

Breeding system and the role of floral visitors in seed production of a 'few-flowered' perennial herb, *Paeonia broteroi* Boiss. & Reut. (Paeoniaceae)

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Abstract: The breeding system and pollination ecology of *Paeonia broteroi* (Paeoniaceae), a self-compatible, perennial herb, which produces few flowers (1-8) per season, are described in two populations in mountains of southern Spain. Experimentally self-pollinated flowers yielded a significantly lower seed set (6-18%) than cross-pollinated flowers (23-29%), but the latter did not differ in seed set from open-pollinated flowers (30-31%). Seeds obtained from self-pollinated flowers were significantly lighter than seeds obtained from open- and cross-pollinated flowers. Pollen and resource limitation, inbreeding depression and allocation to sexual functions are discussed to explain seed set. The role of different floral visitors (including bees, beetles and ants) on reproductive success was examined through exclusion experiments and efficiency tests. Only one species of bee (*Andrena assimilis*, Andrenidae) from the 25 insect species that were recorded during two study seasons seemed to be responsible for successful pollination. Thus, although flower morphology is open and excludes few if any floral visitors, most of the visitors were not suitable pollinators.

Keywords: *Paeonia broteroi*, breeding system, pollinator assemblage, pollinator efficiency, seed set, few-flowered species.

Introduction

Plant species relying on animal (*e.g.*, insects) vectors for pollination may evolve specialized flowers as a result of mutualistic interactions with certain species (see Montalvo & Ackerman, 1986; Herrera, 1989). However, these plant species may be at a greater risk of pollinator limitation to seed production (*e.g.*, Jordano, 1990; Sun, 1997) or pollen removal, if specialized visitors are sometimes scarce or absent (*e.g.*, Herrera, 1990; Ågren, 1996). Other plant species have unspecialized flowers, and the assemblage of floral visitors able to visit these flowers may be highly diverse (see O'Brien, 1980; Herrera, 1989). For these species, the risk of pollinator limitation may be lower. Yet, diversity or abundance of different floral visitors are not the only sources of variation in pollination success, because a variety of visitors is likely to include non-pollinating species which may reduce the efficiency (see Inouye *et al.*, 1994 for a discussion on efficiency vs. effectiveness) of true pollinators (see Schemske & Horvitz, 1984).

In this paper, we explore the effects of breeding system and visitor assemblage on several measures of pollination and reproductive success (seed initiation, maturation and

mass) in *Paeonia broteroi* Boiss. and Reut. (Paeoniaceae). This species is a hermaphroditic, perennial herb, with large (petal length ranges 35-67 mm; see Results) but unspecialized flowers. Its open floral morphology results in a diversity of floral visitors that may differ in their efficiency as pollinators. *P. broteroi* produces flowers that can be considered as providing pollen-reward (nectaries are located outside the corolla, at the base of an elongated sepal and not accessible to flower visitors, while flowers have, on average, more than 160 stamens, producing copious pollen). This may have at least two implications to breeding success. First, although the fertilization success of pollen grains removed from anthers is almost always doubtful, an undefined proportion of plant fitness can be gained through the male function (see Stanton *et al.*, 1992; Thomson & Thomson, 1992). However, *P. broteroi* produces very few flowers/season (usually 1 or 2, rarely more than 8 or 10); thus, unlike many multi-flowered hermaphroditic species, it cannot adjust full functional units (flowers) to act predominantly as males or females, and consequently, both sexual functions must be shared within a flower. Second, if resources for reproduction are limited, investing in male structures related to pollinator attraction may imply that such

resources cannot be allocated to female fitness (seed production). Hence, even under the best pollinating circumstances, one does not necessarily expect maximal conversion of ovules to seeds (see Sutherland & Delph, 1984).

Specifically, we address the following questions: Is seed production limited in *P. broteroi* populations? What factors may be limiting seed production? How efficient is the assemblage of floral visitors as a whole, and the commonest species in particular, at promoting seed initiation?

Material and methods

THE PLANT AND THE STUDY SITES

In the Iberian Peninsula, *P. broteroi* typically grows in the understory of oak and pine woods. It can be found mainly in western areas and it is not present in northwestern Portugal and northern Spain (Castroviejo *et al.*, 1993). Roots are tuberous, but vegetative reproduction seems to be limited (A. Traveset, pers. com.). Plants consist of one to several leafy ramets, each of which can produce a single flower, although not all ramets produce flowers. Flowers are actinomorphic, protogynous (but only for the first day, after which male and female phases overlap for 3-4 d; Sánchez-Lafuente, Rey & Alcántara, unpubl. data), apocarpous and have from 5-8 dark pink-to-red petals, forming a non-tubular corolla. Flowers contain more than 160 stamens (Sánchez-Lafuente, Rey & Alcántara, unpubl. data) with yellow anthers producing copious pollen. Stigmas are wet and fleshy, formed by two 'valves' closed at a suture, and remain receptive for 3-4 d. (Sánchez-Lafuente, Rey & Alcántara, unpubl. data). The stigmas are borne directly on the top of the carpels thus the style is inconspicuous. Nectar is not produced inside the flower and nectaries are located outside the perianth, at the base of an elongated sepal. Seed maturation takes between 10-16 wk.

Our study was conducted at the Sierra de Cazorla mountains (southeastern Spain) in the spring-summer of 1994 and 1995. In this area, *P. broteroi* mostly occurs in small populations (60-130 individuals). In addition, isolated individuals separated from each other and from larger populations by several kms are found. Two populations, located at Roblehondo ($N = 128$ plants, 1300 m.a.s.l.; RH hereafter) and Fuente de la Umbría ($N = 117$ plants, 1650 m.a.s.l., FU hereafter), were selected. Populations are separated by approximately 3 km. Apart from RH, the closest population to FU is located approximately 5 km away, while around RH only small groups of plants (consisting of one to a few individuals) occur, from 2 to several km apart. Plants at RH are consistently subjected to severe herbivory (aerial parts are completely consumed) predominantly by wild Fallow deer *Dama dama*, while plants at FU are in a fenced area, approximately 1000 m² in size. Both populations undergo damage by Noctuidae (Lepidoptera) larvae (*Noctua fimbriata*, *Noctua janthe* and *Orthosia gothica*,

among others) that consume buds and leaves. Flowering begins in mid May at RH, and about ten days later at FU.

PLANT REPRODUCTIVE CHARACTERISTICS IN THE STUDY POPULATIONS

To characterize the plants in the study populations we recorded in 1994 the following measures in 121 plants randomly selected ($N = 60$ from RH and $N = 61$ from FU): no. of ramets/plant, no. of flowers/plant, plant height (longest ramet) and flower size (petal length measured from one petal/flower). Reproductive characteristics related to seed production for each flower were assessed by recording the no. of carpels/flower, no. of ovules/carpel and no. of ovules/flower. Measures were recorded with an electronic caliper to the nearest 0.01 mm. Ovule counting was made from the fruits of the measured plants. Variance components of the no. of ovules/flower accounted for by no. of carpels/flower and no. of ovules/carpel were determined by dividing their variances and covariances (multiplied by 2) by the variance of the number of ovules/flower. The results were expressed as percentages.

PLANT TAGGING

In 1994, 80 single-flower plants were tagged in each population. Additionally, in 1995, 50 single-flowered plants were tagged at FU. Single-flowered plants were selected for two reasons. First, single-flowered plants are the closest to the modal condition of the populations (mean \pm SD flowers/plant: 1.2 ± 0.6 ; $N = 121$), while the number of multi-flowered plants available to replicate treatments within plant was very low. Second, although the range in the no. of flowers/plant in the populations is small, differences in allocation of resources for reproduction between flowers in multi-flowered plants may preclude a comparison of the results obtained from plants with a different number of flowers. All flowers were bagged before anthesis to ensure that newly opened flowers were not naturally cross-pollinated. At RH, bags also discouraged herbivores, thus preventing extensive damage to the population.

BREEDING SYSTEM

In 1994, we examined the breeding system by assigning tagged flowers to one of two pollination treatments: self-pollination ($N = 17$ in each population, 'selfed' hereafter) and cross-pollination ($N = 18$, 'crossed'). Flowers were pollinated on the day after anthesis using a thin brush, with pollen from three anthers from the same flower for the selfed treatment and from flowers from different plants in the same population for the crossed treatment. Crossed flowers were emasculated before dehiscence. After hand-pollination, we used dense mosquito net bags to cover manipulated flowers. Bags were removed when stigmas were no longer receptive. Stigmas were saturated

with pollen after a single hand-pollination, thus experimental pollination was not repeated.

When carpels dehisce, three different ovule states can be detected: a) aborted non-fertilized ovules ('aborted ovules' hereafter) are small, black and flat; b) fertilized ovules that have initiated development but are then aborted at different stages ('aborted seeds' hereafter), are larger and flat, ranging in color from red to brown; c) finally, mature seeds are spherical, filled, large and black. Three different estimations of reproductive success were obtained: seed initiation (percentage of fertilized ovules, also used as a measure of pollination efficiency), seed abortion (percentage of fertilized ovules aborting) and seed set (percentage of fertilized ovules finally maturing to seeds). Unless otherwise stated, 'initiated seeds' will refer to aborted plus mature seeds.

ANTS AS POLLINATORS

At least two ant species (*Lasius* sp. and *Camponotus* sp.) were frequently found visiting flowers within corollas. To test for the effect of ants on pollination (e.g., Takahashi, Nishio & Hayashi, 1993; Gómez & Zamora, 1992; Ramsey, 1995), compared to other visitors, two groups of flowers were assigned to one of the following exclusion treatments: ants excluded but access by other floral visitors ($N = 15$ in each population, 'ants excluded') and ants allowed but other floral visitors excluded ($N = 15$, 'only ants allowed'). To exclude ants, we bent a sticky adhesive band around the flowering ramet to trap ants as they climbed up the ramet. To exclude flying visitors, we covered flowers with a mosquito netting circular bag, leaving the base open. Finally, another set of plants were tagged and left as open-pollinated to act as a control for both experiments ($N = 15$ in each population, 'control'). All mature seeds produced by the tagged plants were collected, individually weighed to the nearest 0.1 mg and averaged for each flower.

INSECT CENSUSES

To assess the assemblage of insects visiting *P. broteroi*, 367 censuses were carried out at FU population in 1994 on $N = 38$ single-flowered plants (including control and 'ants excluded' plants). Censuses lasted three minutes/flower and were conducted from 9 h to 16 h (GMT) from the day after anthesis until stigmas were no longer receptive. Each day, a tagged plant was randomly chosen to start with, then the rest of the tagged plants were sequentially monitored following the same order several times a day. The mean \pm SD number of censuses/plant throughout the flowering season was 15.4 ± 2.0 . During each census, we noted the number and species (or genus, if the latter was unknown) of visitors to each focal flower. We considered that a visit started when visitors first contacted male- or female-related reproductive structures and finished when they left the focal flower. During each visit, we recorded the time

spent on anthers (related to pollen removal) and stigmas (related to pollen deposition). Visitation frequency (visits/plant/hour), overall time/visit and time/visit on anthers or stigmas were obtained for each plant from all the 3-minutes censuses made on each particular plant.

POLLINATOR EFFICIENCY

We used 251 of the 367 insect censuses conducted in 1994 (those made on single-flowered plants that did not suffer herbivory in the carpels by Noctuidae larvae) to analyze the effect of flower visitors on seed production ($N = 30$ plants left, we also used 'ants excluded' plants because ant visitation did not affect seed development and maturation; see Results).

Structural equation modeling (SEM; e.g., Mitchell, 1992) was performed on the matrix obtained from census data, seed initiation and seed set produced by censused flowers, to investigate the relationships between seed production and abundance and behavior of different visitor species. The proposed model related the no. of visits/plant/hour of different visitor species (reflecting both species abundance and preferences by individual plants) to the proportion of time/visit spent on the stigmatic surface. The time/visit spent on the stigmas is likely to affect the probability of pollen delivery, and thus may be related to ovule fertilization (i.e., seed initiation). Finally, the percentage of initiated seeds is likely to be related to the final number of seeds produced by a plant, including the effect of seed abortion. Frequency of visits (visits/plant/hour) is allowed to affect seed initiation only indirectly through the percentage of time/visit spent on the stigmas because of two reasons: first, a higher frequency of visits does not imply a higher probability of ovule fertilization if stigmas are not contacted; second, visitor efficiency measured through frequency of visits is not comparable among species if the time/visit spent on the stigmas varies greatly among them. Only the most common visitors (with at least 1 visit/plant/hour on average, see Results) were included in the path model to test the efficiency of floral visitors: *Andrena assimilis* (Andrenidae), *Halictus scabiosae* and *Lasioglossum* sp. (both Halictidae) as bees, and *Malachius* sp. (Malachiidae) as a beetle. Our dataset was submitted to the EPA program (Exploratory Path Analysis; Shipley, 1997), which suggested the same model as we initially proposed. The final analysis was performed using the CALIS procedure (SAS, 1990a), while confidence intervals for path coefficients were determined by means of an accelerated bootstrap method (Efron & Tibshirani, 1993) with 5000 repetitions, using a program written by Carlos M. Herrera (unpubl. data) using SAS macro processing language (SAS, 1990b).

In addition to this analysis, in 1995 we started a long-term experiment at FU, specifically to test the relationships between different visitor's morphology and behavior and their pollination efficiency. The species examined during this year were the most common

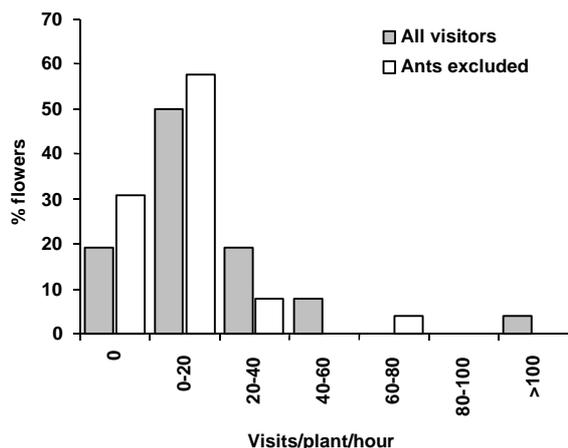


Figure 1. Distribution of the frequency of visits to censused plants ($N=38$).

species that visited *P. broteroi* in 1994, including *A. assimilis* (Andrenidae) and *H. scabiosae* (Halictidae). The efficiency of *Malachius* sp. (Malachiidae) was not tested because it was absent in 1995. Here, we will consider two relative measures of pollinator efficiency: percentage of seeds initiated and seed set due to each visitor after three visits (see Inouye *et al.*, 1994). Buds on $N = 26$ single-flowered plants were bagged, and $N = 13$ plants were assigned to each pollinator. After anthesis, bags were removed and only one insect species was allowed to visit each flower on three occasions. If other visitors were observed around the target flower, they were waved away. For each visit, we recorded the time spent on anthers and stigmas until the visitor left the flower. When the three visits were completed, the flower was emasculated to avoid autogamy and bagged again until the stigmas were no longer receptive. All mature seeds produced by the tagged plants were collected, individually weighed to the nearest 0.1 mg and averaged for each flower.

STATISTICAL ANALYSES

Differences in reproductive parameters (percentage of seed initiation, seed abortion and seed set) among treatments and populations were tested with two-way mixed-model ANOVAs (MIXED procedure in SAS; Littell *et al.*, 1996), with treatment as a fixed effect and population as a random effect. Since seed mass could be affected by flower size (smaller flowers with smaller carpels and ovules may produce comparatively smaller seeds) and ovule number (presumably due to differences in resource allocation among different number of ovules), differences in mean seed mass/flower among treatments and populations were tested with two-way mixed model ANCOVA (MIXED procedure in SAS), using flower size and ovules/flower as covariates. Parallelism was tested to validate the use of ANCOVA.

Variables were transformed (log transformed for measures/counting and arcsine for proportions) when necessary to meet the assumptions of the tests (normality

and homocedasticity). Non-parametric test (NPAR1WAY procedure; SAS, 1990a) were used when there were deviations from such assumptions after transformations. Unless otherwise stated, means are given ± 1 SD.

Results

PLANT REPRODUCTIVE CHARACTERISTICS IN THE STUDY POPULATIONS

Plant size (number of ramets/plant) ranged from 1 – 12 and plant height ranged from 38 – 79 cm. The overall number of flowers/plant ranged from 1 – 8 (mean: 1.2 ± 0.6 ; $N = 121$). Mean (\pm SE) number of flowers/plant at RH (1.4 ± 0.1 , $N = 60$) was higher than at FU (1.2 ± 0.1 , $N = 61$) (Mann-Whitney, $P < 0.02$). Plants at RH had larger flowers than plants at FU (mean \pm SE petal length; RH: 59.3 ± 1.2 ; FU: 47.7 ± 2.4 ; *t*-test: 4.15, $P < 0.001$). Plant height was not correlated with flower size ($r = 0.01$, $P = 0.72$, $N = 121$).

The number of carpels/flower ranged from 1 – 5 (mean: 2.1 ± 0.5 mm, $N = 121$) with a modal value of 2. Again, the mean (\pm SE) number of carpels/flower at RH (2.2 ± 0.1 , $N = 60$) was higher than at FU (1.91 ± 0.1 , $N = 61$) (Kruskal-Wallis: $\chi^2 = 5.10$, d. f. = 1, $P < 0.05$). The number of ovules/carpel was highly variable (range: 10 – 45; mean: 18.8 ± 7.6 , $N = 121$), but it did not differ between populations (mean \pm SE; RH: 18.2 ± 1.5 ; FU: 18.6 ± 0.7 ; *t*-test: 1.53, $P > 0.1$). The mean number of ovules/flower was 39.2 ± 17.6 ($N = 121$), ranging from 10 – 113. The overall number of ovules/flower did not differ between populations (mean \pm SE; RH: 39.5 ± 1.9 ; FU: 36.2 ± 2.0 ; *t*-test: 1.49, $P > 0.1$). The number of ovules/flower mostly depended on the number of carpels/flower (explaining 97.2% and 92.9%, of the variance in ovules/flower, for RH and FU respectively) while the differences in the number of ovules/carpel only explained 2.8% and 7.1%, at each population, of the variance in the no. of ovules/flower.

BREEDING SYSTEM

The percentage of initiated and aborted seeds did not significantly differ among treatments (selfed, crossed and control plants) or between populations (Table Ia). Seed set was significantly different among treatments but not between populations (Table Ia), with self-pollination yielding a lower seed set than open- and cross-pollination. Cross-pollination did not enhance seed initiation or seed set compared to open-pollination, and full seed set was not achieved by any flower. A 46.2% and a 50.0% of the cross-pollinated flowers achieved less than 30% of seed initiation and seed set, respectively. A 73.1% and a 80.8% of the cross-pollinated flowers achieved less than 50% of seed initiation and seed set. Figures for the open-pollinated flowers were similar (40.6% and 43.8% below 30% of seed initiation and seed set; 75% and 87.5% below 50% of seed initiation and seed set). Mean seed mass/flower was correlated with

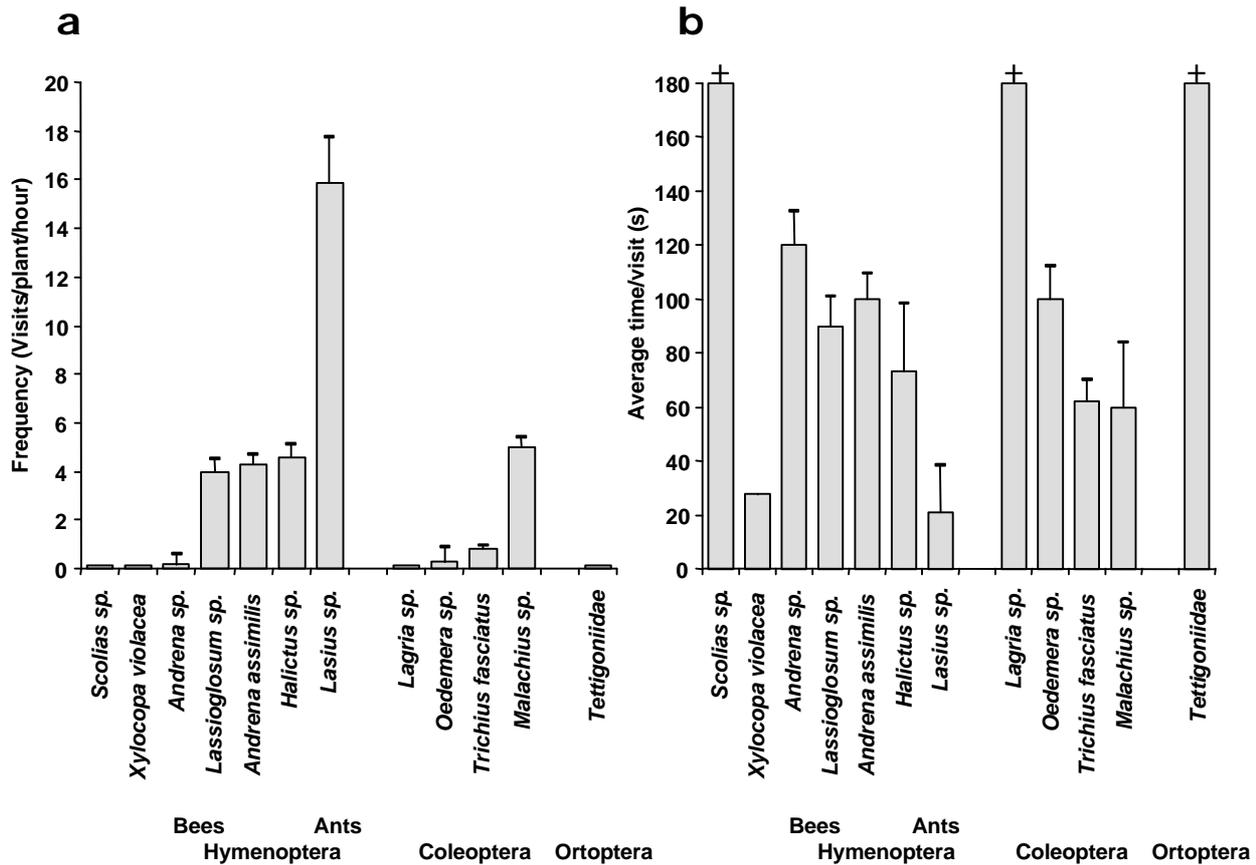


Figure 2. Mean relative abundance (visits/plant/hour) (a) and average time/visit (b) of all floral visitors contacted during regular censuses at FU. + above bars indicate that the average time/visit is longer than the time unit of a census (180 s). Standard errors are shown for species appearing more than once in the censuses. $N=38$ plants.

flower size ($r = 0.68$, $P < 0.001$, $N = 30$) and ovule number ($r = 0.55$, $P < 0.001$, $N = 30$). After controlling for number of ovules and flower size, seed mass significantly differed among treatments (selfed, crossed and control) and between populations. Self-pollinated flowers produced seeds that weighed significantly less than seeds from control and cross-pollinated flowers. In addition, seeds from RH weighed less than seeds from FU (Table Ia). None of the covariates was significant (Flower size: $\chi^2_{1,56} = 0.39$, $P = 0.54$; Ovules/flower: $\chi^2_{1,62} = 0.02$, $P = 0.95$).

ANTS AS POLLINATORS

The percentage of initiated seeds differed significantly among treatments (ants excluded, only ants allowed and control) but not between populations (Table Ib). ‘Only ants allowed’ flowers initiated an extremely low proportion of seeds/flower (3%, see Table Ib) indicating that ants do not appear to have any influence on pollination. Seed set also significantly varied among exclusion treatments and was lower in ‘Only ants allowed’ flowers than in control or ‘Ants excluded’ flowers (Table Ib).

After controlling for number of ovules and flower size, seed mass significantly varied between populations (plants at RH producing significantly lighter seeds than plants at FU) but not among treatments (Table Ib). One of the covariates, flower size, had a significant effect ($\chi^2_{1,56} = 6.76$, $P < 0.01$) but not the other (Ovules/flower: $\chi^2_{1,56} = 0.04$, $P = 0.94$).

INSECT CENSUSES

Preliminary observations at FU, made two days before starting the regular censuses, detected 25 different insect species visiting *P. broteroi* at least once (see Appendix). However, only 12 of these species were later recorded in regular censuses. Considering all visitors together, most plants (80.8%) received at least one visit/hour (mean no. visit/plant/hour: 18.8 ± 6.7), and a relatively high proportion of them (30.8%) were intensely visited (Figure 1). If *Lasius sp.* was excluded (ants of this species did not appear to have any influence on pollination, see above), the proportion of flowers receiving at least one visit/hour is reduced to 70% (mean no. visit/plant/hour: 9.3 ± 2.8). These results indicate that approximately 11% of the flowers were visited only

by ants during regular censuses, while for the rest of flowers the frequency of visits was reduced by half. Thus, *Lasius* sp. appeared to be the most frequent floral visitor (Figure 2a) but was ineffective as a pollinator. Frequency of visits/plant/hour, time/visit (Figure 2a, b) and average percentage of time/visit on the stigmas versus anthers (Figure 3) were very variable among species. Bees, considered together, were more frequent, made longer visits and spent proportionally less time on the stigmas than beetles. Bees, however, were extremely mobile once in a flower, while beetles were static. Overall, the most abundant visitors spent more time on the anthers than on the stigmas (Figure 3).

POLLINATOR EFFICIENCY AND PATH ANALYSIS

A large proportion of variation in percentage of seed initiated ($r^2 = 0.66$) was explained by the path model via pollination services (Figure 4), although only 15% of the variance in seed set was explained. The model fit the data well (Adjusted Population *Gamma* Index = 0.98). Frequency of visits significantly increased the total time/hour spent on the stigmas by each visitor, which was the variable most likely to affect seed initiation. However, only foraging by *A. assimilis* significantly increased seed initiation, while the relationships between foraging by *Lasioglossum* sp. and *H. scabiosae* and seed

initiation were negative. Thus, *A. assimilis* seems to be the only important pollinator for *P. broteroi* during this study. The overall effect of frequency of visits (P1→P5: 0.65; Figure 4) and percentage of time/visit on stigmas (P5: 0.68), on percentage of seeds initiated by *A. assimilis*, was almost two-fold greater than the next most effective visitor. These two steps explained 40% of the overall variance in seed initiation. On the other hand, *Malachius* sp., being as common as the bees and spending a higher proportion of the time/visit on the stigmas, had little importance as pollinator (effects were 0.12 and 0.14 respectively). Nevertheless, confidence intervals for the path coefficients shown in Table II were relatively high, indicating that the effects of the relationships could vary in a certain range.

Regarding the efficiency tests conducted in 1995, we did not find differences in the percentage of time/visit on the stigmas by the two study pollinator species (mean \pm SE s; *A. assimilis* : 30.0 \pm 18.5; *H. scabiosae* : 20.1 \pm 5.9; Mann-Whitney, n.s.), most likely due to the high variability showed by *A. assimilis*. However, *A. assimilis* was more efficient as a pollinator, as the percentage of seeds initiated due to its visits (mean \pm SE: 29.6 \pm 8.6) was much greater than the percentage of seeds initiated by the visits of *H. scabiosae* (mean \pm SE: 3.7 \pm 1.7); differences were significant (Student's *t*-test = 7.03, $P < 0.01$). Consequently, seed set was also higher in flowers

TABLE I. Differences in reproductive success between pollination (a) and exclusion (b) treatments at RH and FU populations. Data are mean (\pm SE). Differences in initiated seeds, aborted seeds and seed set were tested by 2-way mixed ANOVAs. Differences in seed mass (grs) were tested by 2-way mixed ANCOVAs, including flower size and ovules/flower as covariates.

a - Pollination	RH			FU			Population		Treatment		Population x Treatment	
	Selfed	Crossed	Control	Selfed	Crossed	Control	Wald Z	P	$\chi^2_{2,94}$	P	Wald Z	P
	N=17	N=18	N=15	N=17	N=18	N=15						
%initiated seeds	21.2 \pm 4.5	31.2 \pm 7.0	35.3 \pm 7.7	27.5 \pm 9.8	36.3 \pm 7.3	29.1 \pm 6.4	0.27	0.65	1.53	0.22	0.10	0.90
%aborted seeds	15.5 \pm 7.8	8.9 \pm 4.0	7.7 \pm 3.0	10.8 \pm 8.8	7.3 \pm 3.5	3.8 \pm 2.8	0.75	0.38	1.16	0.32	0.05	0.94
Seed set	5.8 \pm 2.7	23.2 \pm 6.2	29.5 \pm 5.6	7.7 \pm 3.7	29.3 \pm 6.8	25.3 \pm 5.3	1.22	0.27	3.99	0.02	0.24	0.78
							Wald Z	P	$\chi^2_{2,62}$	P	Wald Z	P
	N=5	N=17	N=13	N=7	N=16	N=12						
Seed mass ($\times 10^{-3}$)	24.8 \pm 8.3	50.5 \pm 8.8	53.7 \pm 9.4	45.0 \pm 14.3	102.6 \pm 5.3	92.1 \pm 12.7	8.58	0.01	3.41	0.04	0.20	0.82
b - Exclusion	RH			FU			Population		Treatment		Population x Treatment	
	Ants excluded	Only ants allowed	Control	Ants excluded	Only ants allowed	Control	Wald Z	P	$\chi^2_{2,84}$	P	Wald Z	P
	N=15	N=15	N=15	N=15	N=15	N=15						
%initiated seeds	31.4 \pm 7.3	2.9 \pm 1.0	35.3 \pm 7.7	35.7 \pm 8.2	3.0 \pm 0.8	29.17 \pm 6.4	0.66	0.42	6.01	0.01	1.32	0.27
Seed set	29.8 \pm 6.9	2.7 \pm 0.9	29.6 \pm 5.6	30.4 \pm 7.6	2.6 \pm 0.9	25.3 \pm 5.3	0.67	0.42	5.86	0.01	1.52	0.23
							Wald Z	P	$\chi^2_{2,55}$	P	Wald Z	P
	N=13	N=5	N=13	N=14	N=6	N=12						
Seed mass ($\times 10^{-3}$)	46.5 \pm 10.3	57.3 \pm 15.6	53.7 \pm 9.4	60.9 \pm 15.2	56.8 \pm 17.2	92.1 \pm 12.7	5.83	0.02	1.96	0.15	2.01	0.15

pollinated by *A. assimilis* (mean \pm SE: 17.1 ± 6.2 ; for *H. scabiosae* mean \pm SE: 3.5 ± 1.6 ; Student's *t*-test = 2.5, $P < 0.02$).

Discussion

BREEDING SYSTEM AND SEED PRODUCTION

The results of the present study have shown that self-pollinated flowers produced significantly fewer seeds than cross-pollinated and open-pollinated flowers. In addition, the average mass of seeds obtained by selfing was significantly lower than the average mass of seeds obtained by crossing and open pollination. Thus, inbreeding depression following selfing occurred at these two stages of the life cycle. Further, many studies have demonstrated that larger seeds have higher germination success and produce seedlings of better quality (see Stanton, 1985; Martin & Lee, 1993; Ramsey & Vaughton, 1996); therefore, increased levels of inbreeding depression in *P. broteroi* might also be expected at later stages in the life cycle (see Husband & Schemske, 1997). Cross-pollination did not enhance seed initiation or seed set compared to open-pollination. Further, most of the cross-pollinated and open-pollinated flowers achieved less than 50% of seed initiated and seed set. Our seed set values are similar to those found in other studies carried out on *Paeonia* species (e.g., *P. californica*: 2.1% for control plants, 15.9% for selfed plants and 29.8% for crossed plants; see Schlising 1976; *P. cambessedesi*: 35% for control plants, 20% for selfed plants and 40% for crossed plants; A. Traveset, unpubl. data), and all of them are considerably lower than those

TABLE II. Confidence intervals for the path coefficients obtained in path analysis. Intervals were calculated by means of accelerated bootstrap (see Methods). For identification of coefficients see Figure 4.

	Lower 95% interval	Mean value	Upper 95% interval
Path coefficients (P_i)			
P_1	0.883	0.963	0.989
P_2	0.545	0.844	0.975
P_3	0.913	0.969	0.982
P_4	0.480	0.835	0.897
P_5	0.383	0.677	1.000
P_6	-0.831	-0.183	0.067
P_7	-0.610	-0.355	-0.064
P_8	-0.038	0.144	0.352
P_9	0.031	0.387	0.642
Unexplained variance (U_i)			
U_1	0.021	0.073	0.220
U_2	0.052	0.288	0.709
U_3	0.037	0.061	0.166
U_4	0.196	0.303	0.769
U_5	0.284	0.358	0.548
U_6	0.590	0.855	0.999

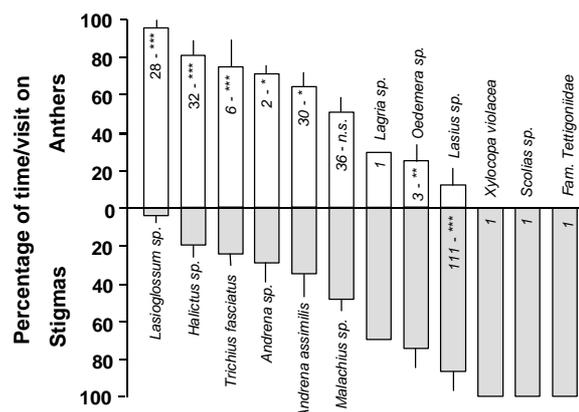


Figure 3. Average % total time/visit spent on anthers and stigmas by each type of floral visitor. Standard errors are shown for species appearing more than once during regular censuses. Sample sizes and significant differences (Mann-Whitney's U test) are shown inside bars (* - $P < 0.05$; ** - $P < 0.01$; *** - $P < 0.001$).

found on average for hermaphroditic flowering plants (see Wiens, 1984; Burd, 1994). Several non-mutually excluding explanations might explain seed set values: (1) Pollen or pollinator limitation could account for low seed initiation in open-pollinated flowers, but it not in cross-pollinated flowers, whose stigmas were saturated with pollen by manipulations. This also demonstrates that open-pollinated flowers were not pollen-limited. (2) If resources available for reproduction are in short supply, a high proportion of ovules might be aborted before enlarging during development. This would be particularly true at RH, where plants are consistently subjected to severe herbivory. Plants at RH tended to initiate and produce less seeds than plants at FU; further, seeds were significantly lighter at RH than at FU. Thus, at least at RH, availability of resources to allocate to seed production may be partly responsible for low seed set. (3) Also, if resources available for reproduction are limited, allocation to male function (pollinator attraction structures and pollen production) may decrease the amount of resources available for female function (seed maturation), thus potential for seed production may be limited by a balance in allocation of resources to sexual functions within the same flower. (4) Genetic disorders, as a consequence of the small population sizes and mating between relatives, could cause the reduced seed set via the expression of recessive deleterious alleles rendered homozygous by inbreeding. However, crossing between populations did not enhance seed initiation or seed set (Sánchez-Lafuente, Rey & Alcántara, unpubl. data), which exclude to a great extent the possibility that a reduced seed set could be due to crossing between close relatives within populations.

THE ROLE OF FLORAL VISITORS

When flying visitors were absent, seed initiation, most likely due to autogamy, was very low (3%), indicating that floral visitors are required for pollination (e.g.,

Ramsey, 1993). Further, our experiments have shown that ant species visiting *P. broteroi* were not acting as pollinators (but see Takahashi, Nishio & Hayashi, 1993; Gómez & Zamora, 1992; Ramsey, 1995; Puterbaugh, 1998).

Bees and beetles were the commonest visitors of *P. broteroi*. Although *P. broteroi* shares several characteristics with other plants that are traditionally described as cantharophilous, *e.g.*, large, flat, non-tubular corolla, protogynous and pollen as reward (Castroviejo *et al.*, 1993, but see Proctor, Yeo & Lack, 1996), we found that beetles were not important pollinators. The results of the SEM analysis (Figure 4) show that *A. assimilis* was mainly responsible for successful pollination of *P. broteroi* during the study season. The negative effects on seed initiation caused by *Lasioglossum* sp. and *H. scabiosae* indicate that these species actually may be removing pollen grains deposited by good pollinators (see Thomson & Thomson, 1992). Pollen foragers were largely more abundant than nectar foragers and spent more time on the anthers than on the stigmas (see Johnson, Delph & Elderkin, 1995). In addition, a likely consequence of the relative large size of flowers (mean \pm SE corolla diameter: 59.34 ± 14.81 mm, $N = 121$; Sánchez-Lafuente, Alcántara & Rey, unpubl. data) compared to the size of the commonest visitors (*e.g.*, mean \pm SE body length: *A. assimilis*: 15.28 ± 0.17 mm; *H. scabiosae*: 13.52 ± 0.19 mm), may be that these insects spend most of the time on the anthers, only occasionally contacting the stigmas. These two findings suggest that pollen removal in *P. broteroi* is likely to be high (Wilson & Thomson, 1991; Thomson & Thomson, 1992). Thus, although *P. broteroi* flowers may produce few seeds, they can be successful as pollen donors. However, *P. broteroi* produces an excess pollen that is not removed (lots of pollen grains are left inside the corolla). Since pollen foragers are usually considered less

efficient, non-legitimate pollinators (*e.g.*, Wilson & Thomson, 1991; Conner, Davis & Rush, 1995; Petanidou, Den Nijs & Oostermeijer, 1995), the excess pollen would ensure that at least a proportion of pollen grains will be successfully deposited onto target flowers.

While the commonest visitors did not vary in frequency of visits, there were differences in duration of visits and the proportion of time/visit on the stigmas. Overall, bees (mainly *Andrena* species) made longer visits than beetles (*Malachius* sp.), while the latter spent more time/visit on the stigmas than any of the bees (Figures 2, 3). Both field and experimental data suggest that pollination efficiency seems to be a combination of the two factors above: (1) A few longer visits, with more time spent on stigmas, may be more important than many frequent but shorter visits. For instance, in a standard visit by *Malachius* sp. of 60 s, about 29.4 s was spent on stigmas, while in a visit by *A. assimilis* of 100 s, about 35 s was spent on stigmas (16% more than the former). In our experiment with two bee species, we also found that *A. assimilis*, with longer visits, was more efficient than *H. scabiosae*. Similar findings that a few long visits may be more efficient than many short ones have been found elsewhere (*e.g.*, Ohara & Higashi, 1994; but see Schemske & Horvitz, 1984; Young & Stanton, 1990). However, the relative importance of frequency of visits vs. time/visit on pollination success may be highly dependent upon particular floral morphologies or pollination systems. (2) When time/visit was similar, as was found for the commonest bees (Figure 2), the percentage of time/visit on the stigmas was the important factor (Figs. 3 and 4). Thus, also experimentally, we found that *A. assimilis* was a 12.5% more efficient than *H. scabiosae* at seed initiation.

The confidence intervals for path coefficients relating time on stigmas to seed initiation were wide (P5 to P8, see Table II). This may be a consequence of the relatively

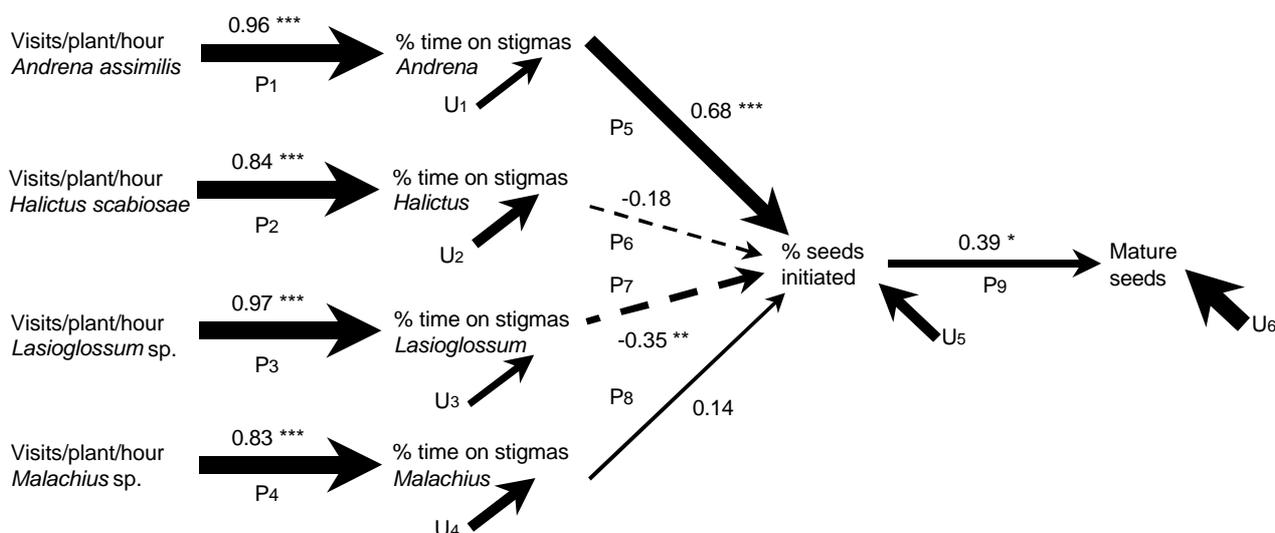


Figure 4. Path diagram for pollinator efficiency. Positive effects are indicated by solid lines, negative effects are indicated by dashed lines. Path coefficients (P_i) significantly different from 0 are shown as * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Width of each arrow is proportional to the standardized path coefficient. See Table II for confident intervals of the path coefficients. % time on stigmas refers to % total time/visit spent on stigmas. $N=30$ plants.

low sample size used in this study ($N = 30$ plants) in relation to the number of direct effects estimated in the path model. However, for *A. assimilis*, this result might also indicate that, although this bee species appeared to be the main pollinator for *P. broteroi*, not all plants were pollinated with the same efficiency, and other reasons not directly influenced by the visitor (*e.g.*, whether mostly selfed or crossed pollen is delivered to flowers) might explain differences in percentage of seeds initiated. Pollen origin could also explain the variation in the significance of the relationships found in the path relating percentage of seed initiation to seed set (P9, Table II, Figure 4). Thus, if self-pollen predominated numerically over cross pollen, the chances of seed abortion due to inbreeding depression would be increased.

Summarizing, although flower morphology did not restrict access to members of a very diverse visitor assemblage, most visitors were probably ineffective at depositing pollen onto stigmas and were probably acting through the male function (pollen removal). Unlike multi-flowered hermaphroditic plants species producing an excess flowers to act as pollen donors (see Sutherland & Delph, 1984), *P. broteroi* cannot adjust full functional units (flowers) to act exclusively as males or females. Also, the range in the number of flowers/plant in *P. broteroi* is too short to think that, even in multi-flowered plants, a number of flowers will be acting solely as pollen donors. Thus, considering that pollen limitation is unlikely and if resources available for reproduction are limited, we suggest that the balance in resource allocation to each male (pollen production and structures attractive to visitors) and female (ovule production and maturation) functions within a flower may be one of the primary reasons explaining seed set in this plant species.

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APPENDIX. List of insects species and groups detected at least once visiting *Paeonia broteroi*. Those recorded during regular censuses are presented in bold

Hymenoptera	Coleoptera	Orthoptera
Fam. Apidae	Fam. Scarabeidae	Fam. Pamphagidae
<i>Bombus terrestris</i>	<i>Cetonia</i> sp.	<i>Eumigus</i> sp.
<i>Apis mellifera</i>	<i>Trichius fasciatus</i>	Fam. Tettigoniidae
<i>Anthophora</i> sp.	<i>Oxythirea funesta</i>	1 species
<i>Xylocopa violacea</i>	<i>Tropinota</i> sp.	
Fam. Formicidae	Fam. Tenebrionidae	
<i>Camponotus</i> sp.	Lagria sp.	
Lasius sp.	Fam. Elateriidae	
Fam. Scoliidae	<i>Drapetes</i> sp.	
Scolias sp.	<i>Ampedus pomorum</i>	
Fam. Andrenidae	Fam. Oedemeridae	
<i>Andrena assimilis</i>	Oedemera sp.	
Andrena sp.	Fam. Coccinellidae	
Fam. Halictidae	<i>Coccinella septempunctata</i>	
Lassioglossum sp.	Fam. Malachiidae	
Halictus sp.	Malachius sp.	
	Fam. Carabiidae	
	Calathus rubeus	