Effects of ants on the reproductive success of *Euphorbia cyparissias* and associated pathogenic rust fungi

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Ants are common visitors to the flowers of *Euphorbia cyparissias*, and also often forage on *E. cyparissias* stems that are infected by rust fungi of the species complex *Uromyces pisi*. These fungi sterilise their host, produce nectar and require insects for their sexual reproduction. Our objective was to determine whether ant visits enhance the sexual reproduction of either *E. cyparissias* or the rust fungi. *Uromyces pisi* is known to be obligately outcrossing, whereas a breeding system experiment established that *E. cyparissias* can self, but sets more seeds when outcrossed. We used insect exclusion experiments to test whether ants fertilise the rust fungi and to determine whether ants are pollinators of *E. cyparissias*. These experiments showed that insect pollination is necessary for seed set and that ants can pollinate the flowers. However, ants do not fertilise the rust fungi.

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Ants and plants are commonly associated, but they only occasionally form mutualistic relationships. For example, the seeds of only a few species are dispersed primarily by ants. Ant pollination is also rare. Only about thirty reports of ant pollination have been published during the last century (reviews by Faegri and van der Pijl 1979, Peakall et al. 1991, Jolivet 1996), and of these studies, there is convincing experimental evidence of ant pollination for only eleven species (Peakall 1989, Peakall and Beattie 1989, Gómez and Zamora 1992, García et al. 1995, Ramsey 1995, Gómez et al. 1996, Puterbaugh 1998).

The best argument to explain the rarity of ant pollination is that some ant species possess metapleural glands whose secretions reduce pollen quality (Beattie et al. 1985). These glands, located on the thorax, secrete antibiotic substances that ants smear on themselves and their larvae for protection against fungal and bacterial infections (Maschwitz et al. 1970, Beattie et al. 1986). Probably due to these secretions, contact with the ant integument is harmful to pollen (Beattie et al. 1984, Gómez and Zamora 1992). However, some pollen species are more resistant than others, not all ant species have metapleural glands, and species without these glands can also reduce pollen viability (Hull and Beattie 1988, García et al. 1995). Nonetheless, although many pollen grains are damaged by ant contact, sometimes enough remain viable to lead to normal seed set (Ramsey 1995).

Ants are also considered to be poor pollinators because their small size allows them to forage on flowers without touching stigmas or anthers, and pollen does not adhere well to the ants’ smooth integument and it is often removed by frequent grooming. However, these hypotheses are usually not tested, and in many of these characteristics ants are not much different from bees (Beattie et al. 1984).

It has also been argued that, because ants do not fly but walk, ant visitation mostly causes self or geitonogamous pollination. This assertion is supported by the evidence collected by Ramsey (1995) and Puterbaugh (1998) but conflicts with that of García et al. (1995) and
Svensson (1985). Crawling may cause self-pollination, but is also the least expensive type of movement (Heinrich 1975) and dependence on pollinators with low energy needs may indirectly benefit the plant by allowing for reduced nectar production.

Any plant-pollinator interaction has a quality and a quantity component (Herrera 1987). The quality term refers, for instance, to the viability of the pollen deposited on a stigma by a pollinator, while the quantity term refers to the frequency of the interaction. The contribution of a particular pollinator group to pollination is then the product of the quality and quantity component of the interaction. From this perspective, all the reasons put forward to explain the rarity of ant pollination are related to the quality component of the interaction. However, the quantity component of the interaction must also be considered. On this point, because ants are social insects and regularly return to a foraging site as long as they are rewarded with nectar (Hölldobler and Wilson 1990), they could surpass the classical pollinators and so compensate for their possible low quality.

*Euphorbia cyparissias* L. is a widespread species in Europe, and is commonly associated with ants, with which it has a known mutualistic relationship for seed dispersal (Dymes 1933 cited in Stahevitch et al. 1988). Not only are ants attracted to the seeds of this species, but ants also represent a large proportion of the insect visitors to the flowers of *E. cyparissias*. In an artificial array experiment conducted in 1997 in Vicques (Switzerland), ants represented 36% of the visitors to the flowers (Pfunder and Roy in press). In the present study, we examined whether ants actually pollinate *E. cyparissias*. We also conducted a hand pollination experiment to determine the breeding system of the plant.

*Euphorbia cyparissias* is also the host for rust fungi of the *Uromyces pisi* (Pers.) Wint. species complex. These fungi produce spores mixed with nectar on the leaves of the infected stems. Insects are required to transport these spores between the different mating types of the fungi, very much like pollination in higher plants (Pfunder and Roy in press). In an artificial array experiment conducted in 1997, 47% of all visitors to infected stems were ants (Pfunder and Roy in press). Because ants were frequent visitors to infected stems, and because some ants actively farm fungi for food (Hölldobler and Wilson 1990, Mueller et al. 1998), we also asked whether ants fertilise the pathogen. If the ants do fertilise the host-specific pathogen, then they may harm the host plant indirectly through this interaction.

In this study, we addressed the three following questions: 1) What is the breeding system of *E. cyparissias*? 2) Do ants pollinate *E. cyparissias*? and 3) Do ants fertilise *Uromyces pisi*?

### Materials and methods

#### Study site

The field site was located at Vicques (Cras de la Combe) in the Swiss Jura mountains (co-ordinates 599 000/245 940) at 550 m above sea level. We examined a population of *E. cyparissias* in a dry calcareous grassland that was formerly grazed and is now mown once a year in the autumn.

#### The plant

*Euphorbia cyparissias* is a common native species in Europe that has been introduced into New Zealand and Eastern North America (Stahevitch et al. 1988). It is a perennial species with vegetative reproduction from root runners. It grows in dry meadows, at ruderal sites, and forest edges.

The flowers of *E. cyparissias* are strongly reduced: the male flower is a pedicelled stamen and the female flower is simply a pistil, both without perianth parts (Stahevitch et al. 1988). One female and several male flowers are enclosed in an involucre (five bowl-shaped fused bracts) that bear four sickle-shaped nectaries and which is subtended by two bracts. This inflorescence is called a cyathium. Two to five (mostly three) cyathia are borne by one ray and the umbel formed by up to 40 rays is termed a pseudo-cyme. *E. cyparissias* was the first described example of protogyne (Sprengel 1793 cited in Proctor and Yeo 1973), in which the stigma is receptive before the pollen is shed. The flowers emit a sweet and strong scent. Fruits are dehiscent when dry and explode to liberate a maximum of three mature seeds each. The seeds bear an elaiosome, a food reserve for the ants that disperse the seeds (Dymes 1933 cited in Stahevitch et al. 1988).

#### The fungi

Rust fungi in the species complex *Uromyces pisi* (Pucciniaceae) often infect *E. cyparissias*, causing drastic changes in plant physiology and morphology (Pilet 1952, Hartwich 1955). Infected stems are mostly unbranched, sterile, and have thick oval leaves instead of the normal needle-like leaves. The upper leaves on these stems are yellow and flower-like, such that the whole infected stem forms a ‘pseudo-flower’. The rust fungi produce spores mixed with sweet smelling nectar on the under side of the leaves. Insects are required to transport these spores (more accurately called spermata) between different mating types (Pfunder and Roy in press). After successful fertilisation, the fungi produce dispersal spores called aeciospores in structures called aecia.

#### The ants

The most commonly observed ant species during the 1998 flowering season was *Lasius alienus* (Förster). This
species undergoes an intensive trophobiotic symbiosis with aphids. In addition, it is a nectarivore, zoophage and also attends lycaenid caterpillars (Seifert 1996). We observed this species foraging on the nectaries of healthy E. cyparissias, and also on infected plants. We also often observed Formica pratensis Retzius, and more seldom, F. cunicularia Latreille, on the flowers of E. cyparissias. All these species possess metapleural glands. Identified specimens are deposited in the insect collection of the Federal Institute of Technology (ETH) Zürich, Switzerland.

Breeding system of E. cyparissias
To determine the breeding system of E. cyparissias, we controlled pollination. We caged 45 plants in bud with florist gauze bags (Kleen Test Products, division of Meridian Industries, Inc., Milwaukee, Wisconsin) to exclude insects. To prevent the bags from collapsing on to the flowers, we put cylinders of chicken wire inside them. On each plant, male flowers on three rays were removed. Each ray had two female flowers. Rays were randomly assigned to one of the three following pollination treatments: (1) no further manipulation (emasculcation), (2) selfing, and (3) outcrossing. For selfed flowers, the receptive stigmas were pollinated with pollen from freshly dehisced anthers from the same plant. Outcrossed flowers were pollinated with pollen from at least three different plants located between three and six metres away. The remaining flowers on the same stem were caged with fine-meshed bags to prevent pollen contamination of the treated flowers; the plants remained caged throughout the whole experiment.

As the fruits matured, we bagged them in dialysis tubing, so they could finish maturing without dehiscing. Seed number was recorded, and we calculated the percentage of ovules that developed into seeds (female fertility). Because each female flower contains three ovules, and two female flowers per plant received the same treatment, female fertility was calculated as the number of seeds produced by this pair of flowers divided by six. Four plants were excluded from the analysis because ants were observed in the cages.

Female fertility following outcrossing and selfing was arcsine square root transformed and analysed with an ANOVA with treatments as a fixed effect and plants as a random effect. Even after transformation, the variances were not exactly equal between treatments, but ANOVA operates well even with considerable heterogeneity of variances provided that sample sizes are similar (Zar 1996). All statistical analyses were performed with the program JMP, version 3 (SAS 1989–1997).

Ant pollination of E. cyparissias
We tested whether ants are pollinators by excluding particular insect groups. At the end of March, we caged 135 plants in bud and assigned them to one of the four following treatments: 1) No visitors – the plants remained caged throughout the whole experiment. 2) Ants only – only crawling insects, which were primarily ants, could visit the flowers (we used the cages described above; however, instead of tying the florist bags around the plant stems, we cut them at the edge of the wire cylinder, leaving a 1-cm gap between the ground and the cage). 3) Winged only – only flying insects could visit the flowers (to prevent crawling insects, we drove in the ground a cylinder of green hard plastic (8 cm high, cross-section of 7 cm) around the about 15 cm tall plant and smeared the top centimetre with an insect trapping adhesive (Tangle-trap from The Tanglefoot Company, Grand Rapids, Michigan). 4) All visitors – all insects could visit the flowers.

The treatments were initiated during the last week of April. We checked the plants regularly to make sure the treatments were excluding and including the proper insects. Seeds were harvested on 23 June, 1998. For each plant, we recorded the number of seeds and the mean seed weight, and estimated the number of female flowers per plant by counting the number of rays.

For analysis we first classified the plants by whether they set seed or not, then we used a G-test of independence to determine whether the formation of seeds depended on the four treatments. To test whether pollinators were necessary for seed set, we performed a G-test between the “no visitors” and the “all visitors” plants. A second G-test was performed between the “no visitors” and the “ants only” treatments. For the treatments where insects were allowed to visit the flowers, we had continuous data and could thus use ANOVA for analysis. Female fertility for these three treatments was arcsine transformed and compared with one-way ANOVA.

Ant-fungus interaction
To test whether ants fertilise the rust fungi, we conducted a second insect exclusion experiment, this time with infected stems. Before the fungi started producing gametes, 125 infected plants were caged and randomly assigned to one of the three following treatments already described above: 1) No visitors, 2) Ants only, and 3) Winged only. There were two kinds of control plants for this experiment: 4) All visitors – infected plants were caged as they emerged and when the treatments started, their cages were removed. Thus the “all visitors” plants act as a control for the other treatments. 5) Continuously open – these infected stems, by contrast, were never caged and received natural visitation. Treatments were initiated during the last week of April. At the end of May, we collected all the infected plants and recorded the number of stems per plant and whether they were bearing aecia (the structures formed after successful fertilisation).

A G-test of independence was used to test whether the formation of aecia was independent of the treatment.
ments. We performed three a priori contrasts with G-tests: “winged only” vs “all visitors”, “continuously open” vs “all visitors”, and “no visitors” vs “ants only”.

Results

Breeding system of E. cyparissias

None of the 52 emasculated flowers set seed, indicating that E. cyparissias needs pollen for seed set (Fig. 1). Of the 20 plants that received the selfing treatment, 10 plants set seed, indicating that E. cyparissias is partially self-compatible. However, female fertility was significantly lower in the selfing treatment than in the outcrossing treatment (ANOVA: F1,18 = 15.56, P = 0.0009). On average, two selfed flowers set 1.45 ± 0.42 seeds vs 2.77 ± 0.41 for two outcrossed flowers.

Ant pollination of E. cyparissias

The exclusion of particular insect groups had a significant effect on seed set (Fig. 2; G = 39.72, P < 0.0001). Only 10% of the “no visitors” plants set seed (n = 20), compared to 86% of the “all visitors” plants (n = 21) and these plants set far fewer seeds (3.5 ± 1.5 vs 10.4 ± 2.1), indicating that the plants did not normally self-pollinate and pollination was essential for full seed set (G = 26.59, P < 0.0001). Ants were able to pollinate the flowers of E. cyparissias; the plants to which only ants had access set seed significantly more often than the ones without visitors (G-test of independence: G = 3.92, P = 0.048). The ANOVA showed that there were efficiency differences among the pollinator groups (F2,58 = 8.33, P = 0.0007). Contrasts showed that female fertility of the plants visited by all insects was higher than that of plants visited only by ants (F1,58 = 6.23, P = 0.015) and similar to that of plants visited only by winged insects (F1,58 = 2.6, P = 0.11). Out of the 23 plants to which only ants had access, eight plants set seed. The female fertility of these eight plants was not significantly different from that of the plants visited by all insects that set seed (Table 1; ANOVA: F1,24 = 0.13, P = 0.72), and their seeds tended to be larger (Table 1; ANOVA: F1,24 = 3.29, P = 0.08).

Ant-fungus interaction

Ants did not contribute to the fungal reproduction, but flying insects did fertilise the fungi (Fig. 3). Treatment had a significant effect on the number of plants that formed aecia (G = 68.44, P < 0.001). Aecia formation did not differ significantly between the “ants only” treatment and the “no visitors” treatment (G = 1.41, P < 0.24), but was more frequent among the plants of the “winged only” treatment than among those that received all visitors (G = 4.45, P = 0.0349). The plants to which insects had continuous access bore aecia more often than the plants that were caged between one and two months prior to unrestricted insect access (the “all visitors” plants), suggesting that aecia formation depends on the length of exposure to insects (G = 20.88, P < 0.0001).

Discussion

Breeding system of E. cyparissias

To understand the interactions between Euphorbia cyparissias and its flower visitors, some knowledge about its breeding system is necessary. Our emasculation treatment established that pollen is necessary for seed set. Further, E. cyparissias is partially self-compatible,
but does not normally self-pollinate: only 2 out of 20 of the plants from which insects were excluded set seed. These results strongly suggest that protogyny is an efficient mechanism to hinder self-pollination and indicate that *E. cyparissias* generally relies on insects for pollination. Although we did not test explicitly for pseudogamy, the fact that few of the self-fertilised flowers set any seed also suggests that they are not pseudogamous, since self-pollen can trigger full seed set in pseudogamous apomicts (Roy 1995).

Either self or outcross pollen can lead to seed set, but manually self-pollinated flowers set less seeds than manually outcrossed flowers did. In a primarily outcrossing species, reduced seed set with self-fertilisation is often an expression of inbreeding depression (Lee 1988). Only about half of the ovules of the flowers outcrossed by hand-produced seeds. Lethal mutations, resource limitation, or pollen limitation could have been responsible for the abortion of the other ovules. Considering that the plants examined in this study grew on a dry calcareous grassland, resource limitation is a plausible explanation. Abortion due to a lack of pollen seems unlikely, because we applied large amounts of pollen on the stigmas. However, the quality of the applied pollen was not tested. In addition, because *E. cyparissias* also reproduces vegetatively, some flowers that were thought to be outcrossed may have been pollinated with pollen from their own clone. Nevertheless, seed set was higher by outcrossed than by selfed plants.

Hand-outcrossed flowers had far higher female fertility than the ones exposed to all visitors (compare Figs 1 and 2), suggesting that reproduction was limited by pollinator activity (Bierzychudek 1981). However, in our study, the number of seeds that hand-outcrossed plants could potentially produce was limited to 12 because the other flowers were not pollinated. The “all visitors” plants had far more flowers to mature, and thus, if resources were limiting, could abort some, leading to lower female fertility as this measure was calculated over all flowers present on a plant. Seed production per plant, and not per flower, should be examined to show pollen limitation (Zimmerman and Pyke 1988). Therefore, our results cannot be interpreted as evidence for pollinator limitation, but neither can they exclude it.

**Ant pollination of *E. cyparissias***

We were principally interested in knowing whether ants, which are often observed on the flowers of *E. cyparissias*, pollinate these flowers or whether they are nectar thieves. The insect exclusion experiment showed that ants pollinate *E. cyparissias*. Plants that were visited only by ants set more seeds than plants from which all insects were excluded. We also observed ants contacting both anthers and stigmas while foraging.

Concerning the extent of the ants’ contribution to pollination, our results showed that the eight plants visited only by ants that set seed had a similar reproductive success as the plants visited by all types of insects. This may be explained by the behaviour of the ants. Worker ants can have a highly specialised foraging pattern and go back to the same foraging site as long as they are rewarded with food (Hölldobler and Wilson 1990). Fowler (1983) showed how workers of *Formica pallidefulva* specialise on the nectaries of another Euphorbia species, *E. esula*, and return to the same flowers over a two-week period. He also observed that some plants are visited daily whereas others are totally ignored. We made similar observations and hypothesise that the “ants only” plants that did not set seed were simply ignored by ants, whereas the ones that set seed were regularly visited. If this hypothesis is true, then it supports the idea that ants may compensate for their low quality by high visitation frequency.

Plants visited only by flying insects had similar female fertility as the plants visited by all insects, including ants. Ants pollinated *E. cyparissias*, but their exclusion did not reduce reproductive success. If a plant is resource limited and not pollen limited, then a small decrease in pollinator visits and therefore pollen supply
may not influence reproductive success. Our experiments clearly showed that ants are pollinators when they are the only visitors, but, from this result, we can only make suppositions about the ants’ contribution to pollination when they co-occur with other visitors. Usually more pollinator visits bring more pollen, but when the pollinator fauna is heterogeneous, the interactions between the pollinators may influence pollen deposition (Thomson and Thomson 1992). For instance, aggressive ants can prevent or shorten the foraging time of better pollinators (we personally observed many such interactions in 1997). In this case, their exclusion would be beneficial to plant reproduction. At our field site, *F. pratensis* workers were very bellicose, while *L. alienus* workers flee rapidly when disturbed (Seifert 1996). Ants can also simply reduce the nectar quantity available to other pollinators, forcing the flying pollinators to visit more flowers to get the same amount of nectar and indirectly affecting the outcrossing rate (Hakwins 1961, Heinrich and Raven 1972).

The fact that the exclusion of ants did not affect reproductive success suggests that pollination caused by ants was not important for *E. cyparissias* in 1998 at our field site. However, ants could still be important pollinators in years and locations where flying visitors are rare and pollination would not occur otherwise. We must stress that the present study was conducted in only one population and year. A similar experiment conducted in another population or year may have a different outcome, depending on the local insect fauna or flowering phenology. Interactions between two species vary among populations and years, and range from antagonism to commensalism or even mutualism (Thompson 1994). Additional studies are needed to clarify the ecological importance of this interaction.

**Ant-fungus interaction**

Finally, we were interested in the role of the ants in moving fungal spores between different mating types. Insects are usually necessary for sexual reproduction in *U. pisi*. In 1997, fungi were able to reproduce on only 5% of the infected stems from which all insects were excluded (Pfunder and Roy in press), and in 1998 on 19% (this study). Here, we showed that ants do not fertilise the fungus. Interestingly, ants were rarely found on infected stems in 1998 although they were almost half of all visitors in 1997. What caused this difference? First, the spring of 1998 was cooler than the previous one, and when the ants began foraging, the healthy plants were already flowering. Perhaps ants switch from pseudoflowers to flowers when these are present. Second, in the 1997 experiment, healthy and infected stems were arranged in artificial plots using florist tubes. In contrast with a natural situation, ants may not have been able to differentiate between flowering and infected stems in the tubes. It is also likely that in the 1997 experiment the location of one plot adjacent to an ant nest increased the overall number of ants observed.

The 1997 experiment also showed that ants only stayed a short time on infected stems (18 s in contrast to 130 s on flowers), indicating that in some way the nectar that they found on infected plants was less interesting than that in floral nectaries. Given that ants forage locally, they probably do little cross-fertilisation. Since the fungi are nearly obligate outcrossers, and usually only one mating type is found per infected plant, inter-plant movement is necessary for fertilisation. In this situation, ants foraging on the infected stems should probably be considered as nectar thieves. A noteworthy difference between the fungi and flowers is that, on infected stems, the nectar and the spores are mixed, but on the true flowers, the nectar and the pollen are produced on separated structures (nectaries or anthers). Perhaps the ants prefer their nectar uncontaminated with fungal spores.

Aecia formation was significantly higher on plants visited only by winged insects than on plants visited by all insects, including ants. These results could be explained by the ants’ aggressive behaviour described above, or perhaps the metapleural gland secretions adversely affected the fungus. Another possibility is that flying insects were attracted by the plastic ring or the tanglefoot glue used to exclude the ants or by the insects accidentally trapped in this glue.

In conclusion, we showed that ants did not help the rust fungus pathogens of *E. cyparissias* reproduce. However, experimental evidence showed that ants pollinated *E. cyparissias* and that plants on which only ants foraged had the same reproductive success as plants visited by all insects. In our field site in 1998, ants were not the main pollinators, but the pollination caused by the ants may be very important in years, or locations when winged pollinators are rare.

**Acknowledgements** – We thank B. Merz for helping prepare the ants, D. Chérix for identifying them, B. Baur for providing the field site, T. Steinger for statistical advice, A. Widmer for comments on the manuscript, and M. Ramsey for advice. This research was supported by the Swiss National Science Foundation (NF 2-77-311-96).

**References**


