on the assumption of a Brownian model of evolution, the method calculates differences (contrasts) between pairs of species and pairs of nodes that share a common ancestor in a phylogenetic tree (for details, see Felsenstein, 1985). To compare data analyses that incorporate phylogeny with analyses that do not, we present results for PIC together with those for cross-species analysis (CSA), a statistical method that does not consider phylogeny. Phylogenetically independent contrasts were calculated with the CAIC software (Purvis and Rambaut, 1995). Before calculation, we excluded clades or parts of clades from the phylogenetic tree that were highly unresolved because simulation studies have shown that the statistical power of correlation analyses diminish when polytomies are present in the phylogenetic tree (Purvis *et al.*, 1994). The analysis of PIC with respect to the assumptions made by the evolutionary and statistical models involved (see Purvis and Rambaut, 1995) revealed a violation of these assumptions, particularly at the within-taxon level, for the tree with adjusted branch lengths. The violations did not occur in the tree with equal branch lengths in most cases, so that we decided to calculate PIC without the available branch lengths information. Purvis *et al.* (1994) showed that equal branch lengths do not substantially affect the validity of correlation analyses compared with 'correct' branch lengths.

Because one of our main objectives was to test the slope of the pollen sizes regressed on the pollen–ovule ratios, it was important to choose the appropriate regression method. Ordinary least square regression underestimates the slope when both variables are measured with error. A variety of methods have been proposed to make inferences about the slope when measurement error exists in the response as well as the explanatory variable. Although there has been debate in the literature when to prefer one of these methods over another, no clear rules can be derived from that literature and different authors recommended different methods. Recently, Warton *et al.* (2006) reviewed the different methods in the context of allometry and summarized and clarified criteria for when to use a particular method. Following Table 1 + 2 in their paper, we chose standardized major axis (SMA) regression as the appropriate method for our analyses. To measure the strength of the correlation between the pollen–ovule ratio and pollen size, we also report correlation coefficients. Warton *et al.* (2006) also modified the method algorithms so they can be used to analyse PIC, which requires forcing the estimated slope through the origin and increasing the residual degrees of freedom from $(n-2)$ to $(n-1)$. For all estimated slopes, 95% confidence intervals were calculated following Pitman (1939). To determine whether the log(pollen-ovule ratio)–log(pollen size) relationship shows the same pattern among different mating systems and taxa, we tested slopes and intercepts for heterogeneity. A common slope was estimated according to Warton and Weber (2002) and tested for heterogeneity by a likelihood ratio test. Given that the common slope was significant, elevations were compared by calculating a common intercept and using the Wald statistic for inference (see Warton and Weber, 2002). We compared elevations for CSA only because the intercept is forced through the origin for PIC.

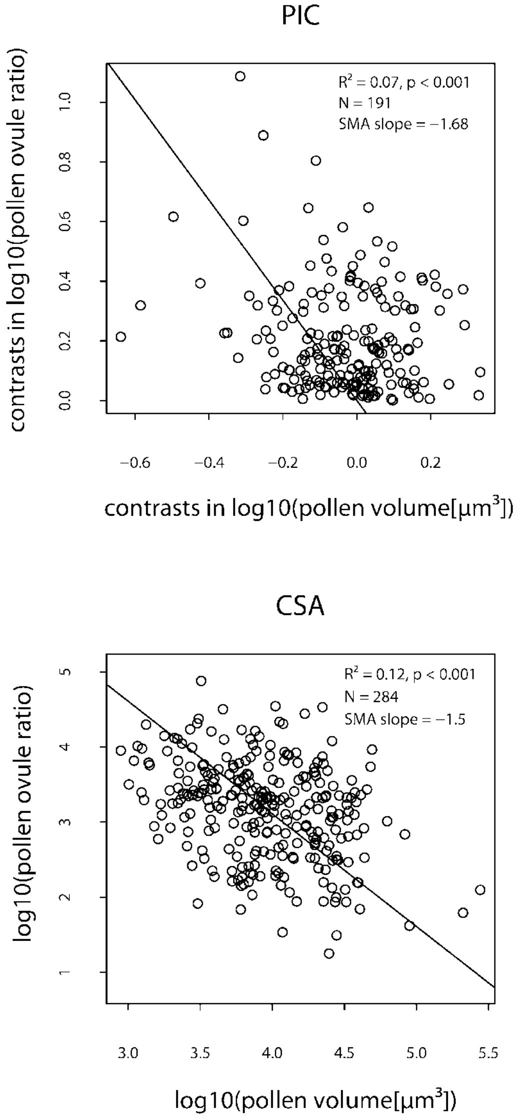
We used first-order partial correlation analysis to establish if the number of pollen grains produced per flower has a confounding effect on the relationship between pollen size and the pollen–ovule ratio. The correlations were calculated for all species but also within the different mating systems, families, and tribes. When multiple tests are applied, it has become standard to 'adjust' critical P-values to control for the fact that the probability of obtaining significant results by chance increases with the number of tests. The commonly applied Bonferroni adjustment and its derivatives (Holm, 1979; Rice, 1989), however, were objected to by Moran (2003) for mathematical, logical, and practical reasons. We followed his suggestions and scanned our results for repeated correlative patterns and deviations thereof instead of searching for statistical significance.

All statistical tests were carried out in the statistical package R (R Development Core Team, 2005). The functions that are needed to calculate the SMA analyses described above are available in the smatr library for R (Warton and Ormerod, 2005).

RESULTS

Both pollen–ovule ratios and pollen volumes showed a manifold variation. Pollen–ovule ratios in the data set ranged from 6 to 75,646 pollen grains per ovule and pollen volumes ranged from 890 to 277,260 μm^3. As expected, pollen–ovule ratios decreased with increasing pollen volume (Fig. 1). This relationship was weak but highly significant for both cross-species analysis (CSA) and phylogenetically independent contrasts (PIC) (CSA: $R^2 = 0.12$, d.f. = 283, $P < 0.001$; PIC: $R^2 = 0.07$, d.f. = 190, $P < 0.001$). The SMA slopes were negative (CSA: −1.5; PIC: −1.68) but the confidence intervals for both CSA and PIC did not encompass a negative unity slope [CSA: confidence interval (CI) = −1.35, −1.68; PIC: CI = −1.46, −1.98).

Of nine families, only three (Lamiaceae, Malvaceae, and Scrophulariaceae) showed a significant relationship for CSA (Table 1, Fig. 2). According to the confidence intervals, the slopes for the Lamiaceae and Scropuhlariaceae were not different from −1. When the PIC data were analysed, the Scrophulariaceae and Solanaceae showed a significant relationship; both had a negative slope of unity according to the confidence limits. Only the



PIC

CSA

Fig. 1. Plot of the pollen–ovule ratio versus pollen volume for phylogenetically independent contrasts (PIC) and cross-species analysis (CSA). Lines depict the SMA regression slope. R^2 and P values from ordinary least square regression.

Scrophulariaceae showed a significant negative relationship with a negative unity slope for both CSA and PIC analyses. Remarkably, within the Solanaceae there was a significant negative correlation between the pollen–ovule ratio and pollen size when the PIC data were analysed, while the results of CSA showed a non-significant relationship. Opposite to the model prediction, the correlation was positive for the Malvaceae for CSA.

Analyses at the tribal level within the Fabaceae, Solanaceae, and Zingiberaceae only revealed significant relationships in Fabaceae tribes. Within the Fabaceae, the results were

Table 1. Results of standardized major axis (SMA) regression between the pollen–ovule ratio and pollen size within families for cross-species analysis (CSA) and phylogenetically independent contrasts (PIC)

Family	SMA slope	Lower CL	Upper CL	Intercept	r^2	d.f.	P
PIC							
Asteraceae	3.60	1.50	8.62	—	0.26	5	0.242
Brassicaceae	−2.33	−0.60	−9.12	—	0.01	3	0.851
Ericaceae	9.87	3.01	32.37	—	0.34	3	0.298
Fabaceae	−1.54	−1.28	−1.84	—	0.02	117	0.183
Lamiaceae	−1.12	−0.52	−2.42	—	0.45	5	0.097
Malvaceae	4.94	3.12	7.82	—	0.13	17	0.124
Scrophulariaceae	−1.18	−0.92	−1.52	—	0.78	16	<0.001
Solanaceae	−1.92	−0.97	−3.81	—	0.59	5	<0.05
Zingiberaceae	−7.21	−3.87	−13.43	—	0.00	11	0.998
CSA							
Asteraceae	1.81	0.80	4.12	0.89	0.16	6	0.334
Brassicaceae	−3.37	−0.86	−13.12	16.30	0.02	3	0.828
Ericaceae	7.87	3.28	18.85	−8.92	0.26	5	0.243
Fabaceae	−1.73	−1.48	−2.02	9.97	0.01	158	0.149
Lamiaceae	−1.41	−0.77	−2.58	9.25	0.69	5	<0.05
Malvaceae	7.14	4.87	10.46	−29.63	0.18	23	<0.05
Scrophulariaceae	−1.06	−0.80	−1.39	7.21	0.28	39	<0.001
Solanaceae	1.80	1.15	2.82	−2.87	0.00	20	0.994
Zingiberaceae	−8.13	−4.66	−14.17	17.37	0.05	13	0.435

Note: P and r^2 values are from Pearson correlation tests. Intercepts for PIC are not given because regression is forced through the origin. CL = 95% confidence limit.

inconsistent when comparing tribes as well as when comparing results for CSA versus PIC (Table 2). For CSA, we found a significant relationship between the pollen–ovule ratio and pollen volume for the Fabeae (syn. Viceae), Loteae, and Trifolieae. For the Fabeae, the relationship was positive and only the Loteae had a negative unity slope. For PIC, only the Fabeae showed a significant positive relationship.

When comparing slopes and elevations among different taxa, we only included groups that at least had a marginally significant ($P < 0.10$) correlation and a negative slope. Thus, at the family level we could only compare the Lamiaceae, Scrophulariaceae, and Solanaceae for PIC and the Lamiaceae and Scrophulariaceae for CSA. At the tribal level, the Loteae and Trifolieae could be compared for CSA. The SMA regression slopes of the tested families were homogeneous for both CSA and PIC (CSA: likelihood ratio = 2.06, $P = 0.36$; PIC: likelihood ratio = 0.93, $P = 0.34$). With respect to elevation we could only compare the Lamiaceae and Scrophulariaceae for CSA. The elevations of the Lamiaceae and Scrophulariaceae were significantly different from each other (Wald statistic = 16.95, $P < 0.001$). Slopes of the regression of pollen–ovule ratio on pollen size among the tested Fabaceae tribes were heterogeneous (likelihood ratio = 6.69, $P = 0.01$) and thus could not be tested for a common elevation.

Species with an allogamous mating system showed a negative relationship between the pollen–ovule ratio and pollen size for both CSA and PIC (Table 3). The confidence intervals

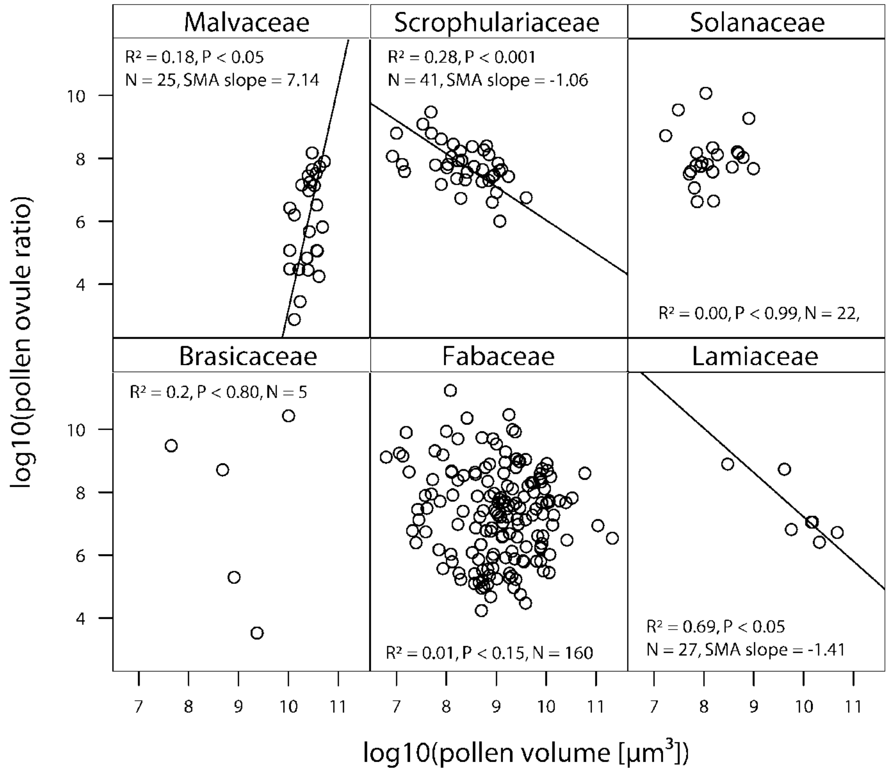


Fig. 2. Plot of the pollen–ovule ratio versus pollen size within families to demonstrate different forms of relationships observed. Only families for which data on pollen size were available as pollen volume measures are shown. Data points are recent species (CSA). Lines depict the SMA regression slope. R^2 and P values from ordinary least square regression. Lines are absent when the regression model was insignificant.

for CSA encompassed a negative unity slope, whereas those for PIC did not. Within the autogamous species there was a significant negative relationship for CSA but not for PIC. Because the SMA regression slopes of allogamous and autogamous species differed from each other (likelihood ratio = 10.3, $P = 0.0013$), we could not test if the slopes share a common elevation.

Results of partial correlation analyses revealed that the overall significant relationship between the pollen–ovule ratio and pollen size is not a direct relationship. We could not detect a significant correlation when removing the effect of pollen grain number (Fig. 3). For both CSA and PIC, the correlations between the pollen–ovule ratio and pollen grain number and between pollen size and pollen grain number remained significant when controlling for the effect of pollen size and pollen–ovule ratio, respectively (Fig. 3).

We summarize the results of (partial) correlations within the mating systems and taxa in Table 4 (view detailed results at http://evolutionary-ecology.com/data/2142app3.pdf). The most recurrent pattern was a strong and highly significant correlation between the pollen–ovule ratio and pollen grain number, when the effect of pollen size was accounted for (Table 4). Both mating systems and 11 of 16 taxa analysed showed this pattern. The

Table 2. Results of standardized major axis (SMA) regression between the pollen–ovule ratio and pollen size within tribes for cross-species analysis (CSA) and phylogenetically independent contrasts (PIC)

Tribe	SMA slope	Lower CI	Upper CI	Intercept	r^2	d.f.	P
CSA							
Tribes within Fabaceae							
Fabeae	1.85	1.33	2.57	−4.48	0.48	21	<0.001
Galegeae	−3.11	−1.67	−5.77	15.37	0.01	12	0.726
Genisteae	1.45	1.00	2.11	−2.23	0.01	29	0.619
Loteae	−1.06	−0.74	−1.50	7.01	0.24	26	<0.01
Trifolieae	−1.86	−1.46	−2.38	10.07	0.14	57	<0.01
Tribes within Zingiberaceae							
Alpinieae	1.98	0.86	4.54	−1.22	0.35	5	0.164
Hedychieae	−16.55	−7.08	−38.70	32.65	0.31	5	0.194
'Subgroups' within Solanaceae							
A	1.48	0.67	3.26	−1.97	0.21	7	0.588
B	−2.44	−1.38	−4.32	11.60	0.60	9	0.050
PIC							
Tribes within Fabaceae							
Fabeae	1.80	1.25	2.60	—	0.50	16	<0.01
Galegeae	−3.18	−1.55	−6.53	—	0.08	8	0.435
Genisteae	1.11	0.77	1.60	—	0.05	28	0.223
Loteae	−1.22	−0.72	−2.06	—	0.15	13	0.149
Trifolieae	−1.47	−1.05	−2.06	—	0.02	35	0.467
Tribes within Zingiberaceae							
Alpinieae	2.09	0.86	5.04	—	0.24	5	0.261
Hedychieae	−14.51	−4.13	−51.03	—	0.23	3	0.417

Note: Results for PIC of Solanaceae tribes are presented in Table 1 because only subgroup B was analysed. P and r^2 values are from Pearson correlation tests. Intercepts for PIC are not given because regression is forced through the origin. CL = 95% confidence limit.

pollen size–number trade-off could also be detected for half of the analyses within taxa. Often for those groups the trade-off was not significant when the pollen–ovule ratio variable or phylogeny was accounted for. All groups that showed a simple negative correlation between pollen size and the pollen–ovule ratio did not show this relationship when controlling for the effect of pollen grain number, with the exception of the Scrophulariaceae. Four taxa in particular showed a deviation from expected correlations. The Malvaceae and the Fabeae had significant positive correlations between pollen size and pollen grain number – that is, apparently no trade-off between size and number of pollen takes effect within the Malvaceae and the Fabeae. For both families, the association between the pollen–ovule ratio and pollen size was positive, significantly so for the simple correlation of CSA within the Malvaceae and for CSA and PIC within the Fabeae. The Scrophulariaceae was the only taxon for which the predictions of Charnov's model were met, as the negative correlation between the pollen–ovule ratio and pollen size was present for both partial and

Table 3. Results of standardized major axis (SMA) regression between the pollen–ovule ratio and pollen size within mating systems for cross-species analysis (CSA) and phylogenetically independent contrasts (PIC)

Mating system	SMA slope	Lower CI	Upper CI	Intercept	r^2	d.f.	P
PIC							
Autogamous	−1.79	−1.33	−2.41	—	0.00	48	0.843
Allogamous	−1.36	−1.13	−1.64	—	0.12	104	<0.001
CSA							
Autogamous	−1.75	−1.37	−2.23	9.54	0.06	63	0.057
Allogamous	−1.08	−0.92	−1.27	7.70	0.12	129	<0.001

Note: P and r^2 values are from Pearson correlation tests. Intercepts for PIC are not given because regression is forced through the origin. CL = 95% confidence limit.

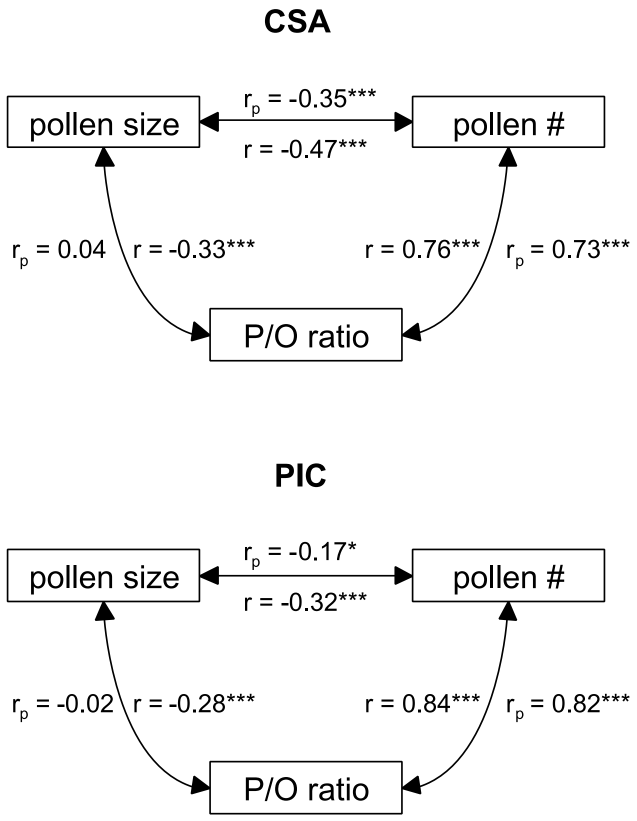


Fig. 3. Path diagram showing simple (r) and partial (r_p) correlations between the pollen–ovule ratio (P/O ratio), pollen size, and pollen grain number (pollen #) across all species (CSA) and across all independent contrasts (PIC). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 4. Summary of the simple (SC) and partial correlation (PC) analyses between the pollen–ovule ratio (po), pollen size (ps), and pollen grain number (p) among mating systems and several taxa

		CSA		PIC				CSA		PIC	
		PC	SC	PC	SC			PC	SC	PC	SC
Allogamous mating system	po~ps		–		–	Fabaceae, tribe Trifolieae	po~ps		–		
	po~p	+	+	+	+		po~p	+	+	+	+
	ps~p	–	–	–	–		ps~p	–	–		
Autogamous mating system	po~ps					Lamiaceae	po~ps		–		
	po~p	+	+	+	+		po~p	+	+	+	+
	ps~p		–				ps~p		–		
Asteraceae	po~ps					Malvaceae	po~ps		+		
	po~p		+	+	+		po~p	+	+	+	+
	ps~p						ps~p	+	+		
Brassicaceae	po~ps					Scrophulariaceae	po~ps	–	–	–	–
	po~p	+	+	+	+		po~p	+	+		+
	ps~p						ps~p		–		–
Ericaceae	po~ps					Solanaceae	po~ps	+			–
	po~p						po~p	+			+
	ps~p						ps~p	–	–		–
Fabaceae	po~ps	+				Solanaceae ‘Subgroup A’	po~ps				
	po~p	+	+	+	+		po~p				
	ps~p	–	–				ps~p	–	–		
Fabaceae, tribe Fabeae	po~ps		+		+	Solanaceae ‘Subgroup B’	po~ps				
	po~p	+	+	+	+		po~p	+	+		
	ps~p		+		+		ps~p		–		
Fabaceae, tribe Galegeae	po~ps					Zingiberaceae	po~ps				
	po~p	+	+	+	+		po~p	+	+	+	+
	ps~p						ps~p				
Fabaceae, tribe Genisteae	po~ps					Zingiberaceae, tribe Alpinieae	po~ps				
	po~p	+	+	+	+		po~p				
	ps~p						ps~p				
Fabaceae, tribe Loteae	po~ps		–			Zingiberaceae, tribe Hedychieae	po~ps				
	po~p	+	+	+	+		po~p	+	+	+	+
	ps~p	–	–				ps~p				

Note: ‘+’ and ‘–’ indicate significant ($P < 0.05$) positive and negative correlations, respectively. CSA = cross-species analysis, PIC = phylogenetically independent contrasts.

simple correlation analyses. The CSA correlations between the pollen–ovule ratio and pollen size for the Solanaceae were significantly positive and not significant for partial and simple correlation, respectively. For the same family and variables, the PIC correlations were negative but insignificant in the partial correlation analysis and significantly negative in the simple correlation analysis.

DISCUSSION

Surprisingly, we found that the negative correlation between the pollen–ovule ratio and pollen size vanishes when the number of pollen grains is accounted for. This contrasts with results on the relationship between the pollen-ovule ratio and seed size (Götzenberger *et al.*, 2006) that remained significant when controlling for the effect of ovule number. The pollen–ovule ratio/pollen size correlation only exists because of a highly significant positive correlation between the pollen–ovule ratio and pollen grain number, and a trade-off between pollen size and pollen grain number. This holds for both cross-species analysis and phylogenetically independent contrasts, but the correlations are weaker for PIC. Cruden and Miller-Ward (1981) found no pollen size–number trade-off within 19 bee-pollinated species. Queller (1984) concluded from this finding that the negative correlation between the pollen–ovule ratio and pollen size found for the same data could not be explained by such a trade-off. Vonhof and Harder (1995), however, showed that for the data of Cruden and Miller-Ward, a trade-off was shown to exist when they 'removed' the phylogenetic relatedness of the species studied. All further interpretation of the results of the regression analyses has to be seen in the light of the spurious relationship between the pollen–ovule ratio and pollen size that exists for our data. Also, it should be mentioned that the data set is ecologically and taxonomically biased. Most species are insect pollinated or selfed and wind-pollinated species are scarce. The number of species in some families, especially the Fabaceae, is inflated while other families are only represented by a few species. However, we do not expect the general results of the analyses to be susceptible to this bias.

A negative relationship between the pollen–ovule ratio and pollen size exists when all species or PIC are analysed together, but the correlation is weaker for PIC. Given the spurious nature of the correlation of the pollen–ovule ratio and pollen size, such a diminished correlation for PIC compared with that for CSA could be explained by the weaker pollen size–number trade-off for the PIC data. The results within taxonomical groups were inconsistent and not as clear-cut as in the study of the relationship between seed size and the pollen–ovule ratio, in which the positive correlation between these two variables was significant for most taxa with a reasonable sample size, and the results for PIC and CSA were very similar (Götzenberger *et al.*, 2006).

For families and tribes that had a low sample size, non-significant correlations between the pollen–ovule ratio and pollen size could have been caused by low statistical power. The Fabaceae showed no significant relationship despite a large sample size. Within this family, however, we found significant relationships at a lower taxonomical level (tribes), at least for CSA. For five taxa of *Astragalus* (Fabaceae), Gallardo *et al.* (1994) reported a strong negative correlation between pollen size and the pollen–ovule ratio when controlling for the effect of seed size and autofertility. Autofertility – the percentage of fruits formed in the absence of pollinators – was used as a mating system estimate. In contrast, within the *Solanum* (Solanaceae) a negative correlation between pollen size and the pollen–ovule ratio was only observed for one of two subgroups (Mione and Anderson, 1992). For all species together and the second subgroup, there was no significant correlation despite the lack of a systematic relationship between seed size and pollen size and apparently equal mating systems within the second subgroup. The study did not consider the possible effect of pollen grain number. According to the results of an insignificant partial correlation of pollen size and pollen–ovule ratio for subgroup B of the Solanaceae for both CSA and PIC, the relationship is spurious. The lack of a correlation between pollen grain number and the pollen–ovule ratio for subgroup A is in line with Mione and Anderson's (1992) finding that the variation in pollen–ovule ratios is at least partly caused by systematic variation in ovule number. There is a strong significant correlation of the pollen–ovule ratio and ovule number in subgroup A ($r = -0.73$, $P = 0.026$, d.f. = 7).

A positive correlation between pollen size and pollen grain number is responsible for the positive correlation between the pollen–ovule ratio and pollen size for the Malvaceae and Loteae. Except for one species, the data for the Malvaceae consist of *Tarasa* species from a paper by Tate and Simpson (2004), who had already reported the positive correlation between the pollen–ovule ratio and pollen size. In contrast to many other studies (reviewed in Muller, 1979), these authors also found that pollen size was negatively correlated with the level of polyploidy and plant lifespan. Usually, pollen size and polyploidy are positively correlated as an effect of greater genome size in polyploid species. In the case of *Tarasa*, the authors argued that derived annual, tetraploid species colonized colder, drier, and more irradiated habitats at higher elevations than their annual and perennial, diploid ancestors. The new environmental conditions acted as selective forces towards an overall reduced floral morphology and an evolution from a predominantly outcrossing to a selfing mating system. The reduced floral morphology included reduced pollen size and pollen grain numbers for the tetraploid species. In combination with the very strong correlation between the pollen–ovule ratio and pollen grain number within *Tarasa*, a positive correlation between pollen size and the pollen–ovule ratio must follow. It is unlikely that similar evolutionary processes led to the positive correlation of pollen grain size and pollen grain number within the Fabeae. Alternatively, we suggest that it is possible that within the Fabeae pollen was selected for both increased size and number through pollination by pollen-feeding insects to increase the reward for such pollinators. The Fabaceae are not only visited for their nectar but also for their pollen, which contains high proportions of protein (Leppik, 1966). While some taxa within the Fabaceae produce either predominantly nectar or predominantly pollen, *Vicia* produces nectar and pollen in large quantities (Ortega-Olivencia *et al.*, 1997).

In general, the question of how a plant species rewards its pollinators could influence the results of testing predictions from sex allocation theory. Reward in the form of pollen can at least partly be interpreted as a contribution to female sex function. Pollen does not only attract the pollinators to remove pollen for outcrossing but also to fertilize ovules. The predictions from Charnov's model may not work well for plants that only offer pollen as a reward to their pollinators, since pollen does not contribute exclusively to male function.

The correlations within the Scrophulariaceae species were strikingly different to those in the other taxa. Except for one species, the data for this family contain species of the genera *Pedicularis*. Two important features of this genus are a high interspecific variation in pollen size compared with other taxa and the exclusive pollination by bumblebees (Yang and Guo, 2004). Although inter-generic, the data of Cruden and Miller-Ward (1981) also show these features. Re-analysing the data of Cruden and Miller-Ward (1981), we found that, as for the *Pedicularis* data, the negative relation between the pollen–ovule ratio and pollen size is not dependent on pollen grain number. Cruden and Miller-Ward (1981) argued that this relation is caused by 'the critical amount of compounds required for germination and penetration of the stigma' such that 'fewer large grains should be required per seed than small grains' and vice versa. Why this functional relation only seems to work within bee-pollinated species, however, remains unclear.

There was a trend for PIC analysis to be weaker and less significant than the CSA analysis. In particular, only one family and only one tribe within the Fabaceae showed a significant relationship between the pollen–ovule ratio and pollen size for PIC versus three families and three tribes within the Fabaceae for CSA. In general, however, the results of PIC correspond with the results of CSA, and in virtually all correlations the direction for PIC and CSA was the same. Therefore, we believe that weaker and insignificant correlations are not the result of a lack of correlated evolution in the traits but are caused by the working phylogeny. As described in the Methods section, we used a phylogeny with all branch lengths set equal. Although this phylogeny violated assumptions of the PIC procedure less than the other phylogeny tested, it surely fails to represent the 'true' phylogeny of the species under study, especially in terms of branch lengths. Thus, the calculated PIC values and their correlation and regression analyses might be biased.

The only consistent relationship when comparing the results for the two mating systems is the positive correlation between pollen grain number and the pollen–ovule ratio. That the relation between pollen size and the pollen–ovule ratio is dependent on the correlation between each of these two traits with pollen grain number becomes clear also: there is only a weak pollen size–number trade-off for CSA and no significant correlation for PIC for autogamous species, while simple and partial correlation show a significant size–number trade-off within the allogamous species. Consequently, the autogamous species do not show any significant correlation between the pollen–ovule ratio and pollen size but the allogamous species show a significant positive correlation, which is not evident for the partial correlation. The correlative patterns within the mating systems are strongly influenced by the subsets of species that are included in the mating system categories. The allogamous group contains the most species and these species represent the taxonomy of all species in the study very well. Thus, the results for this group are very similar to those for all species analysed together. The autogamous mating system group consists of many species from taxa that showed no significant or positive correlations between pollen size and pollen grain number (*Tarasa*, Trifolieae, Fabeae).

Barrett and colleagues' (1996) prediction that the elevation of the regression line should be greater for outcrossers than selfers could not be tested thoroughly with our data. Slopes among mating systems were heterogeneous, and thus a comparison of the intercepts was meaningless. Also, it has to be kept in mind that slopes and intercepts of the regression of the pollen–ovule ratio on pollen size do not represent a functional relationship between the two traits because the correlation between them is mediated through a third trait, pollen grain number.

Pulling together the different analyses, we note that their results contradict the predictions made by Charnov's model in three main ways. First, the Malvaceae show a positive correlation between the pollen–ovule ratio and pollen size for CSA, the Fabeae for CSA and PIC. Second, of all families and tribes studied, a negative significant relationship between the pollen–ovule ratio and pollen size for both PIC and CSA is evident only for the Scrophulariaceae. And third, partial correlation analyses revealed that the detected correlations of the pollen–ovule ratio and pollen size arise from correlations of these two variables with pollen grain number.

Götzenberger *et al.* (2006) found support for the analogous prediction from Charnov's model regarding resources allocated to female function by analysing the relationship between the pollen–ovule ratio and seed size. The predicted positive correlation was observed consistently throughout taxonomical groups and mating systems for PIC and for CSA and was not spurious. A joint consideration of the 2006 study and the conclusions drawn in the present study lead us to consider why the relationship between the pollen–ovule ratio and seed size seems to be in line with equation (1), while the relationship between the pollen–ovule ratio and pollen size does not. The answer to this question may lie in the amount of variation expressed by the traits involved in Charnov's model and the percentage of variation in the pollen–ovule ratio that is explained by these variables. Within the data set used in the present study, the variation in pollen–ovule ratios that is explained by the number of pollen grains is much higher than the variation in pollen–ovule ratios explained by the number of ovules in Götzenberger *et al.* (2006). For many families, the correlation (r) between the pollen–ovule ratio and pollen grain number exceeds 0.80. This strong correlation, in combination with the general pattern that pollen size varies much less than pollen grain number (Vonhof and Harder, 1995), may reduce the possibility for a correlation between the pollen–ovule ratio and pollen size to be expressed. On the other hand, the relation between the pollen–ovule ratio and ovule number is generally weaker, making it more likely that a correlation between the pollen–ovule ratio and seed size will be expressed, independent of the number of ovules produced. Barrett *et al.* (1996) reformulated equation (1) so that pollen size appears on the left-hand side of the equation as the dependent variable and the pollen–ovule ratio on the right-hand side as an explanatory variable. Although the authors gave no reasons for switching the variables, a major concern about the relationships between pollen size and the pollen–ovule ratio and between seed size and the pollen–ovule ratio becomes apparent. The usual set-up of statistical models suggests a causal link between 'dependent' and 'independent' variables but the direction of causality and whether the causation is direct or caused by covariates that are not included in the model cannot be inferred from such models (Shipley, 2000). Therefore, future research of the factors that influence pollen–ovule ratios should try to disentangle the ties between cause and correlation and include further possible covariates such as flower size and style length (Barrett *et al.*, 1996).

In summary, we found a general negative relationship between the pollen–ovule ratio and pollen size but this relation was caused by the effect of pollen grain number. Thus, with respect to male function, the pollen–ovule ratio is influenced by the number of pollen grains produced by a flower but not by the size of the pollen. While the model seems attractive and logical, the results presented in this study places its validity in doubt, at least for the male function part. As we have argued in the case of the relation between seed size and the pollen–ovule ratio (Götzenberger *et al.*, 2006), we believe that the two theoretical frameworks used to explain the association between the pollen–ovule ratio and pollen size are working hand in hand rather than are mutually exclusive. On the one hand, the pollen–ovule ratio is dependent on seed size as predicted by Charnov's model; on the other hand, Cruden's 'efficiency theory' is implemented by pollen grain numbers that are adapted to particular pollination conditions.

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