Seed manipulation by ants: disentangling the effects of ant behaviours on seed germination

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Abstract. 1. Seed manipulation by ants can play a key role in seed germination through two main behaviours: elaiosome detachment and seed scarification. Despite the fact that these behaviours are commonplace, their effects have only been quantified independently, and their consequences on seed germination remain controversial.

2. Here we experimentally investigate the effects of ant manipulation on seed germination, disentangling the contributions of these two ant behaviours to this process. To do so, under laboratory conditions, we studied the effects of seed manipulation by *Acromyrmex subterraneus* on germination of seeds from *Mabea fistulifera*, a myrmecochorous plant. We established six experimental treatments: (i) unmanipulated seeds; (ii) ant-manipulated seeds with the elaiosome detached and scarified; (iii) ant-manipulated seeds with the elaiosome detached and non-scarified; (iv) hand-manipulated seeds with the elaiosome remaining and scarified; (v) hand-manipulated seeds with the elaiosome detached and scarified; and (vi) hand-manipulated seeds with elaiosome detached and non-scarified.

3. We found that both elaiosome detachment and seed scarification decreased seed germination rates. Moreover, there was no difference in germination rates whether the seeds were manipulated by researcher's hand or by ants, suggesting a lack of other ant-manipulation effects on seeds.

4. The present study shows that manipulation by *A. subterraneus* can exert negative effects on germination of a myrmecochorous seed. Accordingly, we suggested that studies evaluating the benefits of myrmecochory for plants, based only on seed removal amount and distance, may be overestimating those benefits. Our results contribute to the understanding of the potential mechanisms influencing plant recruitment, especially for myrmecochorous plants inhabiting tropical forests.

Key words. Leaf-cutting ants, microbial infection, myrmecochory, scarification, seed dispersal.

Introduction

Seed dispersal by ants, known as myrmecochory, is a remarkable dispersal syndrome that has evolved in more than 11 000 species of angiosperms worldwide (Lengyel *et al.,* 2009). Myrmecochorous plants produce an elaiosome, a specialised lipid-rich structure attached to seeds that is attractive to ants

(Gorb & Gorb, 2003; Lengyel *et al.,* 2009). Although ants normally remove seeds to their nests for elaiosome consumption, some loss along foraging trails may occur (Detrain & Tasse, 2000; Retana *et al.,* 2004; Leal *et al.,* 2014), favouring germination through decreased competition with the parental plant (Handel, 1976). Seeds that reach the nest are generally discarded after elaiosome consumption (Gómez *et al.,* 2005) either inside the nest, which does not favour germination (Leal *et al.,* 2014), or close to the nest entrance, which usually favours germination (Giladi, 2006; Leal *et al.,* 2007; Farji-Brener & Werenkraut, 2017).

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The advantages mentioned are associated with the relocation of seeds from beneath the parental canopy, often considered to be the only benefit provided by ants during seed dispersal (Giladi, 2006). However, seed manipulation by ants can also play a key role in seed germination through mechanical and chemical behaviours (Leal & Oliveira, 1998; Pizo & Oliveira, 1998; Ohkawara & Akino, 2005; Leal *et al.,* 2007; Prior *et al.,* 2014). When ants manipulate myrmecochorous seeds, two non-mutually exclusive physical behaviours may occur: (i) ants detach the elaiosome for feeding (Servigne & Detrain, 2010; Caut *et al.,* 2013; Prior *et al.,* 2014); and (ii) ants scarify the seeds, damaging the seed coat (Horvitz, 1981; Zettler *et al.,* 2001). Other non-physical manipulation effects may also take place; for instance, ants can release anti-fungal substances that reduce chances of microbial infection of non-myrmecochorous seeds (Ohkawara & Akino, 2005). However, we are not aware of any study investigating non-physical outcomes of ant manipulation on the germination of myrmecochorous seeds.

Even though these different seed manipulation behaviours are commonplace, their relative effects on seed germination remain elusive. For example, elaiosome detachment and seed scarification have been associated with positive (Horvitz, 1981; Ohkawara, 2005; Leal *et al.,* 2007), negative (Imbert, 2006; Hurtado *et al.,* 2012), and neutral (Boyd, 2001; Castro *et al.,* 2010; Prior *et al.,* 2014) effects on seed germination. Additionally, we are not aware of any study investigating the independent effects of elaiosome detachment and seed scarification on seed germination.

Here, we experimentally investigated the effects of ant manipulation on seeds, disentangling the outcomes of the two main ant behaviours: elaiosome detachment and seed scarification on seed germination. We also tested for possible non-physical effects from ant manipulation on seed germination. We used *Acromyrmex subterraneus* ants (Hymenoptera, Formicidae) (Forel, 1912) and seeds of the myrmecochorous tree *Mabea fistulifera* Mart. (Euphorbiaceae) as they occur naturally in our studied remnant of Atlantic rainforest in southern Brazil and have an overlapping and widespread distribution pattern (Lorenzi, 2000; AntWeb, 2018). Furthermore, they frequently interact through myrmecochory and the seeds are carried up to 2 m by ants in field conditions (Peternelli *et al.,* 2004). The aim of this study was to uncover the effects of seed manipulation behaviours on seed germination and consequently discuss their potential influence on plant fitness.

Materials and methods

Ant and seed collection

We collected 25 mature nests of *A. subterraneus* in November 2012 (rainy season), from a secondary successional remnant of Atlantic rainforest in Viçosa, Minas Gerais state, Brazil (Mata do Paraíso reserve; area of approximately 300 ha – 20°48′07″ S, 42°51′31″ W, 648 m asl). We placed each colony in a plastic tray (30 × 40 cm) with inert talc on the borders to prevent ant escape. We placed the fungus garden inside a 750-ml lidded plastic container (fungus chamber), placed inside the tray, which had a 1-cm² hole to allow ant entry. All nests were kept for 12 months inside a laboratory room under a controlled temperature of 25 °C and LD 12:12 h and fed exclusively with *Acalypha wilkesiana* leaves (Euphorbiaceae). For the experiment, we used 20 nests that had reached 750 ml of fungus mass, a proxy for good health conditions of the colony.

In order to keep the natural features of the system, in October 2013, we randomly collected fruits from a single population of *M. fistulifera* (Euphorbiacea) before fruit ripening. This population was located in the same forest fragment from where the ant nests were collected. We later placed the fruits under direct sunlight for seed maturation and release. *Mabea fistulifera* seeds are globular (0.8 × 0.5 mm), with an oleaginous appendix (elaiosome) attached to its upper part (Fig. 1). The elaiosome of *M. fistulifera* is a nutritious lipid-rich appendage that is attractive to many ant species, including leaf-cutting ants (Peternelli *et al.,* 2004, 2008). After fruit maturation, we kept the seeds refrigerated (5 °C) for 1 month to preserve seed viability. In order to balance seed and room conditions, 1 day prior to the commencement of the experiment, we placed all seeds in a plastic tray inside the same room as the experimental ant nests (25 °C and LD 12:12 h). Lastly, we offered part of those seeds to the ants (see Experimental design), taking care to keep both unmanipulated and manipulated seeds under identical conditions (except for the ant manipulation *per se*).

Experimental design

We connected each colony tray to a 250-ml lidded plastic container with a PVC tube (diameter 19 mm). Inside each plastic container, we offered 20 seeds of *M. fistulifera* every 48 h for 10 days. We also fed colonies with fresh *A. wilkesiana* leaves every 24 h after offering seeds. Before each new seed offering, we discarded the seeds that were not removed by the ants and counted the manipulated seeds. We could discriminate between manipulated and non-manipulated seeds as the former were discarded in the nest tray, whereas the latter remained in the attached plastic container. Twelve days after the first seed offering, we separated manipulated seeds into two groups: (I) seeds that had the elaiosome detached and had their coats scarified; and (II) seeds that had the elaiosome detached but with intact coats. This classification was performed by visually detecting damage to the seed coat (Fig. 1).

In order to measure the independent effects of seed elaiosome detachment, seed scarification, and any possible non-physical outcomes performed by ants on seed germination, we set up six experimental treatments: (i) unmanipulated seeds (control); (ii) ant-manipulated seeds with elaiosome detached and scarified; (iii) ant-manipulated seeds with elaiosome detached and non-scarified; (iv) hand-manipulated seeds with elaiosome remaining and scarified (seeds were manually scarified on one side using sandpaper, following Letnic *et al.,* 2000); (v) hand-manipulated seeds with elaiosome detached and scarified; and (vi) hand-manipulated seeds with elaiosome detached and non-scarified (we manually detached the elaiosome). The seeds from treatments (iv), (v), and (vi) were only hand-manipulated and were therefore never offered to ants (Fig. 1).

We sequentially performed a germination test under a controlled temperature (25 °C) and LD 12:12 h using seeds from the
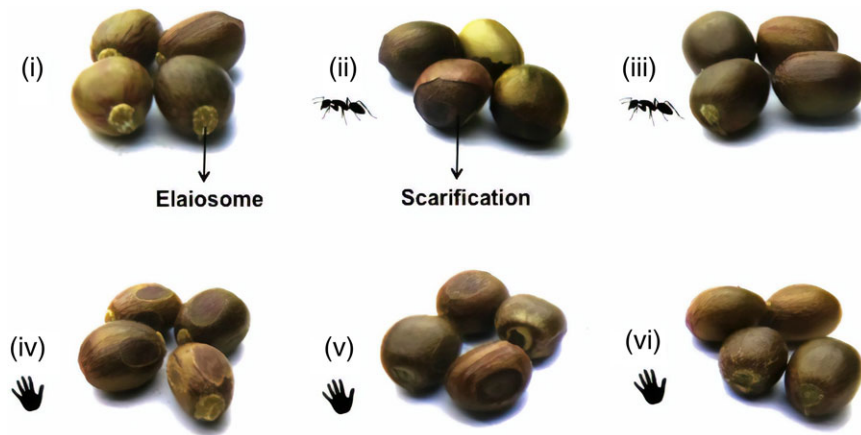
Fig. 1. Images of *Mabea fistulifera* seeds used in the germination experiment. The seeds were separated in six manipulation treatments: (i) unmanipulated seeds (control); (ii) ant-manipulated seeds with elaiosome detached and scarified; (iii) ant-manipulated seeds with elaiosome detached and non-scarified; (iv) hand-manipulated seeds with elaiosome remaining and scarified (the seeds were manually scarified in one side using sandpaper, following Letnic *et al.,* 2000); (v) hand-manipulated seeds with elaiosome detached and scarified; (vi) hand-manipulated seeds with elaiosome detached and non-scarified (we manually detached the elaiosome). Note that seeds from treatments (iv), (v) and (vi) were only hand-manipulated, and therefore were never offered to the ants. [Colour figure can be viewed at wileyonlinelibrary.com].

six treatments. For each treatment, we used 50 Petri dishes covered with filter paper, with four seeds per dish (90×15 mm), totalling 200 seeds per treatment and 1200 seeds divided into 300 Petri dishes for the whole experiment. Filter papers were humidified with 5 ml of distilled water per dish and all dishes were randomly distributed on two germination shelves of 1.39×0.68 m (150 dishes per shelf). On the upper part of these shelves, we placed a set of 10 tubular fluorescent lamps of length 1.20 m, (32 W, 6500 K; NSK, Florianópolis, Brazil), 0.35 m above the Petri dishes. An extra 2 ml of water was added once a week to each Petri dish to maintain moisture. We analysed the dishes daily, counting the number of germinated seeds (sprouting of radicle) per Petri dish, until we recorded no germination for five consecutive days. We marked the end of our experiment on day 32 when we recorded the last seed germination in an interval shorter than five consecutive days.

Statistical analyses

To test whether the proportion of seeds germinated was affected by manipulation treatments, we fitted generalised linear models with the six treatments as explanatory variable, and final proportion of germinated seeds per Petri dish as a response variable, using binomial error distribution (Crawley, 2013). We applied a quasi-correction when overdispersion was detected. To test if the mean time to seed germination differed across treatments, we used survival analysis with Weibull distribution (Weibull, 1951), using treatment as explanatory variable and time to germination as response variable. We set Petri dishes as a frailty random effect (Hougart, 1995) in the survival analysis model with a gamma distribution (Crawley, 2013).

We conducted all analyses using ʀ (R Team, 2016), analysed the residuals, and checked for model suitability in all models. We assessed differences among manipulation treatments

using pairwise contrast analyses, by lumping together similar groups (Crawley, 2013). For survival analyses, we used the sur-vival package v 2.38−3 (Therneau & Lumley, 2015).

Results

Ants removed a total of 2497 seeds, representing 62.73% of 4000 offered seeds. All carried seeds had their elaiosome removed by the ants, while 74.13% (1852 seeds) were also scarified. The proportion of germinated seeds was higher for the non-manipulated seeds (control = 88%), followed by non-scarified seeds with elaiosome detached either by ants (79%) or by hand (78%) [deviance (1, $n = 300$) = 7.74, $P = 0.005$]. Whether the elaiosome was detached by hand or by ants did not affect the proportion of seeds germinated [deviance (1, $n = 300$) = 0.06, $P = 0.81$]. The lowest germination rates were observed for the following three treatments: ant-manipulated with elaiosome detached and scarified (69%), hand-manipulated with elaiosome remaining and scarified (71%), and hand-manipulated with elaiosome detached and scarified (73%) [deviance (1, $n = 300$) = 7.84, $P = 0.005$]. There was no difference in seed germination rates among these last three treatments [deviance (1, $n = 300$) < 0.68, $P > 0.4$; Fig. 2]. We observed that all of the non-geminated seeds had some kind of fungal infection by the end of the experiment, regardless of the treatment.

The mean germination time was lowest for non-manipulated seeds (control; 15.78 ± 0.51 days; mean $\pm$ SE), followed by non-scarified seeds where only the elaiosome had been detached, either by ants (18.09 ± 7.76) or by hand (18.53 ± 0.51) [deviance (1, $n = 1200$) = 21.79, $P < 0.001$]. The agent that performed the elaiosome detachment (ants or hand) did not affect time to germination [deviance (1, $n = 1200$) = 0.44, $P = 0.52$]. Treatments that took longer to germinate were: ant-manipulated with elaiosome detached
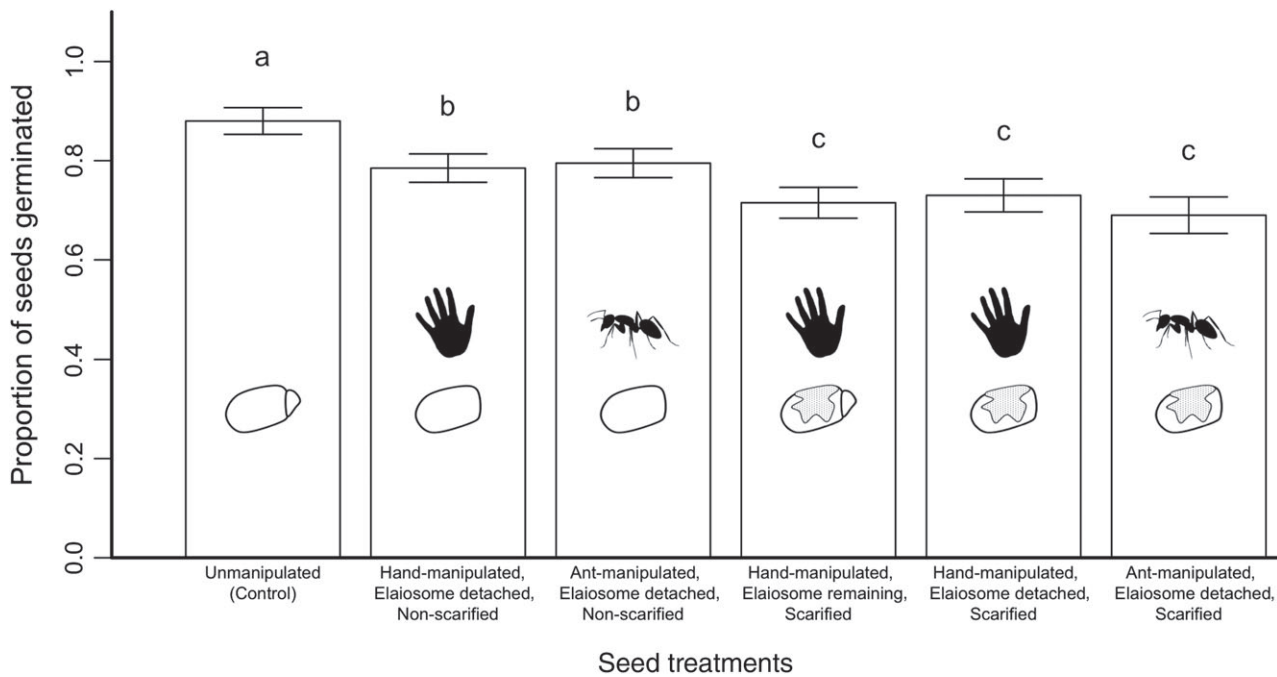
Fig. 2. Mean (±SE) proportion of *Mabea fistulifera* seeds germinated on the 32nd day after the experiment was set up. Seed treatments were as follows: unmanipulated (control), manipulated by *Acromyrmex subterraneus* ants, and/or manipulated by researcher's hand. Different letters indicate significant differences among treatments.

and scarified (20.54 ± 8.7), hand-manipulated with elaiosome remaining and scarified (19.92 ± 8.81), and hand-manipulated with elaiosome detached and scarified (20.01 ± 8.21) [deviance (1, $n = 1200$) = 62.18, $P < 0.001$]. There was no difference in seed germination time among these last three treatments [deviance (1, $n = 1200$) < 0.86, $P > 0.37$; Fig. 3].

Discussion

Here, we experimentally demonstrate that manipulation by *A. subterraneus* ants negatively affects germination of *M. fistulifera* myrmecochorous seeds. This result is fully attributed to physical effects related to manipulation by *A. subterraneus* as there was no difference in seed germination rates between seeds manipulated by ants or by researcher's hand. To our knowledge, this is the first study to disentangle the effects of two important mechanical ant behaviours on seed germination: elaiosome detachment and seed scarification. With regard to the independent effects of those behaviours, we also showed that scarification alone was responsible for the highest decrease in seed germination (17% lower germination than that of control), followed by elaiosome detachment (9% lower germination than that of control). Therefore, ant behaviours might be expected to have a large effect on the *M. fistulifera* population, as ants carried off most of the offered seeds (2497 out of 4000) and from those carried seeds, ants detached all elaiosomes and scarified 70%.

Overall, our findings diverge from most studies which show that ant manipulation increases seed germination rates (Culver

& Beattie, 1980; Horvitz, 1981; Ohkawara, 2005; Ohkawara & Akino, 2005; Leal *et al.*, 2007; Prior *et al.*, 2014). Those studies argue that ants enhance seed germination mainly through two mechanisms: first, ants clean the seeds by releasing antibiotic substances that reduce the chances of microbial infection (Ohkawara & Akino, 2005); and secondly, there is an increase in seed water absorption due to elaiosome removal and seed scarification (Horvitz, 1981; Ohkawara, 2005; Leal *et al.*, 2007). However, the reduction that we found in the germination success of seeds manipulated by *A. subterraneus* might be related to pathogen infection. This was probably favoured by inefficient or absent disinfection of seeds by *A. subterraneus* and boosted by the mechanical behaviours of elaiosome detachment and seed scarification.

More specifically, we suggest that the decrease in seed germination caused by elaiosome removal can be associated with one major cause: elaiosome detachment exposes the seed micropyle (a small orifice at the seed coat border) which can be a gateway to pathogen infections (Kulik & Yaklich, 1991). It has been reported that the presence of the elaiosome could increase water absorption by seeds, facilitating germination for some myrmecochorous species (Lisci *et al.*, 1996). However, any eventual beneficial effect of elaiosome removal, through seed water absorption, might have been masked in our experiment as water was fully available in all treatments. From the results of the present study, we infer that the evidence is stronger for elaiosome removal leading to higher pathogen infection and subsequent lower seed germination. However, as we did not measure pathogen spreading on seeds, this hypothesis would need further investigation.
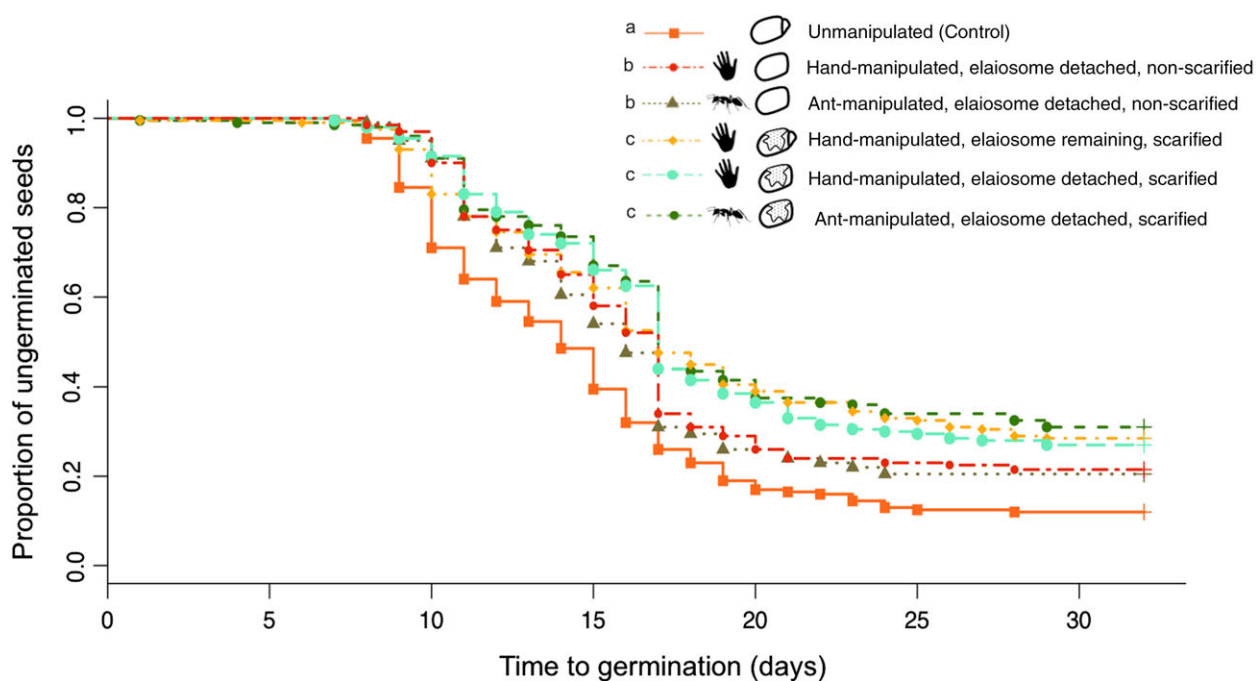
Fig. 3. Proportion of ungerminated seeds of *Mabea fistulifera* at every 24 h during 32 days comparing treatments in which seeds were either unmanipulated (control), manipulated by *Acromyrmex subterraneus* ants, and/or manipulated by researcher's hand. Different letters indicate significant differences among treatments. [Colour figure can be viewed at wileyonlinelibrary.com].

We have shown that seed scarification by ants is the most detrimental behaviour to seed germination. Scarification by *A. subterraneus* of *M. fistulifera* seeds can vary from small scratches to removal of the entire coat (Fig. 1), and this complete coat removal has not yet been documented for any other ant species. However, we did not observe any seed consumption or scarification that breached the seed coat, suggesting that *A. subterraneus* ants do not act as granivores, as do other species, such as *Solenopsis invicta* (Zettler *et al.,* 2001). Seed coats can represent a lignified barrier, with high lipid content and antimicrobial compounds that protect the seed from dehydration and pathogen infections (Moïse *et al.,* 2005). Accordingly, we suggest that the strongest decrease in seed germination promoted by scarification is also likely to be related to pathogen attacks. As we maintained a moist environment throughout the experiment, this may have favoured microbial growth and subsequent infection of coatless seeds (Lamont & Milberg, 1997; Wagner & Mitschunas, 2008). Lastly, when comparing the effects of elaiosome detachment and seed scarification, it is reasonable to suggest that seed scarification exposes a larger area to microbial infections, which could explain why scarification greatly reduced seed germination.

Apart from elaiosome detachment and seed scarification, we did not find any other effect on seed germination from *A. subterraneus* manipulation. In contrast, Ohkawara and Akino (2005) showed that the ant *Pheidole plagiaria* reduced fungal growth on non-myrmecochorous seeds by releasing antifungal substances during seed manipulation, increasing seed germination by 60%. These secretions, usually produced by ants' metapleural glands, can help to prevent microbial infection (Tranter &

Hughes, 2015). Normally, secretions from metapleural glands of leaf-cutting ants have a broad-spectrum effect against microorganisms (Fernandez-Marin *et al.,* 2006; Yek & Mueller, 2011). However, specifically for *Acromyrmex* ant species, there may be a trade-off mechanism between investment in secretions from metapleural glands and an alternative defence provided by a symbiosis with the actinobacteria *Pseudonocardia* sp., which also produces antibiotic secretions (Poulsen *et al.,* 2002). The outcome of this trade-off seems to have led *Acromyrmex* species to base its nest defence mostly on *Pseudonocardia* antibiotics rather than on metapleural gland secretions (Cafaro *et al.,* 2011; Mattoso *et al.,* 2012). As antibiotics produced by this bacteria have a narrower spectrum when compared with metapleural gland secretions (Fernandez-Marin *et al.,* 2009), the lack of non-physical effects in our study could be explained by the narrow spectrum of chemical defences presented by *A. subterraneus*. This ant species might be able to disinfect seeds from nest pathogens, but not from plant pathogens.

Contrary to most studies on seed dispersal by ants (e.g. Culver & Beattie, 1980; Ohkawara & Akino, 2005; Leal *et al.,* 2007; Castro *et al.,* 2010), here we showed that ant manipulation can exert negative effects on germination of a myrmecochorous seed. It is important to note that despite their great ability for seed removal, seed dispersal by leaf-cutting ants is controversial as these ants are not normally considered to be effective dispersers (Leal *et al.,* 2014). Moreover, myrmecochorous plants are uncommon in tropical forests such as the one we studied here (Lengyel *et al.,* 2009). However, the studied species have an overlapping and widespread distribution throughout Brazil, and our results suggest, at least for

leaf-cutting ants and myrmecochorous seeds, that studies based only on quantity of seed removal and distance from the parent plant may be overestimating the benefits of myrmecochory to plants. Moreover, our study highlights the importance of taking into consideration ant manipulation behaviours in studies on seed dispersal. Although myrmecochory may reduce seed germination, there could be overall benefits due to seed dispersal. Advantages of seed reallocation, such as a decrease in seed predation and parent–offspring competition, might outweigh the negative effects of ant manipulation. This emphasises the importance of studying the benefits of seed dispersal by ants under field conditions, which include the effects of intraspecific competition and seed predation, in addition to manipulation effects. Our results help in the understanding of potential mechanisms influencing plant recruitment, especially for myrmecochorous species inhabiting tropical forests.

Acknowledgments

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