



Appetitive and aversive learning of plants odors inside different nest compartments by foraging leaf-cutting ants

Andrés Arenas*, Flavio Roces

Department of Behavioral Physiology and Sociobiology, Biocenter, University of Würzburg, Am Hubland, 97074 Würzburg, Germany



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ABSTRACT

Cues inside the nest provide social insect foragers with information about resources currently exploited that may influence their decisions outside. Leaf-cutting ants harvest leaf fragments that are either further processed as substrate for their symbiotic fungus, or disposed of if unsuitable. We investigated whether *Acromyrmex ambiguus* foragers develop learned preferences for olfactory cues they experienced either in the fungus or in the waste chamber of the nest. Foragers' olfactory preferences were quantified as a choice between sugared papers disks scented with a novel odor and with the odor experienced in one of the nest compartments, before and after odor addition. Odors incorporated in the fungus chamber led to preferences towards paper disks smelling of them. Conversely, odors experienced in the waste chambers led to avoidance of similarly-scented disks. To investigate context-specificity of responses, we quantified learned preferences towards an odor that occurred first in the fungus chamber, and 14 h later in the waste chamber. Foragers initially developed a preference for the odor added in the fungus chamber that turned into avoidance when the same odor solely occurred later in the waste chamber. Avoidance of plants could also be induced in a more natural context, when fresh leaf disks of novel plants, privet or firethorn, were presented in the waste chamber. We conclude that learned acceptance or rejection of suitable plants by foragers depend on the learning context: smells can lead to appetitive learning when present in the fungus garden, or to avoidance learning when they occur at the dump.

1. Introduction

Insect societies display decentralized, collective responses that are not explicitly encoded at the individual level, but emerge from responses of the individuals to relevant local stimuli. Collective tasks such as foraging regularly need to be adjusted in response to changes in food sources and colony requirements. To understand the mechanisms underlying the organization of collective foraging responses, the question of how information about different foraging options and colony needs is conveyed among nestmates is relevant (Deneubourg and Goss, 1989; Camazine et al., 2001; Conradt and Roper, 2005; Gordon, 2010).

As central place foragers, individual foragers always return to the nest after a foraging trip to unload the resources they harvested, and to pass or to obtain information about the available resources (Roces, 2009). Whilst the food collected from a wide range of resource patches converges into the nest, cues available inside the colony can provide nestmates with information about the resources that are being exploited outside. Olfactory cues of the food incorporated into the nest represent

a major component of the decision-making processes leading to collective exploitation of resources (Pankiw et al., 2004; Farina et al., 2005; Arenas et al., 2007, 2008; Molet et al., 2009; Jandt and Jeanne, 2005; Mc Cabe and Farina, 2009; Roces, 1990, 1994; Dupuy et al., 2006).

Studying how workers respond to food-related cues inside the nest and how collective responses arise, is especially challenging in leaf-cutting ants (genera *Atta* and *Acromyrmex*, Formicidae), the success of which depends not only on sharing information among nestmates, but also on acquiring information about the state of their symbiotic fungus *Leucocoprinus gongylophorus*, Lepiotaceae, Basidiomycota (Stahel, 1943; Green and Kooij, 2018). Foraging workers harvest leaf fragments and transport them to the nest' underground fungus chambers, where they are further processed and used to cultivate a fungus that grows on plant material (Weber, 1972). During these steps, plant volatiles might diffuse from the leaf fragments and locally scent the fungus garden. Then, naïve foragers that visit this compartment might learn the odors of those plants currently harvested and use this information to select

* Corresponding author at: Laboratorio de Insectos Sociales, IFIBYNE-CONICET, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Ciudad Universitaria, Buenos Aires, Argentina.

E-mail address: aarenas@bg.fcen.uba.ar (A. Arenas).

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among different plants when foraging outside the nest.

Due to the turnover of the fungus, exhausted plant material and dead fungus are removed from the garden and disposed of at the colony dump (Herz et al., 2007; Jonkman, 1980; Bot et al., 2001; Hart and Ratnieks, 2001; Farji-Brener et al., 2016). If the harvested plant is harmful for the fungus, presumably due to compounds with fungicidal effect, foragers can learn to avoid the cues of the plant in question (Thiele et al., 2014; Ridley et al., 1996; North et al., 1999; Herz et al., 2008; Saverschek and Roces, 2011) and later discontinue its harvesting. This phenomenon starts several hours after the plant was incorporated as substrate for the fungus, and it is known in the literature as delayed avoidance. Although the precise underlying mechanisms for plant delayed avoidance remain elusive, it is likely that ants learn to associate some plant's chemical features, such as its odors (Saverschek and Roces, 2011), with cues available in or signals emitted by the impaired fungus. Consistent with the delayed avoidance at the foraging site, it was recently observed that *A. ambiguus* workers also showed delayed avoidance inside the nest, as gardeners interrupt the processing of already-incorporated unsuitable leaves in the fungus chamber. Interestingly, freshly-cut leaf fragments rejected inside the fungus chamber were subsequently disposed of in the waste chamber (Arenas and Roces, 2016a). The accumulation of unprocessed and partially-decomposed leaf fragments in the waste chamber (Arenas and Roces, 2016a) opens the question if the presence of plant-related odors in the dump provides foragers with information about plant suitability that could be used during plant selection outside of the nest. New evidence indicates that in the dump, waste originating from unsuitable plants induced their avoidance by foragers outside the nest (Arenas and Roces, 2017). However, to what extent the mere presence of odors in the waste chamber provides ants with information of the resources that should be avoided outside the nest remains unknown.

We hypothesize that the relevance of any olfactory cue as a predictor of a suitable or an unsuitable plant strongly depends on the context where it is experienced. On the one hand, odors of freshly-cut fragments experienced by ants under the appetitive context of the fungus chamber might indicate that a putative suitable plant was found, and that more foragers should be recruited for its exploitation. On the other hand, the presence of odors of freshly-cut plant fragments in the waste chamber implies that the resource was disposed of because it did not qualify as suitable for the fungus, thus enabling ants to learn the odors within an aversive context.

Then, we predict that odors recently incorporated into the fungus chamber would increase the acceptance of the resource smelling of it by foragers, but those experienced at the dump should induce avoidance of similarly-smelling plants. To test our hypothesis, we added inert paper disks scented with an odor: i) into the fungus chamber; ii) into the waste chamber, or iii) scented with two different odors, one added in the fungus chamber and the other in the waste chamber. Foraging preferences were evaluated before and after odors were added in the respective nest chambers. Olfactory preferences by single foragers were quantified outside the nest using a pick-up choice test that offered sugared paper disks scented either with the experienced odor, or with an alternative novel one. To investigate whether ants update the meaning of a particular olfactory cue according to the context, we also quantified foraging preferences when a given odor was first experienced in the fungus chamber, mimicking the context of an accepted and readily-incorporated plant into the nest, and later experienced in the waste chamber, recreating a situation in which the recently-incorporated leaf fragments were rejected and disposed of at the dump. Finally, we provide evidence that the avoidance responses observed after the use of controlled amounts of artificial odors also occur in a natural context, when plant cues were directly provided by fresh fragments of suitable leaves placed into the waste chamber.

2. Material and methods

2.1. Ant subcolonies

Experiments were conducted during 2012 and 2013 at the Biocenter of the University of Würzburg, Germany. Sixty-seven queenless subcolonies, composed of 1000 cm³ of fungus (i.e., fungus plus gardeners within the matrix) and about 1000 outside workers were used as experimental units. Subcolonies were obtained from 6 large lab colonies of *Acromyrmex ambiguus* collected in Uruguay in 2002, and reared in a climatic chamber at 25 °C and 50% relative humidity under a 12:12 h light:dark cycle. Subcolonies were organized in three compartments: the foraging box, the fungus chamber and the waste chamber. Each compartment consisted of transparent boxes (19 × 8.5 × 8.5 cm) that connected to each other by clear PVC-tubes (15 cm long, 1.27 cm outside diameter; for further details see Arenas and Roces, 2016a). The fungus chamber housed the fungus garden, the waste chamber the accumulated colony waste, and the foraging box the leaves provided to the ants. According to the availability of plants through the season, subcolonies were fed firethorn (*Pyracantha*), blackberry (*Rubus*), or privet (*Ligustrum*) leaves. Subcolonies also received water and honey-water every day. They remained only unfed at the day of the experiments.

2.2. The influence of plant cues from the nest chambers on foraging decisions

2.2.1. Olfactory cues either in the waste or in the fungus chamber

Here we tested whether the presence of an odor in: i) the waste chamber, ii) the fungus chamber, or iii) in the fungus chamber, but simultaneously presented with a second odor in the waste chamber, influenced odor preferences by foraging workers. To this end, we added 10 standardized paper disks scented with rose essence (0.5 µl odor per disk; Duftöl, Germany) into the waste chamber of 7 subcolonies (first series). In a second series, we added the same number of scented disks into the fungus chamber of 7 additional subcolonies. Finally, we added 10 disks scented with rose into the fungus chamber, and 10 disks scented with lemongrass into the waste chamber of a third group of 7 subcolonies. We added the disks directly on the surface of the fungus garden or on the top of the waste pile.

Foraging preferences in all the three experimental series were tested twice, i.e., before and after giving the scented disks to the respective nest compartments. In all the three series, the first tests were done 22–24 h before adding the disks. In the first series, the second tests were performed 12–14 h after the addition of the disks into the waste chamber. In the second series, choice tests were done 2 h after the addition of the disks into the fungus chamber. In the third series, tests were done 2 h after disks were added into the fungus, and 12–14 h after they were added into the waste chamber. Foraging preferences were evaluated in pick-up dual-choice tests, on a 3 × 3 cm platform located on a bridge 100 cm apart from the entrance of the nest. The bridge worked as the main trail connecting the foraging box of the nest with a second empty box (19 × 19 × 8.5 cm) placed 140 cm apart. Workers could forage on the platform for at least 1 h before the tests, to ensure a well-established foraging column on the bridge. Single foragers choose among four sugared paper disks scented with rose and lemongrass (i.e., 2 with rose and 2 with lemongrass) offered on the platform. Sugared paper disks were soaked in 10% w/w sucrose solution and then dried. They proved to be very attractive to foraging leaf-cutting ants and have been successfully used in previous choice experiments (Roces, 1993; Roces and Núñez, 1993). To scent the sugared paper disks, they were enclosed overnight inside a dish (9 cm in diameter) containing a small cup with 2 ml of the scent essence. Each test extended for 2 h, and

choices were made by 20–30 foragers per subcolony. Once a single ant picked a disk up, a new disk was added onto the platform. The loaded ant was captured on its way back to the nest. Captured ants were returned to their subcolonies when tests finished. The collection of rose (or lemongrass) scented disks over the total number of disks collected was used as an index to quantify the standardized acceptance of one odor over the other. The index ranged from 0.0 to 1.0. A value of 0.5 indicated equal acceptance of both offered alternatives.

We repeated the whole experiment composed of three series using a different pair of odors: blackberry and vanilla (Duftöl, Germany). Blackberry was used to scent the disks added into the waste chamber (first series) and into the fungus chamber (second series). For the third series, we added blackberry-scented disks into the fungus chamber, and disks scented with vanilla into the waste chamber.

2.2.2. Sequential presentation of single odors in the fungus and waste chamber

In this experiment, we evaluated changes in foraging preferences towards an odor that might provide conflicting information as it was first experienced only in the fungus chamber, and 2 days later only in the waste chamber. The experimental procedure recreates the natural situation in which a fragment of an initially-accepted, yet harmful plant, is readily incorporated into a fungus chamber, and later rejected and disposed of at the dump when it proved unsuitable. By offering the same odor sequentially in the two different nest compartments, we investigated the abilities of ants to update the meaning of a cue according to the context where it was experienced. To this end, we measured foraging preferences using pick-up choice tests as described above: *i*) before the odor was added (T0), *ii*) four hours after 10 scented paper disks were added into the fungus chamber (T1), and *iii*) 14 h after 10 new scented disks were given into the waste chamber (T2). Following a similar procedure as in the previous experiments, rose and blackberry essences were used to scent the disks (0.5 µl odor per disk; Duftöl, Germany). Choice tests were performed every two days: T0 was carried out the first day at 09:00 a.m.; T1 on day 3 at 13:00 a.m., and T2 on day 5 at 09:00 a.m.).

2.2.3. Cues from fresh leaves in the waste chamber

Here we investigated the effect of cues provided by fresh leaf fragments in the waste chambers on olfactory preferences by foraging workers, to confirm that the avoidance responses measured after the addition of artificial odors also occur under more natural conditions, namely, when freshly-cut leaf fragments are discarded and dropped among the debris of the waste pile. Considering that unprocessed and partially-decomposed leaf fragments of plants are managed as waste if experienced as unsuitable for the fungus (Arenas and Roces, 2016a), we reasoned that the presence of fresh leaf fragments inside the waste chamber would provide ants with information about the suitability of the harvested plants. As recently described, ants could recover fresh leaf disks suddenly added into the dump if the plant was initially accepted and incorporated as a substrate for the fungus (Arenas and Roces, 2016a). To reduce the attractiveness of the leaf fragments and avoid their potential recovery, we exposed freshly-cut leaf fragments overnight to a small waste sample (10 g) collected from the waste chamber of the same experimental subcolony, so that they were impregnated with waste volatiles (inside a petri dish; 9 cm diameter). In a first group of 8 subcolonies, 10 waste-exposed disks of privet leaves were added into the waste chambers. Foragers' preferences towards privet leaf disks were evaluated in pick-up choice tests against disks of Oregon-grape leaves (*Mahonia aquifolium*). Acceptance was tested twice, 22–24 h before and 12–14 h after adding the leaf disks into the dump. During tests, we used non-exposed disks freshly cut out of

natural fresh leaves. On a second group of 7 subcolonies, we repeated the experiment using waste-exposed leaf disks of firethorn instead of privet. Here, firethorn was offered and compared against privet in the pick-up choice tests.

2.3. Statistics

The effects of factors on standardized acceptances were assessed by means of Generalized Linear Mixed Models (GLMM) with binomial error distribution. Models were fitted in R program (R Development Core and Team, 2016) using the “glmer” function of the “lme4” package (Lenth, 2015). Because data were taken from samples of different size, we included the argument *weight*, which gives more weight to larger samples. For the first experiment, we considered the *test* (a two-level factor corresponding to measurements performed before and after the odor exposure), the compartment or *comp* (a three-level factor corresponding to the waste chamber, the fungus chamber and the fungus + waste chamber), and the *odor* (a two-level factor with rose vs. lemongrass pair and blackberry vs. vanilla pair) as fixed effect. Furthermore, we included their interactions and the subcolony as a random factor. For the second experiment, we considered the *test* (a three-level factor corresponding to T0; T1 and T2) and the *odor* (a two-level factor corresponding to the rose vs. lemongrass pair or the blackberry vs. vanilla pair) as fixed effects, and again, their interactions and the *subcolony* as a random factor. For the third experiment, we included the *test* (a two-level factor as measurements were done before and after adding the leaf disks) and the *plant* (a two-level factor corresponding to the privet vs. Oregon grape or the firethorn vs. privet alternative) as fixed effects, their interactions and the *subcolony* as random factor.

3. Results

3.1. Olfactory cues either in the waste or in the fungus chamber

Foragers rejected sugared paper disks smelling of the odors that scented the waste chamber of their nest. On the contrary, odors learned within the fungus chamber increased acceptance by foragers (Fig. 1). Responses were totally independent of the identity of the odors used to scent the different chambers (*odor*: $F = 2.299$, $p = 0.137$). Standardized acceptances did not differ for the odor identity among the compartments (*comp*odor*: $F = 0.254$, $p = 0.776$), and they were also not different for the odors before and after odor exposure (*test*odor*: $F = 0.281$, $p = 0.599$). The interaction of the factor odor with the two previous factors was not significant either (*test*comp*odor*: $F = 0.6257$, $p = 0.540$).

As hypothesized, standardized acceptances obtained before and after adding the scented disks strongly depended on the nest compartment (*test*comp*: $F = 29.609$, $p < 0.0001$). Before adding scented disks into the waste chambers, foragers showed no preferences for the tested odors (standardized acceptances of rose- and blackberry-scented disks were 0.53 and 0.50, respectively; Fig. 1A and D). After the addition of scented disks to the dump, workers showed a significant lower preference for that odors (indices decreased to 0.26 for rose and 0.35 for blackberry), as compared to the previous day (Simple effects *waste chamber*: $Z = -5.032$, $p < 0.0001$; Fig. 1A and D).

Conversely, foragers significantly preferred the sugared paper disks smelling to the odors that scented the fungus chamber (Simple effects *fungus chamber*: $Z = -4.618$, $p < 0.0001$). When rose scent was present in the fungus chamber, standardized acceptance of rose-scented disks increased from 0.44 to 0.61 (Fig. 1B). In subcolonies with fungus chambers provided with blackberry scent, acceptance of blackberry-scented disks increased from 0.50 to 0.69 (Fig. 1E).

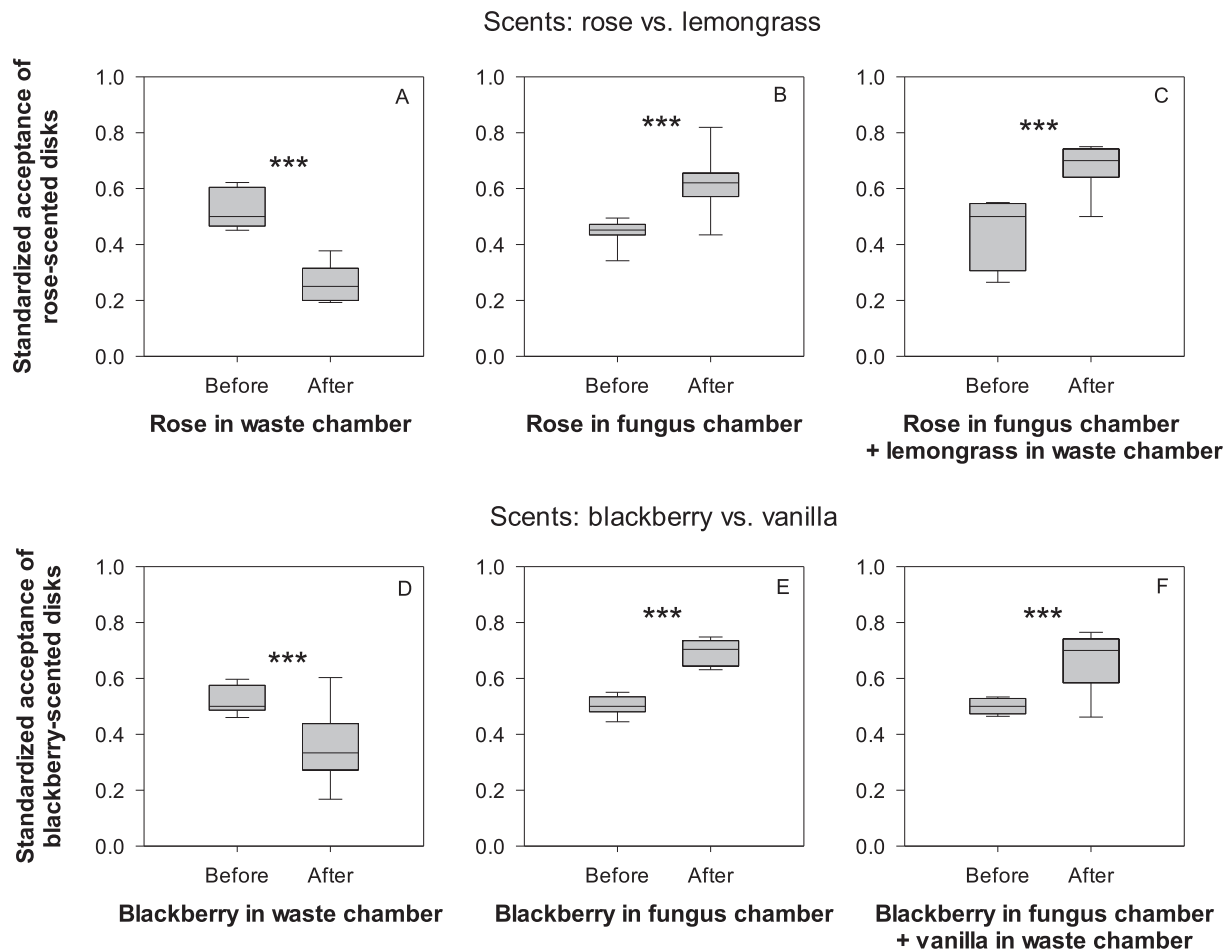


Fig. 1. Olfactory cues either in the waste or in the fungus chamber. Acceptance of scented paper disks before and after giving odors into different compartments of the nest. Standardized acceptances of rose-scented paper disks (A–C) and blackberry-scented paper disks (D–F) (intake of either rose or blackberry-scented paper disks/total intake) were recorded in individual binary choice assays. Rose scent was tested against lemongrass scent (A–C), and blackberry scent against vanilla scent (D–F), 24 h before and 12–14 h after 10 paper-disks scented with rose or blackberry were given into the waste chamber (A and D), or 2 h after disks were added into the fungus chamber (B and E). In C, we gave 10 paper disks scented with rose into the fungus chamber plus 10 paper disks scented with lemongrass into the waste chamber. F, as in C, but using blackberry and vanilla scents instead of rose and lemongrass. The box plots show medians, quartiles and 5th and 95th percentiles from seven subcolonies in each panel, with the exception of those at C and F, which were obtained from six subcolonies. Asterisk indicates statistically significant differences (***) $p < 0.0001$ between indices after simple effects.

A change in the forager's preferences also occurred in the third experimental series, when two different odors were simultaneously presented in the fungus and waste chambers (Simple effects *fungus chamber and waste chamber*: $Z = -4.131$, $p < 0.0001$). For the pair rose and lemongrass, the standardized acceptance of rose scent increased from 0.44 to 0.67 (Fig. 1C), whilst for the other pair, the standardized acceptance of blackberry scent, which initially averaged 0.50, increased up to 0.67 after the exposures (Fig. 1F).

3.2. Sequential presentation of single odors in the fungus and waste chambers

Preferences of foraging workers depended on the olfactory information available in the different compartments of the nest over the three tests of the experiment (*test*: $F = 28.417$, $p < 0.001$), but were independent of the identity of the odors used (*test*odor*: $F = 0.025$, $p = 0.975$; *odor*: $F = 0.225$, $p = 0.645$, Fig. 2).

The standardized acceptance measured at T0, which averaged a

value of 0.46 for rose scent and 0.5 for blackberry scent, indicated a very similar acceptance of both tested pairs of odors before the addition of scented paper disks into the nest. Fifty-three hours later (T1), and four hours after odor cues were added into the fungus chamber, the acceptance of scented disks increased (Simple effects T0 vs. T1: $Z = -4.489$, $p < 0.001$, Fig. 2). It rose up to 0.67 for rose scent, and to 0.71 for blackberry scent. Finally, 35 h after the previous test (at T2) and 14 h after scented disks were given into the waste chamber, the standardized acceptance of scented disks decreased (Simple effects T1 vs. T2: $Z = -7.445$, $p < 0.001$, Fig. 2). The decline to 0.31 in the acceptance for rose scent and to 0.34 for blackberry scent showed a clear avoidance response towards the odors experienced in the waste chamber. Differences were also detected between T0 and T2 (Simple effects T0 vs. T2: $Z = -3.046$, $p = 0.006$, Fig. 2), indicating that responses elicited by scenting the waste chambers not only decreased odor preferences until initial levels of acceptance but statistically beyond, inducing rejection.

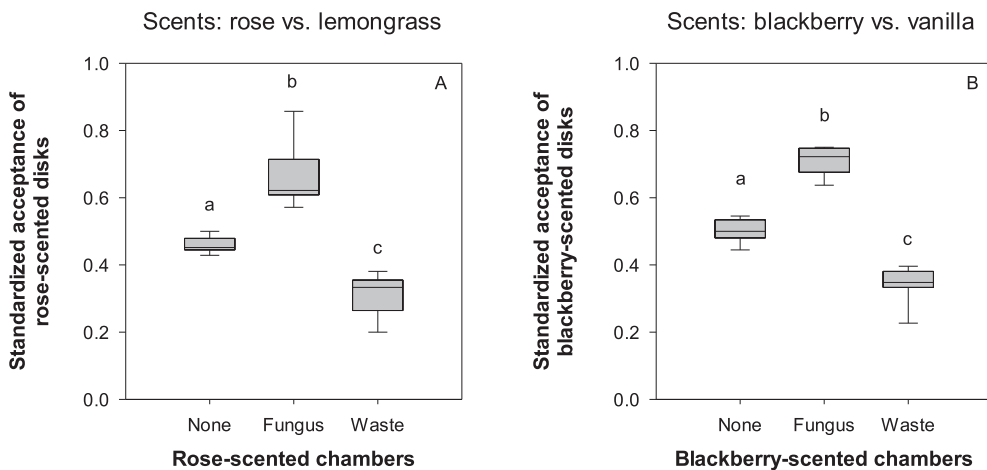


Fig. 2. Sequential presentation of single odors in the fungus and waste chamber. Acceptance of scented-paper disks when no odor was given into the nests, when the odor was given into the fungus chamber, and when it was later relocated into the waste chamber. Standardized acceptances of rose- or blackberry-scented paper disks (intake of rose- or blackberry-scented paper disks/total intake) were recorded in individual binary choice assays. (A) Rose scent was tested against lemongrass scent at three different times: before subcolonies were given the odor (T0), four hours after 10 rose-scented paper disks were added into the fungus chamber (T1), and 14 h after 10 new rose-scented disks were given into the waste chamber (T2). (B) As in A, but using blackberry and vanilla scents instead of rose

and lemongrass. The box plots show medians, quartiles and 5th and 95th percentiles from 5 subcolonies in left panel and from 6 subcolonies in the panel of the right. Different letters indicate statistically significant differences ($p < 0.001$) among indices after simple effects.

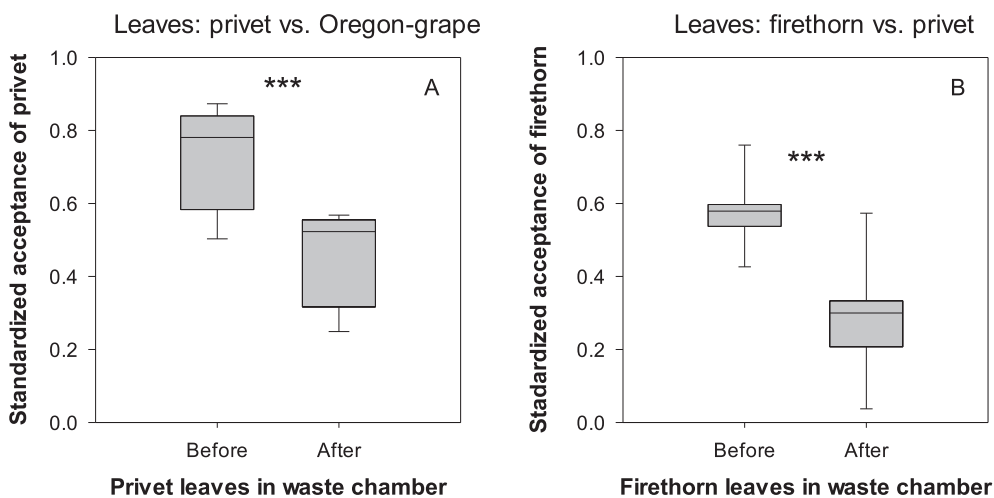


Fig. 3. Cues from fresh leaves in the waste chamber. Acceptance of leaf disks before and after giving waste-exposed, fresh leaf fragments of a single plant into the waste chamber. Standardized acceptance of leaf disks of privet (A), or firethorn (B) (intake of either privet or firethorn leaf disks/total intake) was recorded in individual binary choice assays. (A) Privet leaf disks were tested against Oregon-grape leaf disks 22–24 h before and 12–14 h after subcolonies were given 10 waste-exposed privet disks into the waste chamber. (B) Firethorn leaf disks were tested against privet leaf disks before and after subcolonies were given waste-exposed firethorn disks into the waste chamber. The box plots show medians, quartiles and 5th and 95th percentiles from 8 subcolonies in the left panel, and from 7 subcolonies in the right one.

Asterisks indicate statistically significant differences (***) $p < 0.001$ between indices after simple effects.

3.3. Cues from fresh leaves in the waste chamber

Ants changed their foraging preferences after the addition of waste-exposed leaf disks into the waste chamber ($test: F = 45.931$, $p < 0.001$, Fig. 3). Such changes were independent of the plants used ($test*plant: F = 0.0235$, $p = 0.038$). Foragers showed preferences for privet (index = 0.72) and firethorn (0.58) before the addition of waste-exposed leaf disks into the waste chamber, which both decreased (to 0.45 for privet and to 0.29 for firethorn) in a very similar manner 12–14 h after the leaf exposure. These results indicate that ants learned to avoid suitable plants at the foraging site solely through the presence of their cues inside the waste chamber.

Even though both plants elicited the same pattern of response, the standardized acceptances for privet vs. Oregon-grape and for firethorn vs. privet were indeed different ($Plant: F = 5.190$, $p = 0.038$), as those for privet were always higher than those for firethorn.

4. Discussion

How do sensory cues experienced by foragers inside the nest affect their plant choice when searching outside? Plant cues learned inside the colony could lead to a bias for these stimuli and help foraging workers to decide on a particular resource available in the surroundings. We observed that olfactory foraging preferences changed in a context-specific manner, depending on whether the cues were experienced in

the fungus or in the waste chamber. Particularly, we showed that olfactory cues experienced in the waste chamber were enough for the ants to avoid resources that smell of them. On the contrary, if plant olfactory cues were experienced in the fungus chamber, foragers preferred the resource that smell of those odors. Our results suggest that even when plants (or baits, i.e., sugared paper disks) are suitable, foragers decide to accept or to reject them based on the context within the nest where they experienced their olfactory cues.

In leaf-cutting ants (genera *Atta* and *Acromyrmex*, Formicidae), the smell of the harvested plants can serve either as an orientation cue attracting foragers to the host plants (Littleddyke and Cherrett, 1978; Therrien and Mc Neil, 1990), or as a learned cue used during foraging decision making (Rocas, 1990, 1994; Saverschek and Rocas, 2011; Falibene et al., 2015). Furthermore, it has been shown that recruits are influenced in their foraging choices by the odor of the loads carried by successful ants returning to the nest (Howard et al., 1996; Rocas, 1990, 1994). However, up to now little was known about the effects of food-related cues available inside the nest in shaping plant preferences by foraging workers.

As central place foragers, leaf-cutting ants cut fragments of leaves from a variety of plants available in the surroundings, which are transported into the fungus chamber, processed and finally incorporated (or not) as substrate for the fungus. During these steps, cues of plants that are being processed may be learned by ants inside the nest. In the honeybee, for example, returning foragers transfer the

nectar they collected to their mates via mouth-to-mouth contacts (Pankiw et al., 2004; Farina et al., 2005). As the liquid food can be scented with odors of the visited flowers, food cues shared and learned within the hive are used as informational cues during selection among different foraging options (Free, 1969; Arenas et al., 2007, 2008). The scent of the nectar brought into the nest and deposited in honeypots by successfully-foraging bumblebees (*Bombus terrestris*) can also provide information about the exploited resource (Molet et al., 2009). Wasps *Vespa germanica* that experienced scented food inside the nest are biased toward a similarly-scented food source outside (Jandt and Jeanne, 2005). Stingless bees *Melipona quadrifasciata* show an improved learning performance towards the odors experienced inside the nest (Mc Cabe and Farina, 2009). Our present results go beyond the extended evidence for nectarivorous insects by demonstrating that food-related olfactory cues available inside different nest compartments also have a strong context-specific informational value that underlies foraging decisions in leaf-cutting ants.

We showed that odors experienced in the fungus chamber could lead to memories that bias initial foraging preference, at least in the short-term. As long as the host plants qualify as suitable for the fungus, learning of their cues might lead to a marked preference for them. Then, olfactory information about the food sources available in the fungus chamber might allow not only foragers, but also workers not directly involved in foraging tasks, to obtain information from individuals that actively participate in resource exploitation. On the contrary, ants avoided olfactory cues that are learned in association with the noxious effect of unsuitable substrates on the fungus (Herz et al., 2008). Interestingly, ants are capable of learning to reject plants using solely information obtained from waste particles originating from the impaired fungus, as workers exposed to artificially-scented waste from unsuitable leaves rejected the similarly-scented resources (Arenas and Roces, 2016b). Furthermore, avoidance memories in foraging workers can also be formed at the colony dump, likely through waste particles that contained both the cues of the unsuitable plant and cues of the impaired fungus (Arenas and Roces, 2017). Here, we extended our knowledge about the conditions under which ants could establish avoidance memories, and showed that the mere presence of odor cues in the waste chamber of subcolonies leads to the rejection of resources smelling of it in the foraging context. Therefore, learned avoidance responses are not exclusively mediated by the effects of unsuitable leaves on the fungus, but by other non-specific cues of the waste. Further experiments are required to answer whether or not avoidance memories established under the different conditions are equivalent in strength, i.e., whether they can be recalled or stored over similar time intervals.

Consistent with our suggestion that the waste chamber represents a context in which aversive cues are present, some studies indicated that waste from leaf-cutting ant colonies deters foragers, so that plants surrounded with ants' refuse are protected against herbivores (Zeh, et al., 1999; Farji-Brener and Sasal, 2003). Its deterrent effect might be based on the microorganisms present in the waste pile, which are potentially dangerous to both the ants and their symbiotic fungus (Bot et al., 2001; Hart and Ratnieks, 2001). Ants might be able to associate plant odors with unpleasant cues from debris in the waste-management context, and use this information to avoid learned, aversive stimuli once in the foraging context outside of the nest.

For how long a leaf fragment inside the waste chamber would release volatiles and be therefore identifiable might depend not only on the plant species, but also on the degree of processing by the gardeners and decomposition by the fungus. Observations in waste chambers excavated in the field indicate that fresh, unprocessed leaf fragments may be dropped there (Fig. 4), likely because they were taken into the nest by foragers, yet proved to be unsuitable and were therefore discarded before being incorporated into the fungus garden. Even if not altered by midden workers in the short-term, discarded leaf fragments are expected to be decomposed by the dump's microbiota in the long-term. Although little is known about the architecture of *A. ambiguus*



Fig. 4. Contents of an underground waste chamber of a nest of leaf-cutting ants. Typical view of the waste accumulated inside one large underground waste chamber of a nest of the leaf-cutting ant *Atta laevigata* located at 1.5 m depth, excavated near Botucatu, Brazil. The leaf fragments disposed of on top of the waste pile (white arrows) do not show signs of having been previously incorporated into the fungus garden, i.e., no fungal tufts, changes of color or signs of decomposition are visible, indicating that they were discarded readily after their incorporation into the colony. A whitish film of probable bacterial origin covers the leaf fragments, as well as dead ants and waste particles. Photograph by L. Forti. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nects, Fowler (1985) described that *A. ambiguus* deposits the waste in underground chambers, as a number of *Acromyrmex* spp. does (Haines, 1983; Verza et al., 2007; Farji-Brener et al., 2016). Whether the conditions that prevail in underground dumps enable plant volatiles to last longer than under open air conditions, is unlikely. In fact, some authors argue that external refuse dumps might be advantageous for the ants, as open-air conditions are detrimental for microorganisms involved in the decomposition of discarded leaf fragments (Farji-Brener et al., 2016).

Our first experiment showed that the effect of a single odor experienced in the fungus or in the waste chamber seemed not to differ from the effect elicited by two odors presented in the fungus and the waste chamber simultaneously. For the pair rose-lemongrass, levels of acceptance of the odor presented in the fungus chamber were independent of the presence of the second odor in the waste chamber. Only a small difference (6%) was found on favor of the acceptance measured in the third series (stimulation with two odors) vs. the second series (stimulation with a single odor in the fungus chamber). The difference for the pair blackberry-vanilla was also very small (2%), yet on favor of the acceptance tested in the second series (one odor in the fungus chamber). Such small differences suggest that no additive effect of the cues offered in the different compartments of the nest took place. This finding suggests that foraging ants commonly frequent the two nest compartments, as recently demonstrated (Arenas and Roces, 2017), and that learning of complementary information (i.e., a second odor) did not improve the performance acquired using the information obtained in a single nest compartment.

Furthermore, the effects of the same odor presented in sequence, i.e., first in the fungus chamber and then in the waste chamber, revealed that olfactory memories acquired at the fungus garden are plastic. Preferences towards the similarly-smelling resource could rapidly turn into rejection if the cues are later experienced at the dump, as it may occur when fragments of a given plant are initially accepted, and then disposed of. Based on the evidence that plant avoidance mediated by changes in the fungus garden involves the formation of long-term avoidance memories (they can be recalled up to 18 weeks later, Saverschek et al., 2010), we speculate that reacceptance of plants experienced as harmful both in the fungus garden and in the waste

chamber would not occur as quickly as when avoidance memories are solely formed in the fungus garden. In this regard, it would be interesting to scent both compartments with the same odor and investigate whether scenting the waste chamber overwhelms the effect of scenting the fungus garden.

Leaf-cutting ants (*Atta* and *Acromyrmex*) represent a very serious pest of many crops and plantations in the Neotropics (Cherrett, 1989; Fowler et al., 1986). Different methods, including poison baits, are commonly used to control ant populations with adverse effects on the environment and human health (Cherrett, 1986; Kermarrec et al., 1986; Della Lucia et al., 2014; Bollazzi et al., 2014). Our results open up a new perspective for the biological control of leaf-cutting ants. Even when effectiveness of the addition of species-specific cues at the dump to protect plants from leaf-cutting ant attack deserves detailed studies, our findings highlight the importance of learning and memory in the context of waste management inside the nest to modify ants' foraging preferences.

5. Conclusions

We conclude that both appetitive and aversive learning of plants odors in leaf-cutting ants are context-specific and depend on the nest compartment where learning takes place. The smell of suitable plants can induce plant acceptance by foraging workers if learned within the fungus chamber, where ants grow their own food as a fungus garden. On the contrary, the same odor can trigger plant rejection if learned inside the waste chamber, where exhausted garden, fragments of unsuitable leaves and dead ants are disposed of and decomposed by microorganisms.

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References

- Arenas, A., Fernández, V.M., Farina, W.M., 2007. Floral odor learning within the hive affects honeybees' foraging decisions. *Naturwissenschaften*. 94, 218–222.
- Arenas, A., Fernández, V.M., Farina, W.M., 2008. Floral scents experienced within the colony affect long-term foraging preferences in honeybees. *Apidologie*. 39, 714–722.
- Arenas, A., Rocas, F., 2016a. Gardeners and midden workers in leaf-cutting ants learn to avoid plants unsuitable for the fungus at their worksites. *Anim. Behav.* 115, 167–174.
- Arenas, A., Rocas, F., 2016b. Learning through the waste: olfactory cues from the colony refuse influence plant preferences in foraging leaf-cutting ants. *J. Exp. Biol.* 219, 2490–2496.
- Arenas, A., Rocas, F., 2017. Avoidance of plants unsuitable for the symbiotic fungus in leaf-cutting ants: learning can take place entirely at the colony dump. *PLoS One* 12, e0171388. <https://doi.org/10.1371/journal.pone.0171388>.
- Bollazzi, M., Forti, L.C., Moreira, S., Rocas, F., 2014. Efficiency and soil contamination during underground application of insecticides: control of leaf-cutting ants with thermal foggers. *J. Pest Sci.* 87, 181–189.
- Bot, A., Currie, C.R., Hart, A.G., Boomsma, J.J., 2001. Waste management in leaf-cutting ants. *Ethol. Ecol. Evol.* 13, 225–237.
- Camazine, S., Deneubourg, J.L., Franks, N.R., Sneyd, J., Theraulaz, G., Bonabeau, E., 2001. *Self-organization in Biological Systems*. Princeton University Press, Princeton, N.J.
- Cherrett, M., 1986. Chemical control and bait formulations for leaf-cutting ants. In: Logfren, S., Vander Meer, R. (Eds.), *Fire Ants and Leaf-Cutting Ants: Biology and Management*. Westview Press, Boulder, Colorado, pp. 357–368.
- Cherrett, J.M., 1989. Leaf-cutting ants. In: Lieth, H., Werger, M.J. (Eds.), *Ecosystems of the world 14B: Tropical rain forest ecosystems*. Elsevier, Amsterdam, The Netherlands, pp. 473–488.
- Conradt, L., Roper, T.J., 2005. Consensus decision making in animals. *Trends Ecol. Evol.* 20, 449–456.
- Della Lucia, T.M.C., Gandra, L.C., Guedes, R.N.C., 2014. Managing leaf-cutting ants: peculiarities, trends and challenges. *Pest Manag. Sci.* 70, 14–23.
- Deneubourg, J.L., Goss, S., 1989. Collective patterns and decision-making. *Ethol. Ecol. Evol.* 1, 295–311.
- Development Core Team, R., 2016. R: A Language and Environment for Statistical Computing. The R Foundation for Statistical Computing, Vienna Available at: <http://www.R-project.org/>.
- Dupuy, F., Sandoz, J.C., Giurfa, M., Josens, R., 2006. Individual olfactory learning in *Camponotus* ants. *Anim. Behav.* 72, 1081–1091.
- Falibene, A., Rocas, F., Rössler, W., 2015. Long-term avoidance memory formation is associated with a transient increase in mushroom body synaptic complexes in leaf-cutting ants. *Front. Behav. Neurosci.* 9, 84. <https://doi.org/10.3389/fnbeh.2015.00084>.
- Farina, W.M., Grüter, C., Díaz, P.C., 2005. Social learning of floral odours inside the honeybee hive. *Proc. R. Soc. B* 272, 1923–1928.
- Farji-Brener, A.G., Sasal, Y., 2003. Is dump material an effective small-scale deterrent to herbivory by leaf-cutting ants? *Écoscience* 10, 151–154.
- Farji-Brener, A.G., Elizalde, L., Fernández-Marín, H., Amador-Vargas, S., 2016. Social life and sanitary risks: evolutionary and current ecological conditions determine waste management in leaf-cutting ants. *Proc. R. Soc. B* 283, 20160625. <https://doi.org/10.1098/rspb.2016.0625>.
- Fowler, H.G., 1985. Leaf-cutting ants of the genera *Atta* and *Acromyrmex* of Paraguay. *Dtsch. Ent. Z.* 32, 19–34.
- Fowler, H.G., Forti, L.C., Pereira-da-Silva, V., Saes, N.B., 1986. Economics of grass-cutting ants. In: Logfren, S., Vander Meer, R. (Eds.), *Fire Ants and Leaf-Cutting Ants: Biology and Management*. Westview Press, Boulder, Colorado, pp. 123–145.
- Free, J.B., 1969. Influence of the odour of a honeybee colony's food stores on the behaviour of its foragers. *Nature* 222, 778.
- Gordon, D.M., 2010. *Ant Encounters: Interaction Networks and Colony Behavior*. Princeton University Press.
- Green, P.W.C., Kooij, P.W., 2018. The role of chemical signalling in maintenance of the fungus garden by leaf-cutting ants. *Chemoecology*. 28, 101–107.
- Haines, B., 1983. Leaf-cutting ants bleed mineral elements out of rainforest in southern Venezuela. *Trop. Ecol.* 24, 85–93.
- Hart, A.G., Ratnieks, F.L.W., 2001. Task partitioning, division of labour and nest compartmentalisation collectively isolate hazardous waste in the leaf-cutting ant *Atta cephalotes*. *Behav. Ecol. Sociobiol.* 49, 387–392.
- Herz, H., Beyschlag, W., Hölldobler, B., 2007. Assessing herbivory rates of leaf-cutting ant (*Atta colombica*) colonies through short-term refuse deposition counts. *Biotropica*. 39, 476–481.
- Herz, H., Hölldobler, B., Rocas, F., 2008. Delayed rejection in a leaf-cutting ant after foraging on plants unsuitable for the symbiotic fungus. *Behav. Ecol. Evol.* 19, 575–582.
- Howard, J.J., Henneman, L.M., Cronin, G., Fox, J.A., Hormiga, G., 1996. Conditioning of scouts and recruits during foraging by a leaf-cutting ant, *Atta colombica*. *Anim. Behav.* 52, 299–306.
- Jandt, J.M., Jeanne, R.L., 2005. German yellowjacket (*Vespula germanica*) foragers use odors inside the nest to find carbohydrate food sources. *Ethology* 111, 641–651.
- Jonkman, J.C.M., 1980. The external and internal structure and growth of nests of the leaf-cutting ant *Atta vollenweideri* Forel, 1893 (Hym.: Formicidae). *Z. Angew. Entomol.* 89, 158–173.
- Kermarrec, A., Febvay, G., Decharme, M., 1986. Protection of leaf-cutting ants from biohazards: Is there a future for microbiological control? In: Logfren, S., Vander Meer, R. (Eds.), *Fire Ants and Leaf-Cutting Ants: Biology and Management*. Westview Press, Boulder, Colorado, pp. 123–145.
- Lenth, R., 2015. Package 'lsmeans'. R package version 2.2-2 1–47. <https://cran.r-project.org/web/packages/lsmeans/index.html>.
- Littleldyke, M., Cherrett, J., 1978. Olfactory responses of the leaf-cutting ants *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich) (Hymenoptera, Formicidae) in the laboratory. *Bull. Ent. Res.* 68, 273–282.
- Mc Cabe, S.I., Farina, W.M., 2009. Odor information transfer in the stingless bee *Melipona quadrifasciata*: effect of in-hive experiences on classical conditioning of proboscis extension. *J. Comp. Physiol. A* 195 (2), 113–122.
- Molet, M., Chittka, L., Raine, N.E., 2009. How floral odours are learned inside the bumblebee (*Bombus terrestris*) nest. *Naturwissenschaften*. 96, 213–219.
- North, R.D., Jackson, C.W., Howse, P.E., 1999. Communication between the fungus garden and workers of the leaf-cutting ant, *Atta sexdens rubropilosa*, regarding choice of substrate for the fungus. *Physiol. Entomol.* 24, 127–133.
- Pankiw, T., Nelson, M., Page, R.E., Fondrk, M.K., 2004. The communal crop: modulation of sucrose response thresholds of pre-foraging honey bees with incoming nectar quality. *Behav. Ecol. Sociobiol.* 55, 286–292.
- Ridley, P., Howse, P.E., Jackson, C.W., 1996. Control of the behaviour of leaf-cutting ants by their 'symbiotic' fungus. *Experientia* 52, 631–635.
- Rocas, F., 1990. Olfactory conditioning during the recruitment process in a leaf-cutting ant. *Oecologia* 83, 261–262.
- Rocas, F., 1993. Both evaluation of resource quality and speed of recruited leaf-cutting ants (*Acromyrmex lundi*) depend on their motivational state. *Behav. Ecol. Sociobiol.* 33, 183–189.
- Rocas, F., 1994. Odour learning and decision-marking during food collection in the leaf-cutting ant *Acromyrmex lundi*. *Insect. Soc.* 41, 235–239.
- Rocas, F., 2009. The organization of social foraging in ants: energetics and communication. In: Gadau, J., Fewell, J. (Eds.), *Organization of Insect Societies - From Genome to Sociocomplexity*. Harvard University Press, Cambridge, USA, pp. 289–310.
- Rocas, F., Núñez, J.A., 1993. Information about food quality influences load-size selection in recruited leaf-cutting ants. *Anim. Behav.* 45, 135–143.
- Saverschek, N., Herz, H., Wagner, M., Rocas, F., 2010. Avoiding plants unsuitable for the symbiotic fungus: learning and long-term memory in leaf-cutting ants. *Anim. Behav.* 79, 689–698.

- Saverschek, N., Roces, F., 2011. Foraging leafcutter ants: olfactory memory underlies delayed avoidance of plants unsuitable for the symbiotic fungus. *Anim. Behav.* 82, 453–458.
- Stahel, G., 1943. The fungus gardens of the leaf-cutting ants. *J. New York Botanical Garden* 44, 245–253.
- Therrien, P., Mc Neil, J.N., 1990. The discovery of new resources and subsequent trail formation by *Acromyrmex octospinosus* in Guadeloupe. In: Vander Meer, R.K., Jaffé, K. (Eds.), *Applied Myrmecology: A World Perspective*. Westview Press, Boulder, Colorado, pp. 373–381.
- Thiele, T., Kost, C., Roces, F., Wirth, R., 2014. Foraging leaf-cutting ants learn to reject *Vitis vinifera* ssp. *vinifera* plants that emit herbivore-induced volatiles. *J. Chem. Ecol.* 40, 617–620.
- Verza, S.S., Forti, L.C., Lopes, J.F.S., Hughes, W.H.O., 2007. Nest architecture of the leaf-cutting ant *Acromyrmex rugosus rugosus*. *Insect. Soc.* 54, 303–309.
- Weber, N., 1972. *Gardening ants: The attines*. American Philosophical Society, Philadelphia, PA.
- Zeh, J.E., Zen, A.D., Zeh, D.W., 1999. Dump material as an effective small-scale deterrent to herbivory by *Atta cephalotes*. *Biotropica*. 31, 368–371. **Further reading**
- Gordon, D.M., 1996. The organization of work in social insect colonies. *Nature* 380, 121–124.