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Ant pollination of the palaeoendemic dioecious *Borderea pyrenaica (Dioscoreaceae)*

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Abstract: The importance of ants for pollination in the dioecious *Borderea pyrenaica* (*Dioscoreaceae*), a Tertiary palaeoendemic plant of the Pyrenees (NE Iberian Peninsula) was studied. The frequency of different visitors (ants, lady beetles, and *Diptera*) to staminate and pistillate flowers was quantified, and their effectiveness as pollinators was examined by means of fruit and seed set in selective experimental exclusions. Although ants were less abundant on flowers than other visitors, they were the most effective pollinators. Some qualitative factors of this mutualistic ant-plant interaction may account for their effectiveness: the small size of the ants, their high visitation rate to pistillate flowers, and the lack of reduction in viability of the pollen transported on the integument. In addition, the sedentary nature of ants assures their presence during the flowering period. The most abundant floral visitors of *B. pyrenaica* were therefore not the most effective pollinators.

The flowers of entomophilous species may be visited by a variety of insects, whose pollination ability may vary considerably both in qualitative and quantitative terms (Herrera 1987, 1989). If floral visitors carry different amounts of pollen, deposit on the stigma pollen of different suitability for the fertilization of ovules, or show different visitation frequencies to receptive pistillate flowers, then they differ in their "quality" (effectiveness) as pollinators (Primack & Silander 1975, Arnold 1982, Schemske & Horvitz 1984, Motten 1986, Herrera 1987). However, to assess the relative importance of different floral visitors as pollinators of a particular plant species, it also is necessary to add a "quantity" component to this differential pollinator effectiveness, because insects often differ in flower visitation frequency and in abundance among years and plant populations (Herrera 1989, Schemske & Horvitz 1989, Gómez & Zamora 1992).

Some groups of insects, such as ants, have traditionally been considered as poor or ineffective pollinators, even though some ant species are habitual flower visitors. Ants commonly are regarded as nectar thieves that do not effect pollination because of their small size, smooth integument and frequent grooming (Proctor & Yeo 1973, Faegri & van der Pijl 1979), and some floral structures have been interpreted as adaptations to prevent ant visits (Herrera & al. 1984). Only a small number of plant species have been described as ant-pollinated (see review in Peakall & al. 1991), and only for three of these has it been demonstrated that ant pollination leads to seed-set (Peakall & al. 1987, Peakall 1989, Peakall & Beattie 1989, Gómez & Zamora 1992). Several recent studies have shown that ant secretions have a strong negative effect on potentially pathogenic micro-organisms and disrupt normal pollen function (Iwanami & Iwadare 1978; Beattie & al. 1984, 1985, 1986; Veal & al. 1992). Pollen exposed to secretions of metapleural glands of different ant species exhibited reduced quality, germination, and shorter pollen tubes (Beattie & al. 1984, Beattie 1985, Hull & Beattie 1988, Gómez & Zamora 1992). These results have supported the hypothesis proposed by Beattie (1985) that the evolution of ant pollination may have been prevented by these harmful secretions, hence the paucity of ant pollination systems.

Ants are one of the few groups of insects visiting the small flowers of the dioecious *Borderea pyrenaica* Miégeville (*Dioscoreaceae*), a Tertiary palaeoendemic species of the Pyrenees (NE Iberian Peninsula). The main objective of this study was to determine their importance for pollination in this alpine plant, given the scarcity of ant pollination systems and the possible negative effects of ant secretions on pollen. We examined and compared the visitation frequency of different insects to staminate and pistillate flowers of *B. pyrenaica*, experimentally assessed the effectiveness of different potential pollination vectors, and tested the effect of ants on pollen viability.

Material and methods

The plant. *Borderea pyrenaica* is a small dioecious geophyte occurring on alpine limestone screes of the central Pyrenees, over 1800 m s. m. A subterranean tuber with a strong root system allows the plant to remain attached to the soil below the stones, and a short branched stem with cordate leaves develops from the tuber. The growing season is from June to September. Flowering is usually in June, with female plants flowering for a shorter period of time (both starting later and ending earlier) than male plants. Male plants have several (1–20) many-flowered racemes (mean ± standard deviation of flowers per plant: 66.7 ± 106; range: 1–481; $N = 47$), but with only 3–4 flowers per raceme open at any one time. In contrast, female plants bear only a few flowers (2.1 ± 1.3; range: 1–6; $N = 51$) open at a particular time. Flowers are nearly sessile, simple, about 5 mm wide, and have a six-lobed yellowish-green perianth. Staminate flowers have six stamens (1–2 mm high) with anthers that have introrse dehiscence. Pistillate flowers have six staminodes, a pistil (1–1.5 mm high) with three stigmas reflexed outwards, and a 3-locular ovary with six ovules. Preliminary observations suggested that pistillate flowers are functional (receptive) for a longer time (about 7–9 days) than staminate flowers (2–3 days). Both staminate and pistillate flowers secrete, at the base of the flower, small amounts of nectar which is fully accessible to insects. Fruits are relatively large and mature slowly, remaining green for approximately two months until late August or early September, when they open. A maximum of six seeds per fruit is possible.

Field work was done at Pineta Valley (42°41′ N, 0°06′ E), at 2100 m elevation. The Pineta population contains several thousands individuals and approximately 2.3 times more males than flowering females (García 1993, García & Antor 1995). Plant density in this population is high (up to 230 individuals in 0.25 m^2): leaves and inflorescences of neighbouring plants are often intermingled.

Insect visitors and floral sex ratio. Preliminary observations of flower visitors were made in 1990, and potential pollinators were identified or categorized according to form taxa: beetles, ants, and *Diptera*. Pollination censuses were done on June 26 and 28 of 1991, during the peak of flowering, in dense patches of *B. pyrenaica*. We recorded all flower visitors seen in 15 min periods in 42 randomly chosen 1 m^2 quadrats. We slowly scanned the quadrat and recorded floral visitors and the sexual expression of flowers visited. To determine the floral sex ratio at the time of the study, we counted the number of staminate and pistillate flowers in 13 quadrats of 0.5 × 0.5 m, located in the same area as the pollination censuses.

To confirm the occurrence of the same insects or to record new pollinators, additional observations were made during the flowering in 1992 and 1993 in the same population, as well as in another population 50 km away (Gistain, 1900 m s. m. 42°33′ N, 0°19′ E).

Effectiveness of different pollinating vectors. To evaluate the effectiveness of the different potential pollinating agents, we made several selective exclusion experiments on female plants before flowers had opened, in the same area where insect censuses were made. (A) Several female plants (N = 15) were transported to a botanical garden to test whether or not flowers would set fruit in the absence of pollination (hereafter "isolated"), which will inform on the possibility of agamospermy in this species. (B) On three groups of plants (N = 60 female plants), a small ring of a colourless, odourless and non-drying glue (made by "Rata-Stop") was placed around the peduncle of the pistillate buds to prevent access by walking insects ("flying insects" treatment), which will serve to assess the importance of anemophily + pollination by flying insects. (C) Two other groups of male and female plants (N = 62 females) were covered with a mesh exclosure (1 × 1 × 0.2 m; 1.1 mm mesh), and the buds of some pistillate flowers (N = 25 female plants) also were treated as in B. In these plants, only airborne pollen could result in fruit-set ("wind" treatment), therefore, this treatment will inform on the importance of anemophily. Although the exclosures could slow down air movement and hence the likelihood of wind pollination, there were staminate plants near the treated female plants, and we noted that leaves and flowers inside the exclosure could move due to wind. (D) Flowers of the remaining female plants within the exclosures could only be visited by very small insects capable of passing through the mesh (small ants), providing information on the effectiveness of walking arthropods less than 1.1 mm wide ("ant treatment"). It was not possible to design any exclusion to test the isolated effect of beetle pollinations. Female plants surrounding each treatment (one group for B and a second group for C and D) served as controls.

At the end of the fruit maturation period, before fruit dehiscence, we recorded the number of control and treated pistillate flowers that had set fruit (fruit-set), and the number of ovules per fruit that had matured into seeds (seed-set). Fruit- and seed-set of untreated flowers also were recorded in the two subsequent years (with a different pollinator assemblage).

Quality of pollen carried by ants. To investigate the effect of ants on pollen viability, we collected live specimens of *Leptothorax tuberum* Fabr. ants from the Pineta population in 1992. This small ant (2.4–3 mm length) with metapleural glands, was seen visiting flowers and transporting pollen of *B. pyrenaica*. The viability of pollen carried by this ant was assessed by the fluorochromatic procedure (FCR; Heslop-Harrison & Heslop-Harrison 1970, Heslop-Harrison & al. 1984).

Several stamens, removed using forceps, from recently opened staminate flowers of a plant, were placed in a glass tube, into which one worker ant was introduced. Cotton was gently pushed behind the ant to force it into contact with the anthers. After one hour, the pollen carried by the ant (S-ant) and untreated pollen from anthers of the same plant (S-control) were transferred to a fluorescein diacetate in 20% sucrose solution for fluorochromatic bioassay. The percentage of bright green fluorescing and non-fluorescing grains (viable and inviable, respectively) was determined in a fluorescence ORTHOLUX 2 microscope (Leitz; filter system D: BP 355–425 LP 460). Five bioassays were performed, using five different plant and worker ant combinations.

In ten other glass tubes, we introduced two staminate flowers from a single inflorescence and one worker ant, so that ants could forage on nectar and contact anthers in a situation similar to that encountered in the field. After 30 min, pollen carried by ants (F-ant) and Control (F-control; taken from the same flowers visited by ants) were transferred to the fluorescein diacetate solution, and the number of viable and inviable grains counted as above.

Results

Insect visitors and floral sex ratio. In 1991, we found floral visitors in 37 of the 42 quadrats inspected. The types of floral visitors, number of insect visits, and the sexual expression of flowers visited are summarized in Table 1. All visitors of *B. pyrenaica* flowers searched for nectar, they neither foraged on pollen nor destroyed floral organs. The most frequent insect visiting *B. pyrenaica* flowers was the lady beetle *Coccinella septempunctata* (*Coccinellidae*), occurring in 94% of the quadrats and in 53.8% of those flowers visited by insects. Lady beetles are slightly bigger than the floral perianth, and always make contact with the anthers during visits to staminate flowers. A large quantity of pollen could be seen on their integument, despite frequent grooming. They usually walked on leaves and stones and climed up the stems to reach male inflorescences, though they sometimes moved by flying. Medium-sized *Diptera* (usually between 5–6 mm long), belonging to fam. *Calliphoridae* (as *Lucilia illustris* Meigen), and *Muscidae* also were frequent visitors. Their legs, however, are usually longer than the height of the stamens and the pistil, reducing the possibility of contact with the reproductive parts of the flowers. Pollen sometimes adhered to their proboscis when they sucked nectar, but frequently they groomed and removed attached pollen by throwing away a smaller pellet of pollen. Therefore they carry little, if any, pollen. We observed other smaller files transporting pollen on their body. *Diptera* usually approached *B. pyrenaica* from above, landing directly on the flowers, and then often walking across to nearby flowers. The ant *Leptothorax tuberum* Fabr. (*Formicidae*) was responsible for 8.5% of insect visitation. We clearly observed that the body of this small ant touched the floral reproductive organs while it fed on nectar, and that it transported pollen on its integument despite frequent grooming. Because *B. pyrenaica* is almost the only inhabitant of these screes, all the pollen transported by ants was assumed to be from this species.

The flower sex ratio was strongly male-biased: 98.8% of the open flowers were staminate (N = 3324 flowers examined; average ± s.d. of the ratio staminate/pis-

Table 1. Ratio of male to female flower visits of different potential pollinators. From censuses conduced in 1991

	Lady beetles	*Diptera*	Ants
N	321	225	51
Male : female	35 : 1	224 : 1	16 : 1

Table 2. Percentage of female flowers that set mature fruits (fruit-set), and number of fruits with 1–6 seeds per fruit. Results of fruit-set for each treatment are compared with control fruit-set with a χ^2 test (d.f. = 1; ***: $p < 0.001$; n.s.: $p > 0.05$)

Treatment	Examined flowers	Fruit-set (%)		Frequency of fruits according to the number of seeds 1	2	3	4	5	6
Wind	45	2.2	***	1	–	–	–	–	–
Control	293	81.9		11	13	6	13	37	159
Flying insects	81	7.4	***	–	–	1	–	–	5
Control	412	82		30	21	28	23	55	182
Ants	73	82.2	n.s.	2 (3.3%)	1 (1.7%)	4 (6.7%)	8 (13.3%)	6 (10.0%)	39 (65.0%)
Control	293	81.9		11 (4.6%)	13 (5.8%)	6 (2.5%)	13 (5.4%)	37 (15.4%)	159 (66.3%)

Table 3. Pollen quality (percentage of pollen grains fluorescing with FCR) of *Borderea pyrenaica* and results of paired-sample t-test for differences between control pollen and pollen carried by the ant species *Leptothorax tuberum* (original data were arcsine transformed, following the modification of Freeman and Tuckey, see Zar 1984: 240). For each bioassay, the probability of the Fisher exact test is also indicated

	Ant treatment		Control			t
	N	% Fluorescence	N	% Fluorescence		
Pollen from cut stamens						
1	30	100	105	81	**	
2	74	94.6	161	78.3	**	
3	81	98.8	137	75.9	***	
4	55	87.3	131	78.6	n.s.	
5	29	93.1	85	80	n.s.	
Total	269	94.8	619	78.5		4.82**
Pollen from flowers						
1	13	92.3	158	81.6	n.s.	
2	14	100	52	94.2	n.s.	
3	19	94.7	60	93.3	n.s.	
4	7	100	64	95.3	n.s.	
5	76	77.6	21	85.7	n.s.	
6	8	100	51	96.1	n.s.	
7	27	100	77	100	n.s.	
8.	22	100	86	96.5	n.s.	
9	23	95.7	116	98.4	n.s.	
10	44	88.6	142	76.1	n.s.	
Total	253	90.1	827	89.4		2.33*

tillate open flowers 116.7 ± 96.0: N = 13 quadrats). Relative to this sex ratio, *Diptera* visited relatively more staminate flowers (though not statistically significant, Fisher exact probability test: $p = 0.518$), whereas *Coccinella septempunctata* ($p = 0.038$) and *Leptothorax tuberum* ($p = 0.028$) visited relatively more pistillate flowers (Table 1).

In 1992 and 1993 we observed *Diptera* and *Leptothorax tuberum* in the same populations as in 1991, but we did not find any lady beetles in the Pineta or the Gistain populations. These same years we discovered two other ant species visiting *B. pyrenaica: Formica fusca* L. in the Pineta population (in places near meadows), and *Formica decipiens* Bondr. at the Gistain population. In this latter population, medium-sized *Diptera* (fam. *Calliphoridae* and *Muscidae*) were also observed visiting *B. pyrenaica*.

Effectiveness of pollen vectors. None of the plants transported to a botanical garden (treatment A) produced seeds (N = 67 pistillate flowers) despite the fact that 40% of the flowers had enlarged ovaries. Among non-fruiting flowers in the field, enlarged ovaries without seeds also frequently are observed. Other female plants that were collected after anthesis and transplanted set fruit in the botanical garden, indicating that reproductive failure of "isolated" plants was not caused by

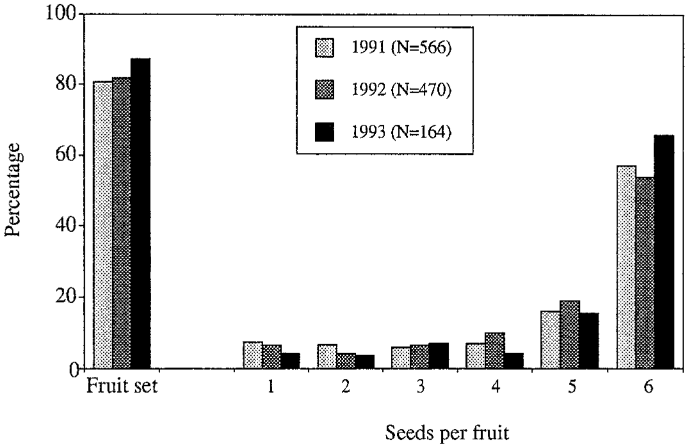

Fig. 1. Fruit- and seed-set of *Borderea pyrenaica* at Pineta for three years of study (N number of pistillate flowers examined)

the transplanting itself, but due to a lack of pollination vectors. Therefore, agamospermy is unlikely in *Borderea pyrenaica*. Only one of the 45 pistillate flowers of the "wind" treatment (C) set fruit, suggesting that this species is not anemophilous. In addition, some morphological characteristics of the pollen suggest that *B. pyrenaica* is entomophilous: it shows a conspicuous ornamentation (sexine clavate-gemmate), is covered by pollenkitt, and is relatively large with a polar axis about 32 µm (García 1993). Flowers of the "flying insects" treatment (B) set significantly fewer fruits than did control flowers (Table 2), suggesting that *Diptera* are probably not so important as pollinators. Fruit-set of flowers that only could be visited by ants (treatment D) did not differ from that of control flowers (Table 2), and a similar number of seeds per fruit was produced in both (mean ± s.d.: 5.2 ± 1.3 and 5.2 ± 1.4, respectively; Mann-Whitney U-test: $Z = -0.3$, $p = 0.763$; see also Table 2).

Quantity and qualtity of pollen carried by ants. Ants placed in glass tubes with cut stamens for one hour carried between 29 and 81 pollen grains (average ± s.d. = 53.8 ± 24.1 pollen grains; N = 5), of which 95% were viable. Ants placed in tubes with two flowers for 30 min carried between 7 and 44 pollen grains (25.3 ± 20.8; N = 10), of which 90% were viable (Table 3). In both experiments the quality of the pollen carried by ants was generally higher than that of control pollen (Table 3). Therefore, there was no detrimental effect to pollen after contact with the integument of *L. tuberum*.

Interannual viability in fruit- and seed-set. In the three years studied fruit-set was greater than 80%, and in most fruits all six ovules developed into seeds (Fig. 1). Fruit- and seed-set were not statistically different among years (fruit-set: $\chi^2 = 3.63$, $p = 0.16$, d.f. = 2; seed-set: $\chi^2 = 14.14$. $p = 0.17$, d.f. = 10).

Discussion

Many dioecious plants have small, unspecialized flowers pollinated by wind or small and generalist insects such as bees or flies (Bawa & Opler 1975, Bawa

1980 a, Freeman & al. 1980, Fox 1985, Muenchow 1987, Charlesworth 1993; but see Renner & Feil 1993). The floral biology of *B. pyrenaica* fits this pattern, having inconspicuous flowers pollinated by unspecialized insects. All floral visitors to *B. pyrenaica* search for the nectar reward offered in a small and apparently similar quantity in both staminate and pistillate flowers. Therefore, pistillate flowers of this dioecious plant are not deceitful, and pollination does not occur by "mistake" (see Willson & Ågren 1989) as it does in some other insect-pollinated dioecious species (Baker 1976, Bawa 1980 b, Little 1983, Ågren & al. 1986, Armstrong & Irvine 1989, Renner & Feil 1993).

In a dioecious species pollinators must visit both staminate and pistillate flowers, thus, not only the visitation rate, but also the ratio of staminate to pistillate flowers visited are important factors for pollination efficiency. *Diptera* were the worst pollinators of *B. pyrenaica*, visiting relatively fewer pistillate flowers per unit time than the other floral visitors, and yielding low seed-set (as exclusion experiments indicated). Lady beetles are also not faithful pollinators as they are not permanent habitants of screes, migrating each year between high and low elevations on mountains (Hagen 1962, Kreiter & Iperti 1984). Their migration to *B. pyrenaica* populations may be too late to coincide with the peak flowering period, as observed in 1992 and 1993. Therefore, these insects can be categorized as unpredictable pollinators, and their importance may be variable in time. In spite of their local abundance and high visitation rates to pistillate flowers in 1991, their absence in 1992 and 1993 did not affect the reproductive success of *B. pyrenaica* as fruit- and seed-set were similar in these three years.

Unlike lady beetles, *Lepthorax tuberum* resides permanently on screes, where it nests in rock crevices. Its small size allows the ant to be an effective pollen vector of *B. pyrenaica*, and flowers that only could be visited by ants produced approximately the same number of seeds as flowers exposed to unrestricted pollination. The relatively high fruit- and seed-set, and the constant presence of ants during the flowering period, make ants predictable and reliable pollinators of *B. pyrenaica*. In addition, our study demonstrated that the quality of pollen carried on their bodies is not reduced. On the contrary, *L. tuberum* transports primarily viable pollen from anthers of *B. pyrenaica*. These results contrast with those obtained in other studies on plants and their natural ant pollinators (Peakall & al. 1990, Gómez & Zamora 1992), and on ants and plants not involved in natural pollination systems (Beattie & al. 1984, 1985; Beattie 1985; Hull & Beattie 1988). A similar absence of negative effect of ant transport on pollen viability has previously been found only in the case of an orchid and its ant pollinator (Peakall & Beattie 1989), though in this latter case natural pollination does not involve a direct contact between pollen and ant integument.

Borderea pyrenaica exhibits many traits described by Hickman (1974) as characteristic of ant pollination syndrome: short plants, interdigitation of branches from adjacent individuals, sessile flowers, an accessible and small quantity of nectar, and few ovules per flower. Although *B. pyrenaica* grows in alpine rather than hot and arid environments (another trait noted by Hickman 1974 for ant-pollinated species), screes are totally exposed to sun radiation and stone surfaces are usually very hot on clear days of summer. Several other ant-pollinated species are alpine, as *Eritrichium aretioides* (*Boraginaceae*), *Oreoxis alpina* (*Umbelliferae*), *Thlaspi* *alpestre* and *Hormathophylla spinosa* (*Cruciferae*), as shown by Petersen (1977 a, b) and Gómez & Zamora (1992). This could be related to the scarcity of other pollinators, such as *Hymenoptera* and *Lepidoptera*, in the alpine environment (Arroyo & al. 1982, Warren & al. 1988).

In one of the few well documented studies on the importance of ants as pollinators, Gómez & Zamora (1992) found, that, although *Hormathophylla spinosa* (*Cruciferae*) was visited by a diverse assemblage of pollinators and the metapleural secretion of ants reduced pollen viability, the reproductive success of this plant species mainly depended on the presence and abundance of ants visiting its flowers ("quantity" component of pollination). In contrast, in *B. pyrenaica* ants were less frequent on the flowers than other floral visitors, but they were the most effective and predictable pollinators. Their effectiveness for pollination ("quality" component) is related to their small size (which ensures contact between the ant body and plant reproductive organs), their relatively high visitation rate to pistillate flowers (probably due to their walking behaviour), and the absence of reduction of viability of the pollen transported on the integument. However, the "quantity" component of pollination also is insured through the sedentary nature of ants contributing year after year their fidelity as pollinators. In both our study and that one of Gómez & Zamora (1992), pollination by ants was shown to be essential for the reproductive success of these alpine species. However, these two studies illustrate how contributions by the same kind of pollinator can differ as to quality and quantity components of pollination.

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