

## Research article

# Ant-seed interactions: combined effects of ant and plant species on seed removal patterns

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**Abstract.** Seed dispersal by ants (i.e. myrmecochory) is usually considered as a mutualism: ants feed on nutritive bodies, called elaiosomes, before rejecting and dispersing seeds in their nest surroundings. While mechanisms of plant dispersal in the field are well documented, the behaviour of the ant partner was rarely investigated in details. Here, we compared in laboratory conditions the foraging behaviour of two ant species, the omnivorous *Lasius niger* and the insectivorous *Myrmica rubra* to which seeds of two European myrmecochorous plants (*Chelidonium majus* and *Viola odorata*) were given. Ant colonies were simultaneously presented three types of items: entire seeds with elaiosome (SE), seeds without elaiosome (S) and detached elaiosomes (E). The presence of elaiosomes on seeds did not attract workers from a distance since ants first contact equally each type of items. Although ants are mass-recruiting species, we never observed any recruitment nor trail-laying behaviour towards seeds. For ants having contacted seed items, their antennation, manipulation and seed retrieval behaviour strongly varied depending on the species of each partner. Antennation behaviour, followed by a loss of contact, was the most frequent ant-seed interaction and can be considered as a “hesitation” clue. For both plant species, insectivorous *Myrmica* ants removed items in larger number and at higher speed than *Lasius*. This fits with the hypothesis of a convergence between odours of elaiosomes and insect preys. For both ant species, the small *Chelidonium* seeds were retrieved in higher proportion than *Viola* ones, confirming the hypothesis that ants prefer the higher elaiosome/diaspore-ratio. Thus, in these crossed experiments, the ant-plant pair *Myrmica/Chelidonium* was the most effective as ants removed quickly almost all items after a few antennations. The presence of an elaiosome body increased the seed removal by ants excepting for *Myrmica* which retrieved

all *Chelidonium* seeds, even those deprived of their elaiosome. After 24 h, all the retrieved seeds were rejected out of the nest to the refuse piles. In at least half of these rejected items, the elaiosome was discarded by ants. Species-specific patterns and behavioural differences in the dynamics of myrmecochory are discussed at the light of ant ecology.

**Keywords:** Myrmecochory, elaiosome, *Chelidonium majus*, *Viola odorata*, *Myrmica rubra*, *Lasius niger*.

## Introduction

Myrmecochory, or seed dispersal by ants, is a worldwide mechanism involving more than 3,000 plant species (Beattie and Hughes, 2002) and hundreds of ant species across many ecosystems such as dry sclerophyll woodlands of Australia (Berg, 1975), sclerophyll shrubs in south Africa (Bond and Slingsby, 1983), tropical regions (Horvitz and Beattie, 1980) or temperate deciduous forests of Europe (Sernander, 1906) and North America (Culver and Beattie, 1978). Generally, ants remove intact diaspores, bring them back to the nest, eat the nutrient-body appendage called elaiosome and reject seeds within or outside the nest.

Depending on each ecosystem, many benefits are listed for plants and can be multiple: directed dispersal towards nutrient-enriched microsites (Hanzawa et al., 1988; Passos and Oliveira, 2002), reduction of parent-offspring or sibling competition (Higashi et al., 1989; Boyd, 2001), predator avoidance (Heithaus, 1981; Ohkawara et al., 1997) or fire avoidance as seeds are quickly retrieved within the ant nest (Berg, 1981; Bond and Slingsby, 1983). Myrmecochory can also facilitate germi-

nation due to elaiosome removal or seed testa scarification by ants (Culver and Beattie, 1980; Gomez and Espadaler, 1997). Some negative effects of myrmecochory on plant fitness were also reported such as increased competition due to a clumping of seeds in the refuse piles around ant nests (Culver and Beattie, 1980; Davidson and Morton, 1981) as well as a decreased germination in a few plant species due to elaiosome removal or due to seed scarification (Imbert, 2006).

As it is often the case in research dealing with animal-plant mutualisms, there is an imbalance in our current knowledge about the evolutionary ecology of each partner (Cushman and Beattie, 1991; Bronstein, 1994). Indeed, benefits due to myrmecochory are far less known for ants. While more than two hundred papers tell us about plant benefits, only seven studies have shown so far that ants can benefit from elaiosomes by using them to feed their larvae (Handel, 1976; Morales and Heithaus, 1998; Bono and Heithaus, 2002; Fischer et al., 2005; Gammans et al., 2005; Marussich, 2006; Fokuhl et al., 2007) what may increase gynes production in some ant species (Morales and Heithaus, 1998) or increase larval weight (Gammans et al., 2005).

Myrmecochory involves a variety of taxa, often a guild of ants and a guild of plants, and is thus considered as a diffuse mutualism (Beattie and Culver, 1981; Giladi, 2006). This does not mean that all ant-plant pairs are equivalent since as argued by Giladi (2006), “*the overall fitness gain for plants resulting from myrmecochory significantly depends on the identity and behaviour of the seed disperser*” (p. 482). In this respect, species-specific quantitative differences, even small ones, can have major consequences on the overall seed dispersal when multiplied by the number of ant-seed interactions occurring during a fructification season.

The aim of this paper is to dig into some aspects of ant behaviour likely to influence seed dispersal and thus to be a possible source of variability of myrmecochorous ant-plant interactions. In controlled conditions we shall see how ant traits such as diet and foraging behaviour may influence seed retrieval and elaiosome removal, and conversely how seed type may alter ants' response.

Therefore, we compared the foraging behaviour of the aphid-tending ant *Lasius niger* to that of the more insectivorous species *Myrmica rubra*. These ant species were given two different types of seeds quite common in their natural biotopes: *Viola odorata* seeds and smaller *Chelidonium majus* seeds. We assessed how seed species as well as the presence of an elaiosome influence the ants' behaviour by following up all steps from seed retrieval to seed dispersal out of the nest.

## Materials and methods

### *Ant species: collection and rearing of colonies*

Mature colonies of the black garden ant *Lasius niger* (L.) are monogynous and contain up to 13,000 workers (Stradling, 1970). These workers are 3 to 4 mm in length and mainly feed on honeydew (Pontin, 1958). This aphid-tending ant species can however be considered as omnivorous because it occasionally eat aphids (Pontin, 1958; Offenberg, 2001), dead or small alive insects (Pontin, 1961) as well as seed elaiosomes (Sernander, 1906; Oberrath and Böhning-Gaese, 2002).

The red ant *Myrmica rubra* L. is 4 to 6 mm in length, forming polygynous colonies which can reach 6,000 workers and more than 50 queens (Elmes, 1973). It is a common predator of small and soft-bodied animals, also scavenging upon large dead prey items (Le Roux et al., 2002). Whereas *Myrmica rubra* is considered as a carnivorous species, it can also consume sugared solutions (Cammaerts, 1977) or elaiosomes (Bülow-Olsen, 1984; Gorb and Gorb, 2000).

Both ant species are not granivorous and are becoming reference species for the study of myrmecochory in temperate regions (see e.g. Oberrath and Böhning-Gaese, 2002; Prinzing et al., 2007). They are found in open and forested habitats. We collected eight colonies of *Lasius niger* and eight colonies of *Myrmica rubra* from earth banks in Brussels (Belgium) and La Gorgue (France). In the laboratory, colonies were reared in plaster nests (Janet type, 20 × 20 × 0.5 cm) and placed in arenas (50 × 38 × 10 cm) with Fluon-coated borders to prevent ants' escape. Nests were regularly moistened and kept at 21 ± 2 °C, 35 ± 5% relative humidity and a constant photoperiod of 12 h per day. We covered the nest chamber with a red glass plate through which the ants could be observed. All colonies contained 1000–1500 workers. Nests with brood contained similar numbers of larvae, which covered 10–15% of the nest area. *Myrmica rubra* colonies contained 5–10 gynes. *Lasius niger* colonies were queenless. Foraging activity of queenless and queenright colonies may differ or not depending on the ant species (see e.g. Vienne et al., 1998; Della Lucia et al., 2003; Brightwell and Silverman, 2007). Not too much weight should however be given to the presence/absence of a queen in the present research. Indeed, we simply investigate the basic responses of ant individuals facing food items without addressing any development or social regulation issues. Moreover, in the case of *Lasius niger*, using queenless colonies allowed us to make reliable comparisons with previous studies on foraging towards different food types (sucrose solution or prey: see e.g. Portha et al., 2004; Devigne and Detrain, 2006). We supplied ants *ad libitum* with water and sucrose solution (1M), and twice a week with cockroaches (*Periplaneta americana*) and an artificial diet with proteins, sugars and vitamins enriched (Bathkar and Withcomb, 1970). Colonies were collected in September and experiments were carried out in the laboratory between October and January. As for previous studies with *Lasius niger* (e.g. Portha et al., 2004) or *Myrmica rubra* (e.g. Cammaerts, 1978, pers. comm.) that were carried out in winter, our tested colonies kept a high foraging activity towards sugary food sources (21 to 25 workers of *Lasius niger* and 17 to 20 workers of *Myrmica rubra* present after 15 min at a source of 1M sucrose solution). Hence, the absence of colony overwintering does not seem to significantly lower down the foraging response of ant workers.

### *Plant species and seed storage*

*Viola odorata* L. is a perennial plant of temperate forests, edge bushes, open or ruderal habitats (Lambinon et al., 1992), widespread in Belgium, capable of vegetative reproduction. Plants flower from February to September with a peak in early spring and produce seeds from May to October (Oberrath and Böhning-Gaese, 2002; pers. obs.). Inflorescence stems bend down to the litter layer and capsules open barely without exploding. The peduncle carrying the diaspores is weak and prostrated towards the litter level, thus considered as an adaptation for ant removal (Beattie and Culver, 1981). Seeds are then clustered within the capsule and available for ants. It is considered as an obligate

myrmecochorous plant. Seeds are yellow-brown, measured  $3.34 \pm 0.21$  mm length and  $2.24 \pm 0.14$  mm width ( $n=10$ ) with white-coloured and soft cone-like basal elaiosomes, smaller than the seed as they measured  $2.01 \pm 0.25$  mm length and  $1.65 \pm 0.16$  mm width ( $n=10$ ). Seed mean weight (4.231 mg;  $n=10$ ) and elaiosome mean weight (0.772 mg,  $n=10$ ) gives a elaiosome/seed ratio of 0.18.

*Chelidonium majus* L. is a perennial plant present in ruderal habitats, edges or small ruderal forests (Lambinon et al., 1992). It is common in Belgium, flowers from April to October and produces seeds from May until November in a more important and continuous way (without peak) than *Viola odorata*. It is considered as a diplochorous plant, of which seed dispersal involves two steps: after fruit dehiscence, seeds are scattered around the parent plant on the ground (autochory) and are then removed by ants from the soil surface (myrmecochory). Seeds are dark brown and small:  $1.95 \pm 0.06$  mm length and  $1.43 \pm 0.11$  mm width ( $n=10$ ). Elaiosomes are white-coloured, soft, larvae-like and measured  $1.66 \pm 0.06$  mm length and  $0.81 \pm 0.12$  mm width ( $n=10$ ). Seed mean weight (0.985 mg;  $n=10$ ) and elaiosome mean weight (0.309 mg;  $n=10$ ) gives a elaiosome/seed ratio of 0.31.

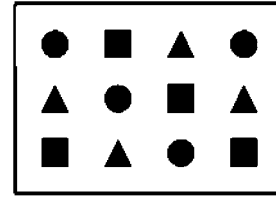
Mature seeds were collected and stored at  $-18^\circ\text{C}$  before being used in behavioural experiments. Nevertheless, we checked in preliminary experiments whether this preservation mode might have altered seed attractivity and potential for removal by ants. Therefore, for each plant (*Chelidonium* and *Viola*), three colonies of *Lasius* and three colonies of *Myrmica* were presented with simultaneously six fresh seeds and six seed stored at  $-18^\circ\text{C}$  during 6 months. We compared the number of contacts, the removal rates for 45 minutes as well as the mean time before removal of fresh seeds to those of frozen items. On average, the number of contacts were not significantly different between frozen and fresh seeds (*Lasius/Viola*:  $P=0.7436$ ; *Lasius/Chelidonium*:  $P=0.4212$ ; *Myrmica/Viola*:  $P=0.0833$ ; *Myrmica/Chelidonium*:  $P=0.7819$ ; NS for  $\alpha=0.05$ ;  $n=18$ , Wilcoxon paired test). After 45 minutes, all *Chelidonium* seeds were removed by ants and the mean time before removal were not significantly different for fresh and cold preserved seeds (*Lasius/Chelidonium*:  $P=0.0887$ ; *Myrmica/Chelidonium*:  $P=0.4683$ ; NS for  $\alpha=0.05$ ;  $n=18$ , Wilcoxon paired test). Concerning *V. odorata* seeds, nearly all of them (94.4%,  $n=72$ ) were removed by both ant species except two seeds (one fresh and one frozen). Furthermore, on average, the time before removal did not significantly differ as both fresh and frozen seeds were as likely to be removed (*Lasius/Viola*:  $P=0.6441$ ; *Myrmica/Viola*:  $P=0.1089$ ; NS for  $\alpha=0.05$ ;  $n=18$ , Wilcoxon paired test). All these results strongly suggest that the seed attractivity to the ants was not altered by our method of seed storage.

### Experimental procedure

Most of the previous studies carried out on at least one of the four tested species were done in the field and looked at myrmecochory from a global point of view (such as global rate of seed retrieval, cafeteria experiments; see e.g. Beattie and Lyons, 1975; Gorb and Gorb, 2000; Peters et al., 2003). Here, for the first time, we detail ant-seed interactions in a standardized way by comparing the influence of seed/ant traits on myrmecochory in a  $2 \times 2$  species combination.

In order to highlight species-specific differences in the myrmecochorous process, we investigated the possible four pairs of ant-plant interactions: *Lasius/Chelidonium* (LC), *Lasius/Viola* (LV), *Myrmica/Chelidonium* (MC) and *Myrmica/Viola* (MV). We carried out 24 experiments for each ant-plant pair: eight colonies with three replicates per tested colony. A colony was tested weekly and starved four days before each experiment. One day before, the colony was connected by a bridge to a foraging arena ( $50 \times 38 \times 10$  cm) with Fluon-coated borders. The seed source was a square plastic film ( $3 \times 4$  cm) placed in the centre of the arena and used once. We presented to ants three types of items: entire seeds (i.e. with elaiosome) (SE), seeds without elaiosome (S) and detached elaiosomes (E). Elaiosomes were cut off with a scalpel under a binocular microscope. *V. odorata* elaiosomes were discarded easily contrarily to *C. majus* ones, for which we took care to keep their turgescence unburst. Moreover, we checked that no elaiosome amounts (white tissue) were left attached at the insertion point and that only *C. majus* seed embryo (grey, foamy tissue) appeared at the tiny

cutting wound (wound size of around  $0.4 \text{ mm} \times 0.2 \text{ mm}$ ). Items were placed on the source following the pattern shown in Figure 1. Each experiment started with the first contact between an ant and a seed. The ants' behaviours at the source were video-recorded for 45 minutes (magnification 10 xs).



**Figure 1.** Spatial distribution of items at the food source. Entire seed (SE) (circle), seeds without elaiosome (S) (square) and elaiosome detached (E) (triangle).

### Recruitment

We counted the number of ants at the seed source every 15 minutes after the start of the experiment. We noted possible trail-marking behaviour by directly observing ants going from and to the food source. Mean numbers of ants exploring the foraging area before seed introduction were compared between ant species with a Mann-Whitney Test ( $n=24$ , unpaired data). In order to detect any increase or decrease over time in the number of foraging ants, we compared measures done at 0, 15, 30, and 45 min by using the Page test for ordered alternatives ( $k=4$ ,  $n=24$ ).

### Seed attractivity

The attractivity of an item from a distance was given by its probability to be contacted by the first ant reaching the seed source. Besides, as long as no item was removed, we observed whether each food item had equal chances to be contacted by any approaching ant. This was done by comparing the relative frequencies of contacts directed to each item type. Statistical tests used were Chi-square Goodness-of-fit, comparing our three probabilities with random (0.33).

### Behavioural parameters

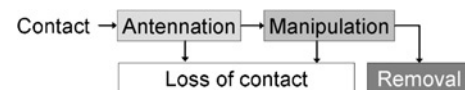
For each contacted seed item, we quantified the following three types of ants' behaviours:

1. *Antennation*: the ant merely touched the item with antennae, and then continued to explore the foraging area.
2. *Manipulation*: the ant contacted the item with antennae, held it between mandibles, bit it and then released it, without any removal.
3. *Removal*: after having antennated and manipulated an item, the ant took it away out of the camera field (1 cm around the seed source).

These behaviours can be summarized in the following sequence (Fig. 2).

In order to compare behavioural means between the four ant-plant pairs, we took into account only scores of entire seeds (SE) and used a Kruskal-Wallis Test followed by a Dunn Post-hoc test.

For each ant-plant pair, we compared the mean number ( $n=24$ ) of behaviours (antennations, manipulations, removals) displayed by ants



**Figure 2.** Sequence of behaviours.

after contacting the three types of items (SE, S and E). This was done by using Friedman Test followed by a Post-hoc Test.

The removal dynamics were compared for each type of seed item. Slopes of the regression lines (calculated on Log transformed data) were compared with a Test of Comparison of several Slopes followed by a Post-hoc Test (q).

### Seed fate

Twenty-four hours later the experiment, we noted the location of seeds in the whole setup as well as the presence (or absence) of elaiosomes on rejected seeds (SE or S). No elaiosome-item (E) was found out neither in the nest nor in the refuse piles, either because we overlooked them due to their minute size or because they were eaten.

## Results

### Recruitment

We never observed any recruitment towards seed sources. At the beginning of the experiment, the numbers of exploring ants were low and similar whatever the seed species for *Myrmica rubra* (*Viola*:  $2.2 \pm 1.73$ ; *Chelidonium*:  $1.58 \pm 0.91$ ; N.S.,  $p=0.2855$ , Mann-Whitney test,  $n=24$ ) and for *Lasius niger* (*Viola*:  $1.33 \pm 1.07$ ; *Chelidonium*:  $0.87 \pm 1.33$ ; N.S.,  $p=0.0677$ , Mann-Whitney test,  $n=24$ ). Every 15 min, we counted the number of ants at the source and did not detect any increase in ant population even after 45 minutes (Table 1). On the contrary, there was a decrease in the number of foraging ants, this trend being significant for all pairs but the L/C pair (MV  $p=0.0455$ ; MC  $p=0.0202$ ; LV  $p=0.0307$ ; LC  $p=0.2090$ , N.S.; Page test for ordered alternatives,  $k=4$ ,  $n=24$ ). For both *Lasius* and *Myrmica*, no foraging ant was seen laying a chemical trail neither along its home-ward nor its outward journey. Even successful individuals that were transporting seeds were never seen laying a trail on their way back to the nest.

**Table 1.** Mean number of ants ( $\pm$ S.D.,  $n=24$ ) on the source square at the start of the experiment, after 15 min, 30 min and at the end (45 min).

|                            | Start         | 15 min        | 30 min        | End           |
|----------------------------|---------------|---------------|---------------|---------------|
| <i>Lasius/Viola</i>        | $1.3 \pm 1.1$ | $1.3 \pm 1.8$ | $1.0 \pm 1.3$ | $0.6 \pm 0.9$ |
| <i>Lasius/Chelidonium</i>  | $0.9 \pm 1.3$ | $0.6 \pm 1.2$ | $0.5 \pm 0.8$ | $0.6 \pm 0.7$ |
| <i>Myrmica/Viola</i>       | $2.2 \pm 1.7$ | $1.8 \pm 1.9$ | $1.6 \pm 1.7$ | $1.1 \pm 1.0$ |
| <i>Myrmica/Chelidonium</i> | $1.6 \pm 0.9$ | $1.1 \pm 1.2$ | $0.8 \pm 0.9$ | $1.0 \pm 1.0$ |

### Seed attractivity

For all pairs, each type of item (SE, S and E) was as likely to be contacted by the first ant approaching the food source. Indeed, approaching ants showed a probability to first contact one of the three item types that did not significantly differ from a random choice (0.33) (Table 2) (N.S.; Chi-square goodness-of-fit test;  $\alpha=0.05$ ,  $n=24$ ).

Likewise, as long as no food item was removed, each item remained as likely to attract foraging ants from a distance with probabilities to be contacted not different from random (N.S.; Chi-square goodness-of-fit test;  $\alpha=0.05$ ). However, while food items were not selected by ants from a distance, their first removal strongly depended on whether an elaiosome was present or not (Table 2). Seeds without elaiosomes (S) were less frequently first removed, this trend being significant for all pairs but the M/C pair (LV,  $p<0.01$ ; LC,  $p<0.01$ ; MV,  $p<0.05$ ; MC, N.S.; Chi-square goodness-of-fit test;  $\alpha=0.05$ ). This demonstrates that the perception of elaiosome chemicals plays a key role in the triggering of seed removal. Furthermore, we noticed that for *Viola*, two thirds of items first removed were elaiosomes alone (E) (0.64 for *Lasius*; 0.58 for *Myrmica*). This suggests that, in addition to the elaiosome chemicals, the small size of one item is another factor that may enhance removal rates by the ants (comparison of SE versus E results). Hence, the choice of removing seeds was done only after the ants had contacted the item and was driven by the perception of elaiosome chemicals and to a lesser extent by the food item size.

### Ant behaviour at the seed source

#### The species effect

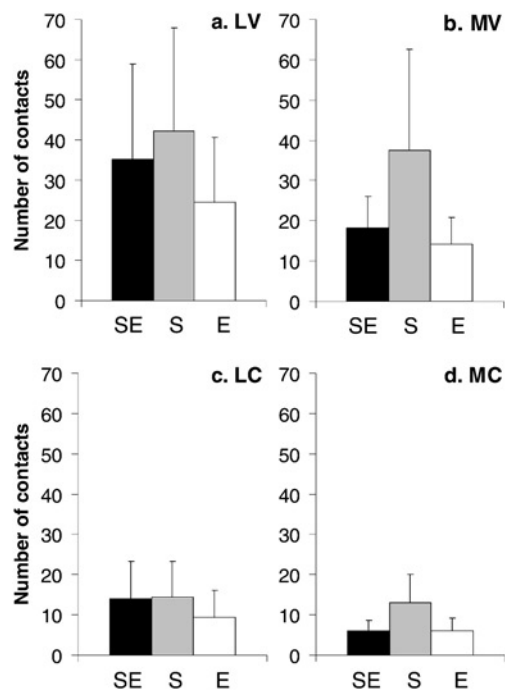
For the entire seeds (SE), the number of contacts, antennations and manipulations during the whole experiment differed greatly among pairs (Figs 3 and 4) (all  $p$  values  $<0.0001$ , Kruskal-Wallis Test,  $n=24$ ).

*Viola* seeds were three times more contacted than *Chelidonium* ones by both ant species (Figure 3) (MV vs. MC,  $p<0.001$ ; LV vs. LC,  $p<0.01$ ; Dunn Post-Hoc Test). As regards ants, *Lasius* usually performed more contacts than *Myrmica*, although this trend was only significant for *Chelidonium* (LC vs. MC,  $p<0.01$ ; LV vs. MV, N.S.; Dunn Post-Hoc Test).

Any ant contacting a seed item always started to antennate it. In most cases, ants just antennated the food item and then left it to further explore the foraging area. Indeed, "antennation" behaviour (followed by a loss of contact) accounted for 49% to 63% of all the observed contacts for the three following ant/seed pairs (LV, LC and MV). Only *Myrmica* foragers had lower antennation scores (11% to 14%) when they encountered *Chelidonium* seeds (Fig. 5). As previously reported for contacts, *Viola* seeds were always more antennated than *Chelidonium* ones (Fig. 4) (MV vs. MC,  $p<0.001$ ; LV vs. LC,  $p<0.01$ ; Dunn Post-Hoc Test). Likewise, we found out the same trend of *Lasius* workers to antennate seeds more frequently than *Myrmica*, especially when faced with *Chelidonium* seeds (LC vs. MC,  $p<0.001$ ; LV vs. MV, N.S.; Dunn Post-Hoc Test). One should notice that a low number of contacts or antennations does not reflect a lack of interest for a seed item but, on the contrary, results

**Table 2.** Proportions of each food item (SE: seed with elaiosome; S: seed without elaiosome; E: elaiosome alone) to be first contacted or first removed by ants. The proportion of contacts with each food item was also calculated before any seed removal as an indicator of the relative attractiveness of these items. These proportions were compared to the expected values ( $0.33 * n$ ) when items are assumed to be equally attractive (Chi-square test goodness of fit,  $\alpha = 0.05$ ).  $\chi^2$  theoretical = 5.991)

| Pair                       |                                   | SE   | S    | E    | n   | $\chi^2$ | p    |
|----------------------------|-----------------------------------|------|------|------|-----|----------|------|
| <i>Lasius/Viola</i>        | First contacted                   | 0.33 | 0.17 | 0.5  | 24  | 4        | N.S. |
|                            | Contacted before any seed removal | 0.32 | 0.35 | 0.33 | 440 | 0.88     | N.S. |
|                            | First removed                     | 0.27 | 0.09 | 0.64 | 24  | 10.2     | **   |
| <i>Lasius/Chelidonium</i>  | First contacted                   | 0.29 | 0.42 | 0.29 | 24  | 0.75     | N.S. |
|                            | Contacted before any seed removal | 0.42 | 0.29 | 0.29 | 150 | 5.08     | N.S. |
|                            | First removed                     | 0.5  | 0.04 | 0.46 | 24  | 9.25     | **   |
| <i>Myrmica/Viola</i>       | First contacted                   | 0.29 | 0.29 | 0.42 | 24  | 0.75     | N.S. |
|                            | Contacted before any seed removal | 0.33 | 0.29 | 0.38 | 157 | 1.62     | N.S. |
|                            | First removed                     | 0.33 | 0.08 | 0.58 | 24  | 9        | *    |
| <i>Myrmica/Chelidonium</i> | First contacted                   | 0.25 | 0.46 | 0.29 | 24  | 1.75     | N.S. |
|                            | Contacted before any seed removal | 0.29 | 0.45 | 0.26 | 42  | 2.71     | N.S. |
|                            | First removed                     | 0.42 | 0.21 | 0.38 | 24  | 1.75     | N.S. |



**Figure 3.** Mean number of contacts ( $\pm$  S.D.,  $n = 24$  experiments per pair) for entire seeds (SE, black), seeds without elaiosome (S, grey) and elaiosomes (E, white). a. *Lasius niger/Viola odorata*. b. *Myrmica rubra/Viola odorata*. c. *Lasius niger/Chelidonium majus*. d. *Myrmica rubra/Chelidonium majus*.

from their shorter lasting availability due to their quick removal by ants (see below).

Some ants that discovered a seed item went further than a simple antennation as they manipulated the seed by biting it, rolling it up or seizing it up in their mandibles for a while. Manipulation behaviours accounted in all pairs for a similar proportion (between 23.3% and 32.3% of all contacts) (Fig. 5). But the mean number of manipulations was different for plant species: *Viola* seeds were three times more manipulated than *Chelido-*

*nium* ones (Fig. 4) (MV vs. MC,  $p < 0.001$ ; LV vs. LC,  $p < 0.001$ ; Dunn Post-Hoc Test). However, there was no significant difference between ant species (LC vs. MC, N.S.; LV vs. MV, N.S.; Dunn Post-Hoc Test).

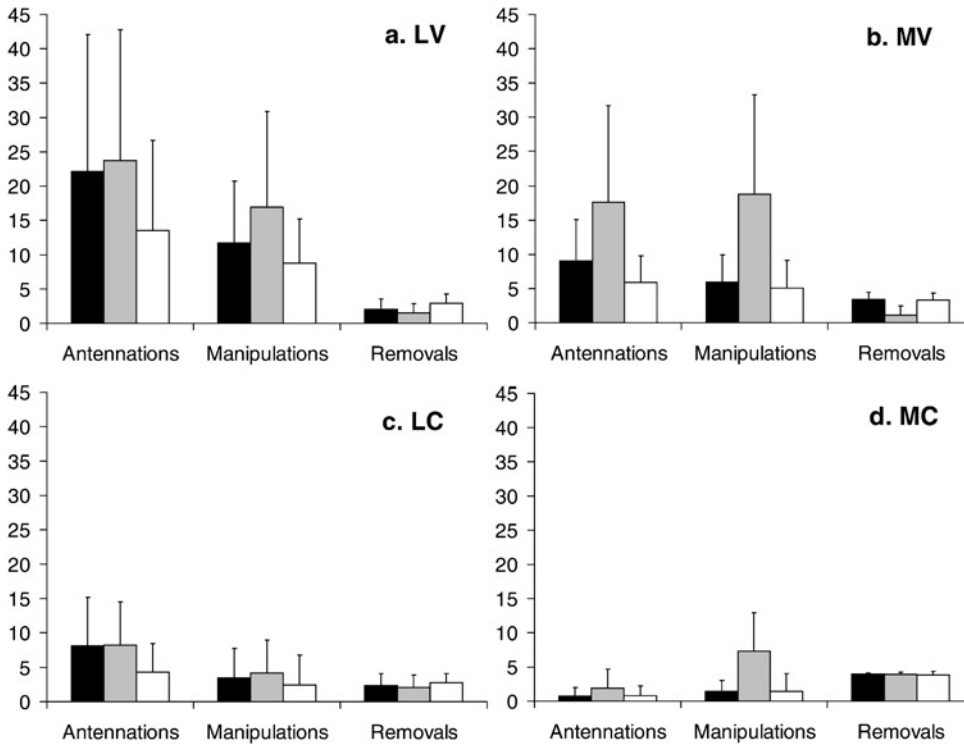
This shows that seed inspection and/or difficulty to handle a seed is dependent on plant species, but not on the manipulating ant.

#### The elaiosome effect

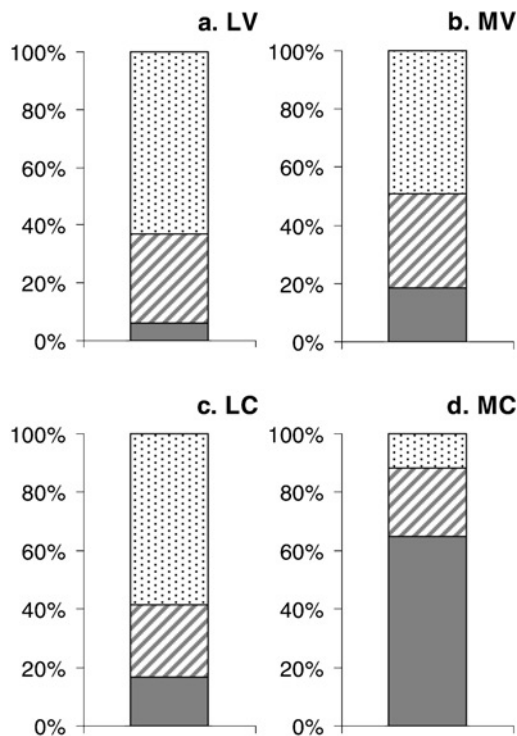
The tearing out of the elaiosome may deeply influence the behaviour of ants contacting a seed item. Indeed, as regards *Myrmica* ants (Figs 4b,d), items without elaiosomes were always more frequently antennated than items with elaiosomes (*Viola*:  $p = 0.0005$ , Post-hoc test: SE vs. S,  $P < 0.05$ ; *Chelidonium*:  $p < 0.001$ , Post-hoc test: SE vs. S,  $P < 0.05$ ; Friedman test,  $n = 24$ ). The discarding of the elaiosome had the same effect on manipulations by *Myrmica* ants (Figs 4b,d) (*Viola*:  $p = 0.0002$ , Post-hoc test: SE vs. S,  $p < 0.01$ ; *Chelidonium*:  $p < 0.0001$ , Post-hoc test: SE vs. S,  $p < 0.001$ ; Friedman test,  $n = 24$ ).

*Lasius* antennations and manipulations displayed almost the same trends. The only difference was that they antennated more frequently large items (SE and S) than smaller ones such as elaiosomes (E) (Figs 4a,c) (*Viola*:  $p = 0.0038$ , Post-hoc test: SE vs. E,  $P < 0.01$ ; S vs. E,  $P < 0.05$ ; *Chelidonium*:  $p = 0.0009$ , Post-hoc test: SE vs. E,  $P < 0.05$ ; S vs. E,  $P < 0.01$ ; Friedman test,  $n = 24$ ). Furthermore, they manipulated these large items more frequently, especially *Viola* ones (Figs 4a,c) (*Viola*:  $p = 0.0020$ , Post-hoc test: S vs. E,  $p < 0.05$ ; *Chelidonium*:  $p = 0.2686$ , N.S.; Friedman test,  $n = 24$ ).

The high number of pre-removal behaviours (antennations and manipulations) showed that ants were interested in items but could not go further in the removal process when they did not find an elaiosome attached to the seed. Three non-exclusive explanations can be



**Figure 4.** Mean number of behaviours ( $\pm$  S.D.,  $n=24$  experiments per pair) for entire seeds (SE, black), seeds without elaiosome (S, grey) and elaiosomes (E, white). a. *Lasius/Viola*. b. *Myrmica/Viola*. c. *Lasius/Chelidonium*. d. *Myrmica/Chelidonium*.



**Figure 5.** Proportions of antennations (dotted), manipulations (hatched), and removals (full) displayed by ants contacting entire seeds (SE).

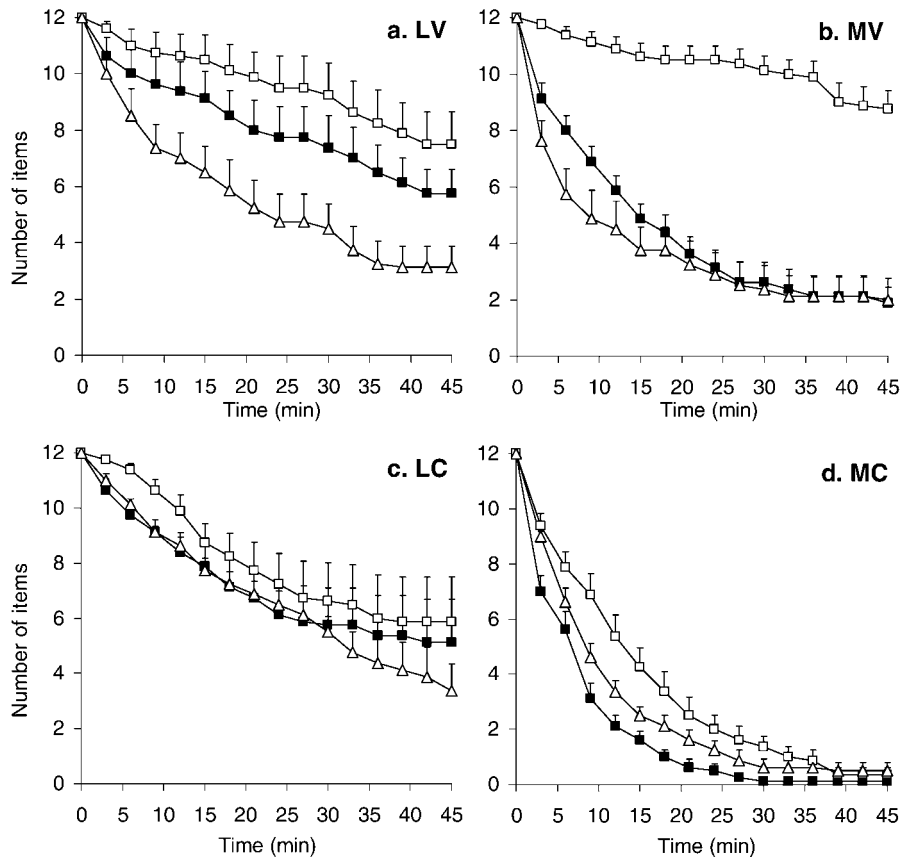
evoked: the lack of a grasping point facilitating the items' transport, the large size of the food items (SE and S of *Viola*) or possibly some minute remains of elaiosome

chemicals that elicit a prolonged inspection of the seeds by the ants.

#### Seed removal

##### The species effect

We also found differences among pairs in seed removal patterns. Proportions of seeds (SE) removed were higher for *Myrmica* ants and higher for *Chelidonium* seeds (Fig. 5). When looking at the mean number of removals (Fig. 4), global differences still occurred ( $p < 0.0001$ , Kruskal-Wallis Test,  $n=24$ ), but the plant-effect was not significant (MV vs. MC, N.S.; LV vs. LC, N.S.; Dunn Post-Hoc Test). Only the ant-effect was confirmed, showing that seed removal depend strongly on the ant species: entire seeds (SE) were more removed by *Myrmica* than *Lasius* (LC vs. MC,  $p < 0.01$ ; LV vs. MV,  $p < 0.05$ ; Dunn Post-Hoc Test). Indeed, *Myrmica* ants removed more than 90 % of items in 54 % of experiments, with a mean of  $9.7 \pm 2.9$  items removed per experiment ( $n=48$ , both pairs pooled), whereas *Lasius* removed more than 90 % of items in only 27 % of experiments, with a mean of  $6.9 \pm 4.3$  items removed per experiment ( $n=48$ ). This ant-effect is also clear in removal dynamics (Fig. 6). Each ant species had a different removal-curve pattern, in which *Myrmica* removed more seeds (SE) after 45 min. All these curves had an exponential shape, which means that the probabilities of seed removal per minute were constant values given by the slopes of the



**Figure 6.** Removal dynamics of the three items: seed with elaiosome (SE, full square), seed without elaiosome (S, open square) and elaiosome alone (E, triangle), during the experiment (45 min). The three replications were pooled for each colony (i.e. 12 items at the beginning of the experiment). Each point corresponds to a mean ( $\pm$  S.E.) of the number of colonies ( $n=8$ ). a. *Lasius/Viola*. b. *Myrmica/Viola*. c. *Lasius/Chelidonium*. d. *Myrmica/Chelidonium*.

regression lines (calculated on Log transformed data). Then, *Myrmica* tended to remove *Chelidonium* seeds (SE) five times faster than *Lasius* did (*Myrmica*,  $p=0.109$ ; *Lasius*,  $p=0.023$ ), and *Viola* seeds three times faster (*Myrmica*,  $p=0.049$ ; *Lasius*,  $p=0.017$ ).

#### The elaiosome effect

Besides the species effect, the presence of elaiosomes also influenced seed removal patterns. Firstly, *Lasius* ants removed more items with elaiosomes (SE and E) than seeds without elaiosomes (S) (Figs 4a,c) (*Viola*:  $p<0.0001$ , Post-hoc test: S vs. E,  $p<0.0001$ ; *Chelidonium*:  $p=0.0092$ , Post-hoc test: S vs. E,  $p<0.05$ ; Friedman test,  $n=24$ ). Comparing seed-removal dynamics, *Lasius* ants removed 25% of *Viola* seeds with elaiosomes (SE) after only 15 min, whereas it took around 33 min to remove the same percentage of seeds when they were deprived of their elaiosome (S) (Fig. 6a). This was confirmed by slopes of the regression lines of seed-removal curves that differed among items (Test from comparison of several slopes:  $F_{0.05,2,46}=405.9$ ,  $P<0.0001$ ; Test post-hoc:  $q_{GE-G}=10.79$ ,  $p<0.001$ ;  $q_{G-E}=39.01$ ,  $p<0.001$ ;  $q_{GE-E}=28.22$ ,  $p<0.001$ ). Indeed, seeds with elaiosomes (SE) had a twice higher probability ( $p=0.017$ ) to be removed per minute than seeds without

elaiosomes ( $p=0.010$ ). We also noticed that the size of the item could play a role as small elaiosome items (E) were removed faster than large items (SE and S). For *Chelidonium* (Fig. 6c), we found out a similar enhancing effect of the elaiosome on the seed removal as slopes of seed-removal curves were quite different (Test from comparison of several slopes:  $F_{0.05,2,46}=45.96$ ,  $P<0.0001$ ; Test post-hoc:  $q_{GE-G}=7.85$ ,  $p<0.001$ ;  $q_{G-E}=13.50$ ,  $p<0.0001$ ;  $q_{GE-E}=5.64$ ,  $p<0.001$ ). This effect was however smaller than for *Viola*.

Concerning *Myrmica* ants, they showed the same choice pattern on *Viola* items than *Lasius*, removing significantly more items with elaiosomes (SE and E) (Fig. 4b) ( $p<0.0001$ , Post-hoc test: SE vs. S,  $p<0.0001$ ; S vs. E,  $p<0.0001$ ; Friedman test,  $n=24$ ). The effect on removal dynamics was also very clear as they removed 25% of *Viola* SE seeds after only 3 min, compared with 45 min when they were deprived of their elaiosome (S) (Fig. 6b). This is confirmed by slopes of the regression lines (Test from comparison of several slopes  $F_{0.05,2,46}=147.33$ ;  $P<0.0001$ ; Test post-hoc:  $q_{GE-G}=20.86$ ,  $p<0.001$ ;  $q_{G-E}=21.18$ ,  $p<0.001$ ;  $q_{GE-E}=0.33$ , NS), giving a probability per minute to be removed seven times higher for SE ( $p=0.05$ ) than for S ( $p=0.007$ ).

Curiously, this pattern is totally absent on *Chelidonium* items (Fig. 4d) ( $p=0.4437$ , N.S.; Friedman test,  $n=24$ ). Indeed, nearly 100% of all *Chelidonium* items

—even those deprived of elaiosome— were removed after 45 min. As a corollary, slopes of seed-removal curves were not significantly different between items (Test from comparison of several slopes; N.S.), and probabilities of being taken per minute were always high, independently of the presence or not of an elaiosome (SE,  $p=0.1$ ; S,  $p=0.08$ ) (Fig. 6d).

### Seed fate

After 24 h, for all tested ant/plant pairs, no difference was found out since all seed items that bore or not an elaiosome were removed and recovered outside the nest in the refuse piles. Approximately half of elaiosomes from SE items were discarded by ants ( $L/V$ : 44.8%;  $L/C$ : 44.8%;  $M/V$ : 45.8%) except for the pair  $M/C$  for which 100% of elaiosomes were removed.

## Discussion

### Recruitment

Ant recruitments are rarely reported in the whole myrmecochory literature (see Bond et al., 1991; Gorb and Gorb, 1999). In our case, we never observed any recruitment nor any significant increase of the foragers' population, which confirms the field results obtained with the pair *Myrmica rubra* / *Viola odorata* (Gorb and Gorb, 1999). Mass-recruiting ants such as *Lasius* and *Myrmica* species can nevertheless display all intermediate strategies between solitary foraging and collective food exploitation through trail recruitment (Hölldobler and Wilson, 1990). The lack of recruitment towards elaiosome-bearing seeds can thus be explained by the two following characteristics of food source. Firstly, the number of food items was not enough to trigger recruitment. Secondly, elaiosomes could be considered by foragers as small dead-insect preys (Carroll and Janzen, 1973) and as such, did not elicit recruitment as they could be easily individually retrieved, as shown in *Myrmica rubra* (Cammaerts, 1978) and *Pheidole pallidula* (Detrain and Deneubourg, 1997).

### The elaiosome effect

As expected, the presence of an elaiosome influenced positively seed removal of *Viola odorata* for both ant species. The obligate myrmecochorous plant *Viola odorata*, which produces a few dozens of seeds during a season, blooms in the early spring like most of the other European myrmecochorous plant species (Oberrath and Böhning-Gaese, 2002). This pattern differs from *Chelidonium majus* which flowers later and produces thousands of seeds during a season. In this species, the presence of an elaiosome on *Chelidonium majus* seeds

does not seem to enhance its removal since the seed itself, even deprived of its elaiosome (E), remains very attractive to ant workers, especially *Myrmica* ones. Differences —even slight ones— in removal dynamics between pairs or between items can have major consequences on plant fitness at the time scale of the whole season. This is especially true for *Chelidonium majus*, since one single medium-sized plant produces more than 75,000 seeds per year (Servigne, unpubl. data). Hence, by “flooding the market” and being very appreciated by ants, seeds of *Chelidonium majus* will increase their chances to be scattered everywhere, what may compensate the lack of vegetative reproduction. As a result, *Chelidonium majus* is very efficient in colonizing ruderal places in which ants are used to forage such as pavements or cracks in walls.

The attractiveness of a seed deprived of its elaiosome (S) has already been reported for non-granivorous ants (Weiss, 1909; Bond and Breytenbach, 1985; Pemberton, 1988; Oostermeijer, 1989; Espadaler and Gomez, 1997; Orivel and Dejean, 1999). However, these studies never reached the unexpected level of our pair *Myrmica* / *Chelidonium* (97%). In our case, it is likely that the testa itself was appreciated by workers. An additional explanation may be that ants were interested in the embryo tissue that can be reached by workers at the tiny wound (around 0.4 mm × 0.2 mm) due to the cutting of the elaiosome (as also reported for *Aphaenogaster iberica*; Boulay et al., 2005). Another consideration could be that, as *Chelidonium majus* seeds are mainly present during summer, they have to compete with insect preys which are abundant at this time and need to be more attractive than early spring seeds such as *Viola odorata* seeds. Further investigations will focus on what could make it attractive and how its attractiveness varies in the field with different food spectrum, like Boulay et al. (2005) did it with *Helleborus foetidus* in a Mediterranean ecosystem.

The fact that every seed item has the same probability to be first contacted means that ant orientation is not influenced by the presence of an elaiosome. One ant has thus to come into direct contact (antennating, licking, chewing) before taking any removal decision. This confirms that ants either do not perceive elaiosome volatile compounds, or that elaiosomes have no volatile compounds. EAG experiments also showed that other ant species do not perceive elaiosomes from a distance, but only after antennations (Sheridan et al., 1996 but see also Giliomee, 1986).

### The ant effect

Our two ant species are undoubtedly seed dispersers as they retrieve seeds of *Chelidonium majus* and *Viola odorata*, bring them back to the nest, remove the elaiosomes and reject (*i.e.* disperse) them. Nevertheless, seed removal dynamics and elaiosome discarding rates differed depending on partner species and more precisely on ant behaviour, as shown in other species (Hughes and

Westoby, 1992b; Gorb and Gorb, 2003; Cuautle et al., 2005). Such behavioural differences may have been an important selective force in the evolution of myrmecochory (Beattie and Hughes, 2002). Here, *Myrmica* ants removed seeds faster and in higher proportions than *Lasius*. Their response were also more sensitive to the presence of an elaiosome, as the differences of removal rates between seeds with (SE) and without (S) elaiosomes were more clear-cut than for *Lasius niger*. Finally, *Myrmica* detached *Chelidonium* elaiosomes more efficiently than the black garden ant. Because both ant species have approximately the same size, we can assume that their different seed removal responses were mainly due to their diet. Following the hypothesis of a convergence between odours of elaiosomes and insect preys (Carroll and Janzen, 1973; Hughes et al., 1994), one can explain why insectivorous and predatory species such as *Myrmica* ants are more involved in the myrmecochory process.

In our experiments, antennations were the main behaviour displayed by ants contacting seeds since they accounted for 41% to 63% of all contacts in three following pairs: M/V, L/V and L/C. Conversely, in the field, one can find out markedly lower rates of antennations ranging from 1% to 33% for ten ant species and seeds of *Sanguinaria canadensis* (Pudlo et al., 1980). These values are closer to those obtained in our study for the M/C pair that less antennate seeds and remove them more quickly than the others. We interpret antennations as an examination process without further interest towards seeds. For *Myrmica rubra*, the different levels of antennations between the seeds of two plant species indicate that ants have a clear preference for *Chelidonium majus* and that foragers can be very efficient in seed removal. It is different for *Lasius niger*, that typically antennated both seed species at the same rate. Levels of antennations could thus be a measure of the appropriateness of a seed to an ant.

As regards the manipulation behaviour, it accounts for an increased interest towards seeds. But since contact is finally lost after manipulation, scouts could be not “satisfied” with the nutritional compounds (protein, sugars, etc.) of the seed. Moreover, as manipulations were more frequent for seeds without elaiosomes (S), ants may have difficulties to handle the seed. The handle function of the elaiosome has already been highlighted and could be an important evolutive force in these ant-seed interactions (O’Dowd and Hay, 1980; Horvitz, 1981; Byrne and Levey, 1993; Gomez et al., 2005).

### *The seed size effect*

We found out that the removal rates by both *Lasius niger* and *Myrmica rubra* were higher for the smaller seed (*Chelidonium majus*) than for the larger one (*Viola odorata*). Conversely, *Viola odorata* seeds were more quickly removed than *Chelidonium majus* when foraged

by a larger ant species *Formica polyctena* (Gorb and Gorb, 2000). One could evoke a *size-matching* effect to account for this difference: small ant species (*Lasius* and *Myrmica*) transported preferentially smaller seeds than the large ant *Formica polyctena*. This seed-size dependent selectivity is well known for harvester ants, which remove the largest seeds as possible. Below their seed-size scale, the ant does not detect the seed as a potential food, and above, seeds are too large to be removed (Davidson, 1978; Retana and Cerdá, 1994; Kaspari, 1996; Willott et al., 2000; Heredia and Detrain, 2005).

Currently, the seed-size effect on ants’ removal is quite controversial. Some studies showed that removal rates were seed-size dependent, not elaiosome-size dependent (Gorb and Gorb, 1995, 2000): for an ant species, the bigger the seed, the higher its removal rate. However, other studies found that removal rates were elaiosome-size dependent (Oostermeijer, 1989, Mark and Olesen, 1996) or dependent on the ratio between elaiosome and diaspore sizes (Gunther and Lanza, 1989, Hughes and Westoby, 1992b, Bas et al., 2007). Our results corroborate this latter elaiosome/diaspore-ratio hypothesis: *Chelidonium majus* has smaller seeds, smaller elaiosomes, but a higher elaiosome/diaspore ratio than *Viola odorata* (*Viola*: 0.18; *Chelidonium*: 0.31). Seed retrieval by myrmecochorous ants may obey to more complex decision criteria. While the reward of harvester ants is the entire seed, for seed-dispersing ants, it is the elaiosome. A large diaspore could then benefit to harvester ants (more transport and more food) whereas it could cost to myrmecochorous ants (more transport without any supplementary food) (Schoener, 1971; Hughes and Westoby, 1992a). Selective forces of myrmecochory should favour increasing elaiosome size or elaiosome/seed size ratio. Hence, the efficiency of myrmecochorous ant-plant interactions may depend on two factors: the seed-retrieving ability of ants depending on seed-size matching, and within their seed-size scale, ants choose those bearing the largest elaiosome or showing the highest elaiosome/diaspore ratio.

In addition to these size effects, seed selectivity may also depend on differences in seed-surface compounds as well as ant sensitivity to these chemicals (Gunther and Lanza, 1989). Finally, removal rates are dependent on ant’s historical constraints (Peters et al., 2003). By historical constraints, we mean the seed-specific satiation level of ants (Heithaus et al., 2005), their individual foraging experience coupled to the availability of alternative resources (Boulay et al., 2005). As regards the effect of individual experience, it has been shown that ants can learn how to handle some available seeds and thus increase their removal rates (Johnson, 1991; Gorb and Gorb, 1999). These historical constraints are an important source of variability in field experiments and can be better controlled in laboratory experiments.

This work has evidenced differences in the interaction patterns between four crossed ant-plant pairs in controlled conditions. In those conditions, *Myrmica rubra*

appears to be the best potential dispersal vector and *Chelidonium majus* seems to be very attractive to this ant. But this mutualism is not exclusive as other plants or ants may also interact with different removal dynamics. In natural communities, those differences in plant-ant interactions should be added to changing relative abundances and species combinations in a diversity of landscapes. This may create local spatial shifts in ant-plant interaction intensity and change the outcome across space and time. Traits of interacting species will be well matched in some communities and mismatch in others, forming a geographical mosaic of coevolution (Thompson, 1999, 2005; Garrido et al., 2002).

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