



Gardeners and midden workers in leaf-cutting ants learn to avoid plants unsuitable for the fungus at their worksites



Andrés Arenas^{*}, Flavio Rocés

Department of Behavioral Physiology and Sociobiology, Biozentrum, University of Würzburg, Germany

ARTICLE INFO

Article history:

Received 22 September 2015

Initial acceptance 17 November 2015

Final acceptance 5 February 2016

MS. number: A15-00821R

Keywords:

Acromyrmex ambiguus
colony organization
leaf-cutting ant
learned plant avoidance
plant suitability
social insect
symbiotic fungus
waste

Plant selection in leaf-cutting ants is not solely based on innate or learned preferences by foragers, but also on their previous experience with plants that have harmful effects on their symbiotic fungus. Foragers learn to avoid plants harmful for the fungus, albeit harmless for themselves. Since harvested leaves are processed inside the nest, it is an open question whether gardeners and midden workers also participate in the process of plant selection, for instance by learning to reject leaves that proved to be unsuitable for the fungus. Besides occasional observations of fresh leaf fragments in the waste dump, nothing is known about how unsuitable plants already harvested are handled inside the nest. To investigate plant avoidance by gardeners and midden workers, we quantified the dynamics of leaf processing and disposal in laboratory subcolonies of *Acromyrmex ambiguus* during and after having offered them fungicide-treated leaves over 3 days. Control subcolonies received water-treated leaves. Both foraging and processing of fungicide-treated leaves dramatically decreased after 24 h, indicating that learned responses were involved. By this time, midden workers handled leaf fragments as waste and transported them to the waste chamber. On day 4, we asked whether foragers, gardeners and midden workers had learned to avoid plants in a species-specific way, by offering them a choice between untreated leaves of the previously treated plant and untreated leaves of an alternative plant at their worksites. They all rejected the plant previously experienced as harmful for the fungus, indicating that delayed avoidance inside the nest represents an additional step of quality control to preserve the garden from noxious plants that may have qualified as suitable for foragers. We discuss how plant material that is discarded as waste may provide a source of information about plant suitability inside the colony.

© 2016 The Association for the Study of Animal Behaviour. Published by Elsevier Ltd. All rights reserved.

Social insects live in well-organized societies without central control, yet with mechanisms that enable workers to adjust their responses according to the needs of their colony and to the changing environment. To meet colony requirements for nourishment, for example, related activities such as foraging, food processing and disposal of unsuitable foraged material are decentrally coordinated (Gordon, 1996). Taking into account that collective behaviours are not explicitly programmed at the individual level but emerge from numerous interactions of individuals at their worksites, an important question is whether or not workers engaged in different tasks respond to similar stimuli, yet within their own behavioural repertoires, to improve the success of the group (Deneubourg & Goss, 1989; Rocés, 2002).

Among social insects, leaf-cutting ants (genera *Atta* and *Acromyrmex*) represent an interesting case study since their ecological success is based on a relationship with a symbiotic fungus. Foraging workers collect large quantities of fresh vegetation from different plant species (Cherrett, 1989; Wirth, Herz, Ryel, Beyschlag, & Hölldobler, 2003) that they use to cultivate a symbiotic fungus in underground nest chambers. As leaf fragments reach the fungus chamber, a complex process of preparation and incorporation of the plant material into the fungus garden begins (Weber, 1972; Wilson, 1980). Gardeners lick the leaf fragments, cut them into small pieces (1–2 mm²), incorporate them into the garden structure and place faecal droplets and tufts of fungal mycelium on the leaf pieces (Mangone & Currie, 2007; Quinlan & Cherrett, 1977; Stahel, 1943; Weber, 1972). Finally, workers harvest both hyphae and gongylidia from the fungus garden to feed brood and themselves (Bass & Cherrett, 1995). Due to the turnover of the fungus, exhausted plant material and dead fungus are removed from the fungus garden and transported to specific external or internal waste dumps (Herz, Beyschlag, & Hölldobler, 2007;

^{*} Correspondence: A. Arenas, Laboratorio de Estudio de Insectos Sociales, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, IFIBYNE-CONICET, Universidad de Buenos Aires, Intendente Güiraldes 2160, Ciudad Universitaria, Buenos Aires, C1428EHA, Argentina.

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

Jonkman, 1980). Refuse disposal is a common task among leaf-cutting ants (Bot, Currie, Hart, & Boomsma, 2001; Fowler & Louzada, 1996) that avoids accumulation of waste in the garden and reduces the risk of infection for the fungus (Bot et al., 2001; Fernandez-Marin, Zimmerman, & Wisclø, 2003; Hart & Ratnieks, 2001). Disposal of waste is performed by the so-called midden workers. They remove not only the exhausted plant material and fungus from the garden, but also dead ants, debris and other materials carried into the nest but subsequently not processed (Camargo et al., 2003). As a consequence, foragers, gardeners and midden workers, although engaged in different tasks, might all be involved in the process of plant selection, being able to assess the quality of the harvested host plants at their worksites.

Leaf-cutting ants harvest up to 50% of the plant species available in the area surrounding their colonies (Wirth et al., 2003), yet they show marked preferences in their plant choice. A first step in plant selection occurs at the cutting site based on the foragers' preferences for certain leaf features (e.g. toughness, moisture and nutrient content, presence of attracting or deterring compounds; Cherrett & Seaforth, 1970; Hubbell, Wiemer, & Adejare, 1983; Wirth et al., 2003). Initial preferences can be further modulated by previous experience with the plants, for instance influenced by the odour of the loads carried by successful scout ants returning to the nest (Howard, Henneman, Cronin, Fox, & Hormiga, 1996; Roces, 1990, 1994). An additional step of 'plant quality control' takes place inside the nest after foraging, where workers may discard materials inappropriate as substrates for the fungus before their incorporation into the garden (Camargo et al., 2003).

In addition, plant choice is influenced by the effects of the harvested plants on the symbiotic fungus, via a process that involves avoidance learning in foraging workers (Herz et al., 2008; North, Jackson, & Howse, 1999; Ridley, Howse, & Jackson, 1996). Although the fungus is not specialized on any particular substrate, foragers learn to avoid certain plant species proved to be harmful for the fungus, even when those plant species are harmless for the ants. In response to their deleterious effects on the fungus, ants discontinue the harvesting of initially accepted plants. This phenomenon is called 'delayed avoidance', as discontinuity occurs some hours following the collection of the unsuitable substrate for the fungus, lasts over several weeks and involves the formation of long-term avoidance memory (Falibene, Roces, & Rössler, 2015; Herz et al., 2008; Saverschek, Herz, Wagner, & Roces, 2010; Saverschek & Roces, 2011). Delayed avoidance by foraging leaf-cutting ants has been investigated both in the laboratory (Camargo et al., 2003; Herz et al., 2008; Knapp, Howse, & Kermarrec, 1990; North et al., 1999; Rahbé, Febvay, & Kermarrec, 1988; Ridley et al., 1996; Saverschek & Roces, 2011) and in the field (Ridley et al., 1996; Saverschek et al., 2010), and has also been documented towards plants with induced antiherbivore defences (Thiele, Kost, Roces, & Wirth, 2014). In a number of these studies, the suitability of one plant species offered to foragers as a choice for the fungus was altered by infiltrating the leaf tissue with a fungicide (cycloheximide), which was undetectable to the ants but led to delayed avoidance of the otherwise acceptable plant (Herz et al., 2008; North et al., 1999; Ridley et al., 1996; Saverschek et al., 2010; Saverschek & Roces, 2011). For delayed avoidance of previously accepted plants to occur, foraging workers need to associate the state of the fungus with the characteristics of the incorporated plant (chemical and/or physical features), thus allowing its recognition at the foraging site and its avoidance.

While learned plant avoidance by foragers has been explored in some detail, it is unknown whether experience-based avoidance responses towards unsuitable plants also occur inside the nest, and to what extent information about plant unsuitability is distributed among the workers inside the nest. Interestingly, in laboratory

colonies fed fungicide-treated leaves, we have occasionally observed ants disposing of fresh, unprocessed leaf fragments in the waste chamber, a phenomenon unusual in colonies fed untreated leaves. The presence of unprocessed plant material in the waste chamber suggests that delayed avoidance responses also take place inside the nest, and opens the question whether or not gardeners and midden workers learn to reject unsuitable substrates that foragers fail to reject. Delayed avoidance responses inside the nest may comprise the lack of processing of previously incorporated leaf fragments and their removal from the fungus chamber to the waste dump. Delayed avoidance responses inside the nest are expected to represent an additional step of quality control that preserves the fungus garden from the noxious compounds of plants that may have been assessed as suitable by foragers and were therefore incorporated into the nest.

Our aim in the present study was to investigate whether ants working inside and outside the nest learn to prevent the incorporation and processing of plants unsuitable for the symbiotic fungus. To verify the disposal of fresh leaf fragments from unsuitable plants, we first quantified the dynamics of removal and transport of leaf fragments to the dump after their incorporation in the nests of subcolonies of the leaf-cutting ant *Acromyrmex ambiguus* in laboratory. Secondly, we investigated the existence of learned responses in foragers, gardeners and midden workers that enable them to avoid leaves unsuitable for the fungus. To this end, we carried out experiments lasting 4 days. During the first 3 days, a group of laboratory subcolonies were fed leaves infiltrated with a fungicide that could not be detected by the ants. Another group of subcolonies received leaves that were infiltrated with water as a control. Over this period, we repeatedly quantified the acceptance or avoidance of the offered leaves at three nest compartments: (1) at the foraging box, by counting the number of leaf fragments taken into the nest; (2) inside the fungus chamber, by counting the number of leaf fragments that were processed and incorporated into the fungus garden; and (3) inside the waste chamber, by counting the number of leaf fragments that were removed from the fungus chamber and disposed of as waste. On the 4th day, we investigated whether the ants' avoidance responses were plant specific, and whether experienced foragers, gardeners and midden workers were able to discriminate untreated leaves of the plant previously experienced as unsuitable from untreated leaves of a novel plant at their working sites.

METHODS

Ant Subcolonies and Leaf Suitability

For the experiments, performed during 2012 and 2013 at the Biocenter of the University of Würzburg, Germany, we built queenless, functional subcolonies containing about 600 workers, brood at different developmental stages and 1000 cm³ of fungus garden (i.e. fungus plus gardeners within the matrix). Subcolonies remained active and showed intense foraging activity for up to 8 weeks. Eighteen subcolonies were obtained from six large queen-right colonies of *Acromyrmex ambiguus* (three subcolonies per colony) 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. A single subcolony was organized in three transparent compartments: the foraging box, the fungus chamber and the waste chamber. The boxes containing the fungus and waste (19 × 8.5 × 8.5 cm) remained closed with a sealed cover. The bottom of the fungus box was covered with moistened expanded clay pebbles to maintain high humidity and prevent desiccation of the fungus. The foraging box (19 × 19 × 8.5 cm) remained open. Paraffin oil was applied to the walls to prevent the ants from

escaping. Nest compartments were arranged in a T fashion by means of a T junction connecting the tubes (15 cm long, 1.27 cm outside diameter, clear PVC tubes) leading to the fungus, waste and foraging boxes.

To make leaves unsuitable for the fungus, but keep them acceptable for the ants, we infiltrated leaf disks with an aqueous solution of cycloheximide (0.03% w/w, CHX; Sigma Aldrich, Deisenhofen, Germany), following Herz et al. (2008). Cycloheximide, which is undetectable to the ants, is a potent fungicide (North et al., 1999; Ridley et al., 1996) that causes detrimental changes in the fungus but does not affect the leaf tissue. Thus, 'fungicide-treated' leaves function as 'hidden carriers' of the fungicide into the nest. We used freshly collected blackberry leaves, which were infiltrated with the fungicidal solution, or with water as control. Blackberry leaves were successfully infiltrated in each case, as evidenced by the darkening of the leaves.

Disposal of Partially Decomposed and Unprocessed Leaf Fragments

Motivated by occasional observations of unsuitable leaf fragments being discarded in the nest dump, we first sought to determine the disposal rate of leaf fragments readily incorporated into the fungus chamber during and after feeding subcolonies fungicide-treated leaves, by quantifying the removal and transport of partially decomposed and unprocessed leaf fragments. Unprocessed leaf fragments were whole or half leaf disks with little or no signs of processing by the ants or fungus (Fig. 1a, b). Partially decomposed leaf fragments were easily recognized as clusters of small green fragments (<0.5 mm²) with tufts of hyphae on them, often mixed with fungal hyphae (Fig. 1c, d).

We initially started the experiment by feeding six subcolonies 100 fungicide-treated blackberry disks five times per day (20 disks every 2 h, between 0900 and 1700 hours) at the foraging box, over the first 3 days. We videorecorded the entrance of the waste chamber with a digital video camera covering a 2 cm² area and subsequently quantified the disposal rate of decomposed or

unprocessed leaf fragments into the waste chamber from the video. We counted the number of ants carrying decomposed or unprocessed leaf fragments during a 5 min period every 4 h for a total of 60 h. We compared disposal rates before (time: 0 h) and after unsuitable leaves were offered. Recordings began immediately before the onset of the first feeding event, and thus, before unsuitable leaves had been incorporated into the fungus chamber. Data from one subcolony had to be discarded because of unreliable video recordings.

Delayed Avoidance in the Nest Compartments

We assessed the ants' avoidance of leaves in the fungus chamber and in the waste chamber of the nest during and after the ants were fed leaves that were unsuitable for the fungus. To compare the rejection responses of workers inside the nest with those of foragers, we also measured rejection of unsuitable leaves in the foraging box. Six subcolonies were fed fungicide-treated blackberry disks in five feeding events per day, over 3 days. Data were collected 15 min before the onset of each feeding event. On day 1, we made only four measurements as no blackberry disks were present before the onset of the first feeding event. On day 3, we took measurements during the last survey at 72 h even though no feeding event followed. Six subcolonies were fed water-treated blackberry leaves and used as controls. On day 4, when we expected foragers' rejection responses to be strong, we investigated whether their avoidance responses were plant specific or not. To do so, we tested plant selectivity by foragers, gardeners and midden workers by presenting them with a choice between untreated blackberry leaves and untreated rose leaves at their specific worksites, as described below. Foragers, gardeners and midden workers were functionally defined according to the nest location where they performed their tasks.

For the first 3 days of the experiment, when subcolonies were repeatedly fed treated blackberry leaves, we measured rejection responses using three indices: foraging, processing and disposal. In

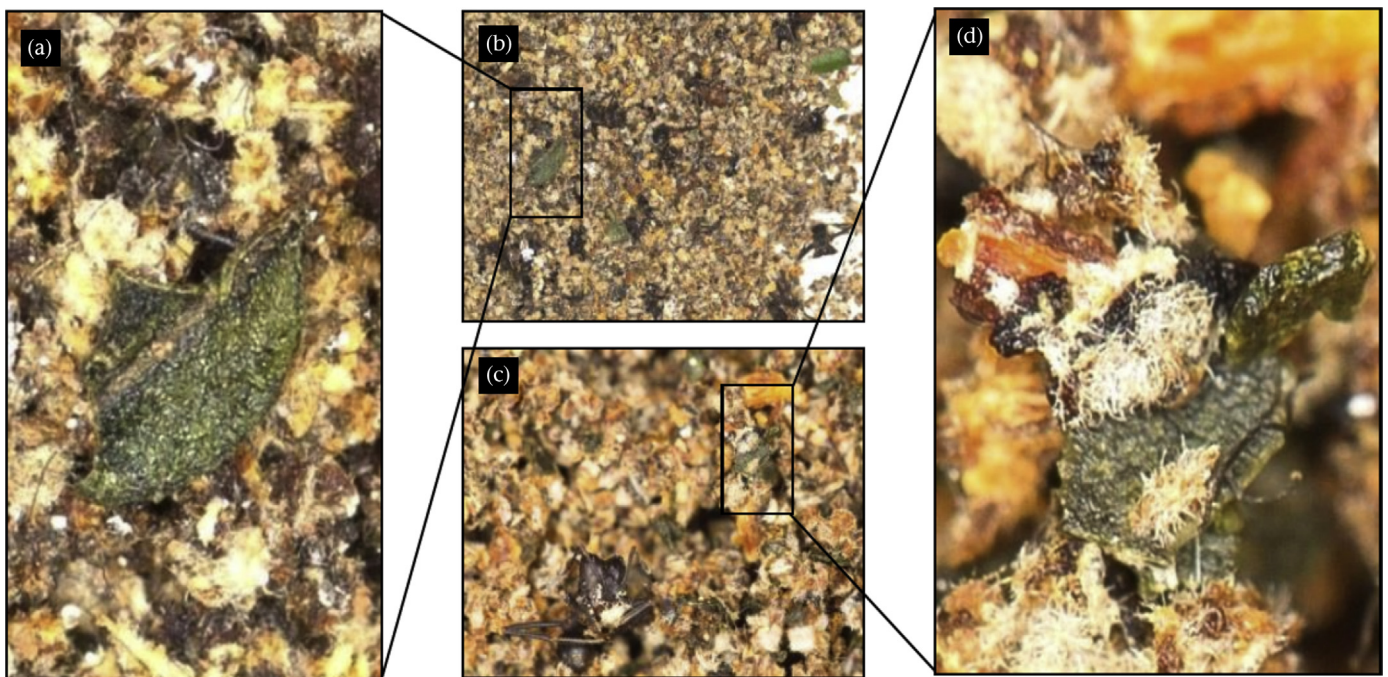


Figure 1. Disposal of partially decomposed and unprocessed leaf fragments into the waste chamber by subcolonies fed fungicide-treated leaves. (a) Magnification of an unprocessed leaf fragment. (b, c) Typical views of the waste pile inside the waste chamber. (d) Magnification of a partially decomposed leaf fragment with tufts of fungus hyphae.

the foraging box, we calculated the foraging index as the ratio between the number of collected disks and the total number of offered blackberry disks: (collected disks)/(collected + uncollected disks). Before the onset of the next feeding event, the remaining uncollected leaf disks from the foraging box were counted and removed. Similarly, in the fungus chamber, we calculated the processing index as the ratio between processed leaf disks and the total number of disks available: (processed disks)/(processed + unprocessed disks). Unlike the processed leaf disks, which were cut into small pieces and incorporated with fungus tufts into the garden, unprocessed disks could easily be recognized since they remained as intact disks lying on the fungus garden or aside in the chamber. We calculated the number of disks processed as the difference between the counts of offered disks and the counts of unprocessed disks. Because we did not remove the unprocessed disks from the closed fungus chamber during the first 3 days (to avoid perturbations), the number of available disks to be processed could accumulate over time after successive feeding events. Thus, the number of unprocessed disks at each counting event included disks that remained unprocessed from previous events. For this index, we also took into account all instances in which the ants removed any unprocessed disks from the garden and transported them to the waste chamber. We used the disposal index to quantify the disposal activity of the subcolonies. It was calculated as the number of leaf disks transported to the waste chamber (i.e. discarded) divided by the number of discarded disks plus the number of unprocessed disks available in the garden, which could potentially be taken to the dump: (discarded disks)/(discarded disks + unprocessed disks).

On day 4, as indicated above, we evaluated whether subcolonies' avoidance responses were specific to the plant they had experienced as being unsuitable for the fungus. For that, workers in all three nest compartments were presented with a choice between 10 untreated blackberry leaf disks and 10 untreated leaf disks of a novel plant (rose). For each nest compartment, we calculated two sets of indices as described above, one for blackberry and one for rose. In each subcolony, we first conducted choice tests in the foraging box, then 10 min later, in the fungus chamber, and immediately thereafter in the waste chamber. Before choice tests, we removed all unprocessed disks that remained in the foraging box, the waste chamber and the fungus chamber.

Statistics

Since the homogeneity of variance assumption was met, we analysed the disposal rates of partially decomposed and unprocessed leaf fragments over time by a repeated measures ANOVA. For post hoc comparisons, we used Fisher's LSD method to create confidence intervals for all pairwise differences in the disposal rate over time. For simplicity and clarity, we made comparisons against the rates obtained at 0 h (i.e. just before fungicide-treated leaves were offered for the first time). We also used repeated measures ANOVA to compare the foraging, processing and disposal indices between control subcolonies and subcolonies fed fungicide-treated leaves during the first 3 days of the experiment. The treatment (feeding fungicide-treated versus water-treated blackberry leaves) was the independent factor, and time was the repeated measure. When significant interactions between these factors were detected, we applied simple effects to evaluate the effect of one factor separately for each level of the other (Quinn & Keough, 2002). In other words, if the treatment*time interaction was significant, we applied simple effects to describe the relation between the factors.

We used two-way ANOVA to compare indices on day 4, when specificity of the avoidance response was studied. The treatment and the foraging option (blackberry versus rose) were the

independent factors. Fisher's LSD tests were performed for post hoc comparisons to determine whether indices across and within treatments differed or not.

RESULTS

Disposal of Partially Decomposed and Unprocessed Leaf Fragments

The disposal rate of partially decomposed and unprocessed leaf fragments significantly varied over the experimental period (repeated measures ANOVA: $F_{15,60} = 3.395$, $P < 0.001$; Fig. 2). The rate measured 12 h after the onset of the first feeding event differed significantly from the initial rate at 0 h (Fisher's LSD: $P = 0.006$). Disposal activity showed the highest peak after 20 h, and remained high for 8 h (Fisher's LSD: 0–16 h: $P = 0.019$; 0–20 h: $P < 0.001$; 0–24 h: $P < 0.001$; 0–28 h: $P = 0.001$). A further peak was detected at 48 h (Fisher's LSD: $P = 0.045$). Changes in the disposal of unprocessed and partially decomposed leaf fragments confirmed our preliminary observations suggesting that workers actively remove unprocessed material and partially processed material (i.e. material that has been incorporated into the garden, and thus, that shows some degree of processing by the gardeners and decomposition by the fungus).

Delayed Avoidance in the Three Nest Compartments

Foraging ants in the control subcolonies collected almost all the offered leaf disks. Therefore, the foraging index was close to 1 and remained relatively constant over the first 3 days (Fig. 3a). In contrast, ants in subcolonies fed fungicide-treated leaves exhibited delayed avoidance. In this group, the foraging index decreased from 1 to 0.2, 26 h after the onset of the experiment, and remained low until the end. The differences in foraging indices between control subcolonies and subcolonies fed fungicide-treated leaves (repeated measures ANOVA: treatment*time interaction: $F_{14,140} = 4.578$, $P < 0.001$) were detectable from 24 h onwards (simple effect analyses: $F_{1,150} = 8.649$, $P = 0.003$; Fig. 3a). On day 4, workers from control subcolonies and those previously fed fungicide-treated leaves also performed differently in dual-choice tests (two-way ANOVA: treatment*foraging option interaction: $F_{1,20} = 4.983$, $P = 0.037$; Fig. 3b). While both tested plants were equally accepted

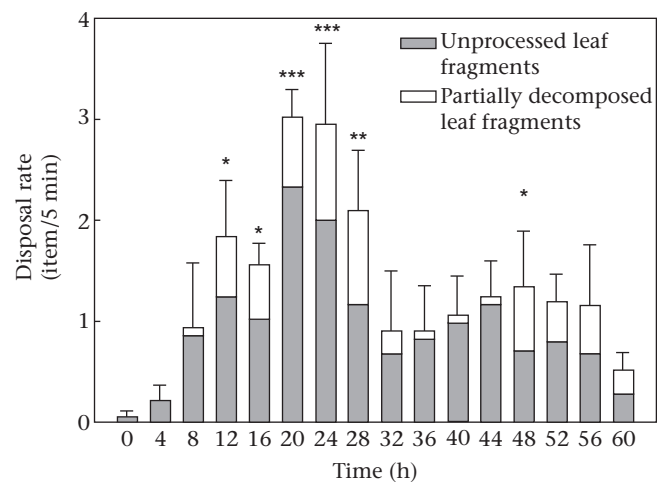


Figure 2. Disposal rate was calculated as the number of items carried into the waste chamber, sampled for 5 min at 4 h intervals over 60 h. Data are means \pm SE. Significant differences are shown relative to counts made at 0 h, before unsuitable leaves were offered (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Five subcolonies were used in the experiment.

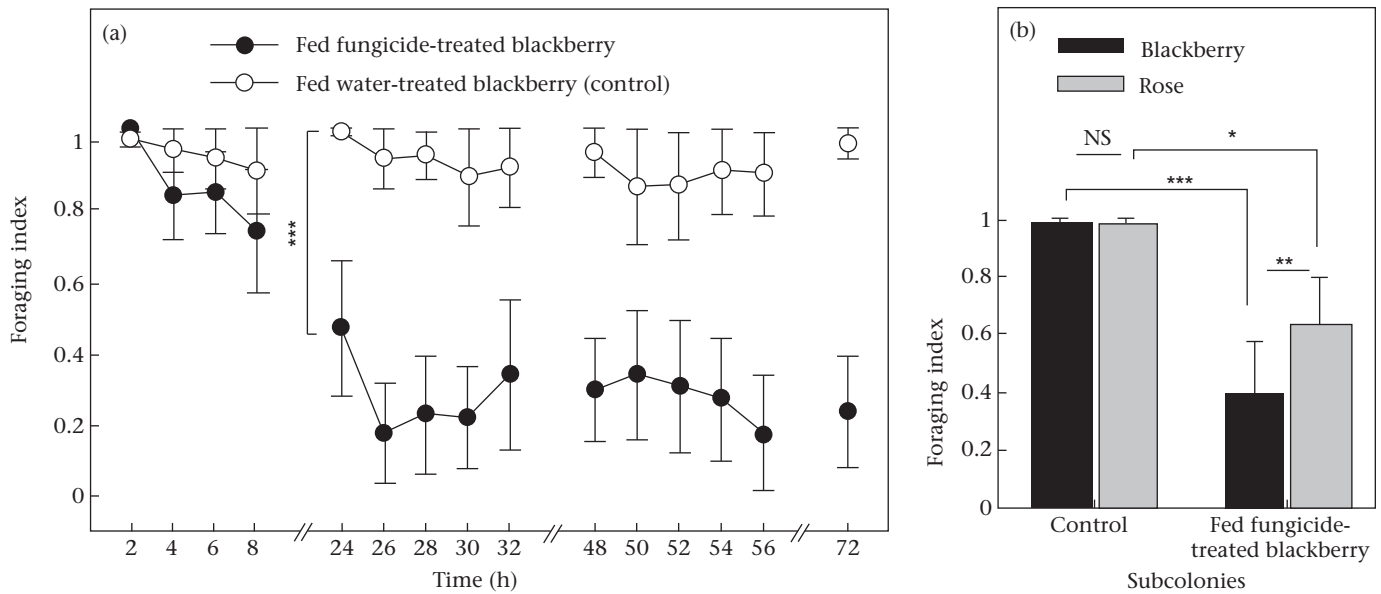


Figure 3. (a) Ratio of the number of leaf disks collected to the total number offered inside the foraging box (foraging index) for subcolonies fed either fungicide-treated blackberry leaves or water-treated blackberry leaves (control) during days 1–3 of the experiment ($N = 6$ subcolonies/treatment). (b) Foraging indices on day 4, when subcolonies were given a choice between untreated leaves of the previously experienced plant (blackberry) and untreated leaves of a novel plant (rose). Data are means \pm SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

in controls, blackberry was less accepted than rose in subcolonies previously fed fungicide-treated blackberry (Fisher's LSD: blackberry index versus rose index: $P = 0.004$). This result indicates that experienced foragers were able to discriminate between untreated blackberry leaves and untreated rose leaves. Despite the species-specific effect, blackberry and rose indices were both lower in subcolonies fed fungicide-treated leaves than in control subcolonies, showing that feeding leaves unsuitable for the fungus affected the acceptance of the two offered plants, but with different strengths (Fisher's LSD: rose index in subcolonies fed fungicide-treated leaves versus rose index in control group: $P = 0.013$; Fig. 3b).

Gardeners inside the nest, like foragers outside the nest, rejected unsuitable leaves that they had previously experienced as noxious for the fungus, by discontinuing their processing (Fig. 4a). Differences in the processing indices between controls and subcolonies fed fungicide-treated leaves (repeated measures ANOVA: treatment*time interaction: $F_{14,140} = 9.203$, $P < 0.001$; Fig. 4a) varied significantly from 24 h after the onset of the experiment (simple effect analyses: $F_{1,150} = 31.679$, $P < 0.001$). On day 4, plant choice inside the fungus chamber also differed between groups (two-way ANOVA: treatment*foraging option interaction: $F_{1,20} = 4.565$, $P = 0.045$). In the control group, novel and experienced plants were equally processed (Fisher's LSD: blackberry index versus rose index: $P = 0.474$; Fig. 4b), as in the foraging context. In contrast, subcolonies previously fed fungicide-treated blackberry leaves processed more disks of rose than of blackberry (Fisher's LSD: blackberry index versus rose index: $P = 0.032$; Fig. 4b).

Disposal of unprocessed leaf disks into the waste chamber by midden workers was observed only in subcolonies fed fungicide-treated leaves. Although disposal averaged 7% of the leaves offered during the 3-day experimental period, it contrasted with the complete lack of disposal of unprocessed disks in control subcolonies. Therefore, we restricted our statistical analysis to changes in the disposal index over time (repeated measures ANOVA: $F_{14,70} = 2.909$, $P = 0.0016$; Fig. 5a). Rates of leaf disposal were highest 24 h after onset of the experiment (Fisher's LSD: 0–24 h:

$P < 0.001$) and at the end of the experiment (0–72 h: $P = 0.005$). On day 4, we offered the ants a choice between untreated leaf disks of the previously experienced plant and untreated leaf disks of a novel plant inside the waste chamber, and then measured the recovery of the offered leaf disks from the waste chamber. Midden workers in control subcolonies recovered all of the offered fragments and transported them to the fungus chamber (Fig. 5b). However, midden workers in subcolonies previously fed fungicide-treated blackberry recovered fewer fragments from the untreated blackberry, as compared to the recovery of fragments from untreated rose (two-way ANOVA: treatment*foraging option interaction: $F_{1,20} = 4.456$, $P = 0.047$). Midden workers in subcolonies that had been fed fungicide-treated leaves exhibited specificity in their recovery response, as they avoided untreated blackberry disks but carried untreated rose disks to the fungus garden (Fisher's LSD: blackberry index versus rose index: $P = 0.007$; Fig. 5b). Together, these results show that experienced foragers, gardeners and midden workers preferred a novel plant species to a plant previously experienced as unsuitable for their fungus, thus indicating that ants inside and outside the nest were able to discriminate plant-specific cues at their worksites.

DISCUSSION

Despite their different behaviours to prevent the impairment of the fungus, foragers, gardeners and midden workers of the leaf-cutting ant *A. ambiguus* were informed about the unsuitability of a specific plant species. After having experienced a plant as unsuitable for the fungus, they responded by avoiding the leaves of that plant at the foraging box, by discontinuing the processing of those leaf fragments already present in the fungus chamber, and by recovering fewer of those leaf fragments from the waste chamber than they did leaf fragments of a novel suitable plant. Our results clearly indicate that the three functional groups (i.e. foragers, gardeners and midden workers) were able to assess the suitability of leaves for the fungus at their respective worksites. This outcome suggests that information about plant suitability is not exclusively

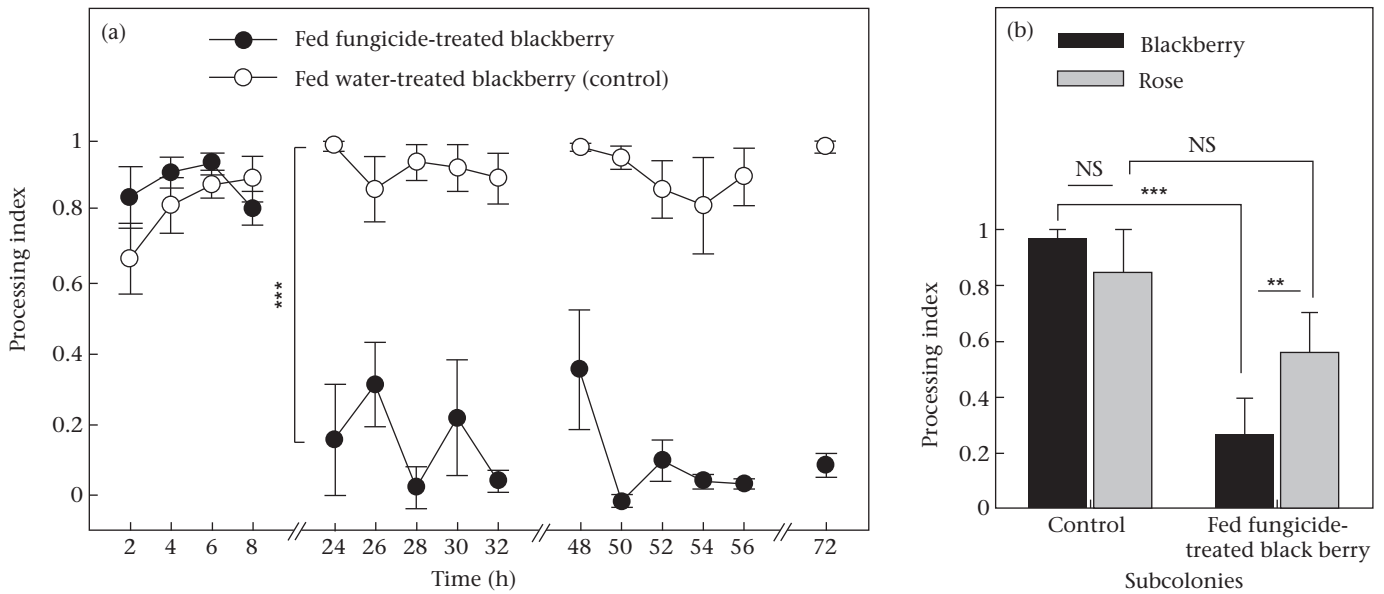


Figure 4. (a) Ratio of the number of leaf disks processed to the total number available in the fungus chamber (processing index) for subcolonies fed either fungicide-treated blackberry leaves or water-treated blackberry leaves (control) during days 1–3 of the experiment ($N = 6$ subcolonies/treatment). (b) Processing indices on day 4, when subcolonies were given a choice between untreated leaves of the previously experienced plant (blackberry) and untreated leaves of a novel plant (rose). Data are means \pm SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

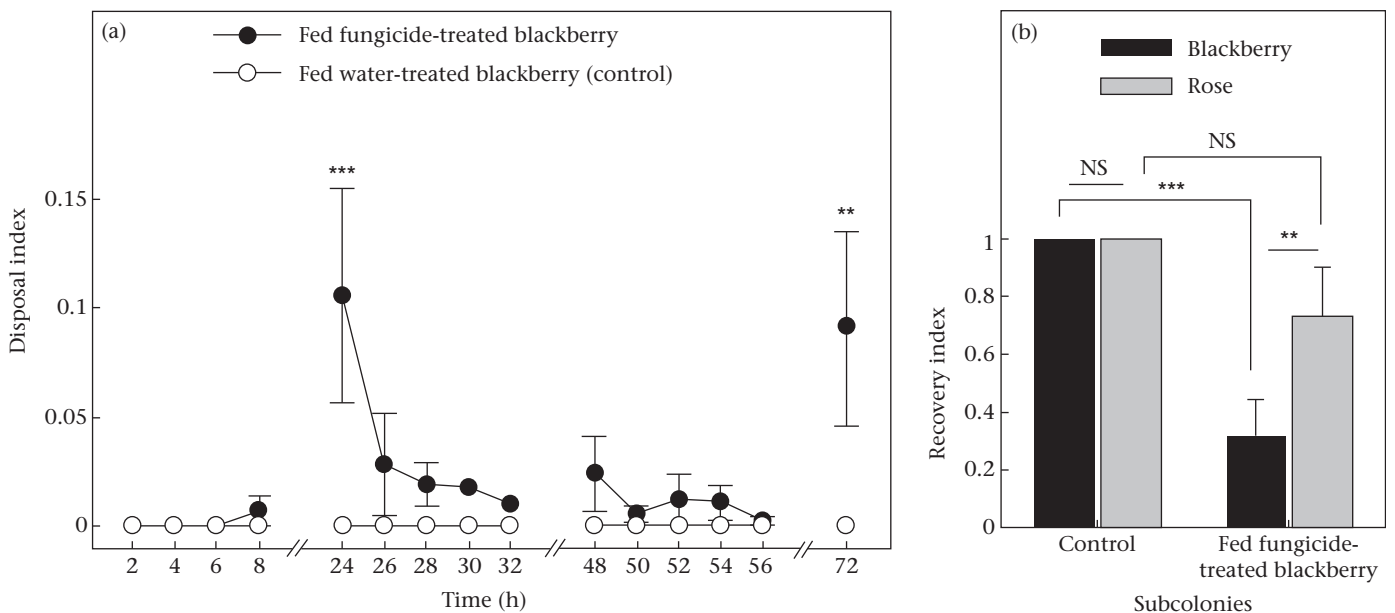


Figure 5. (a) Ratio of the number of leaf disks transported to the waste chamber to the number of discarded + unprocessed disks in the garden (disposal index) for subcolonies fed either fungicide-treated blackberry leaves or water-treated blackberry leaves (control) during days 1–3 of the experiment ($N = 6$ subcolonies/treatment). Note: none of the control subcolonies disposed of unprocessed leaf disks, so statistical analyses are restricted to comparison of disposal rates across time for subcolonies fed fungicide-treated leaves. (b) Ratio of the number of leaf disks transported to the fungus chamber from the waste chamber (recovery index) on day 4, when subcolonies were given a choice between untreated leaves of the previously experienced plant (blackberry) and untreated leaves of a novel plant (rose) inside the waste chamber. Data are means \pm SE. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

managed by any particular group of ants. On the contrary, the three functional groups were able to learn what plants should be avoided inside and outside the nest.

Avoidance Behaviours Inside the Nest

Our study is the first to investigate avoidance responses of leaf-cutting ants towards unsuitable plants for symbiotic fungus inside the fungus chamber. Here, we demonstrate that the suitability of

leaf fragments to be incorporated into the garden is assessed by ants engaged in fungus cultivation. Their most conspicuous response was the decrease in the processing of leaves as substrate for the fungus. In addition, avoidance responses were also observed inside the waste chamber, as midden workers were more likely to recover fresh leaf fragments of novel plants than they were fresh leaf fragments of the plant previously experienced as unsuitable for the fungus. The observation that ants can recover plant material from the dump and transport it back to the fungus chamber

suggests that midden workers are more flexible in their tasks than previously thought (Hart & Ratnieks, 2001). This observation is also consistent with recent descriptions of the behaviour of midden workers in *Acromyrmex echinator* and *Atta sexdens rubropilosa*: midden workers perform diverse activities such as foraging or fungal care when conditions within the nest environment change (Lacerda, Della Lucia, DeSouza, de Souza, & de Souza, 2013; Waddington & Hughes, 2010).

Our results clearly indicate that avoidance behaviours of gardeners and midden workers also rely on a learning process, as already described for foragers (Herz et al., 2008; North et al., 1999; Saverschek et al., 2010; Saverschek & Roces, 2011). Workers might associate cues related to the current state of the fungus (healthy or unhealthy) with cues from the plant, and in fact, the mere perception of the odour of the plant experienced as unsuitable for the fungus suffices to retrieve aversive memories in foraging workers (Saverschek & Roces, 2011). We hypothesize that putative cues or signals from the impaired fungus might act as the negative reinforcement (or unconditioned stimulus), whereas the cues from the harvested plant (i.e. odour, taste, roughness, etc.) might represent the conditioned stimuli (Pavlov, 1927/2010). Whatever the process by which ants learn to associate features of the harvested plant with its harmful effects on the fungus, we show that gardeners and midden workers have the ability to discriminate between untreated leaves of the plant that they have experienced as harmful for the fungus, and leaves from a novel suitable plant. Beyond the species specificity of the described avoidance responses, a nonspecific effect was also evident: subcolonies initially fed fungicide-treated leaves showed reduced acceptance of a novel plant. Such a decrease in acceptance of a novel plant led us to speculate that exposure to fungicide-treated leaves may not only affect associative components of the avoidance-learning process, but nonassociative components too (Menzel, 1999). One plausible explanation for these results is that exposure to the fungicide-treated leaves increased the ants' sensitivity to respond to 'common green volatiles of leaves' or even to plant sap, thus leading to the rejection of an otherwise suitable plant species.

Disposal of Plant Material

Despite reduced collection of fungicide-treated leaves 24 h after their initial harvesting, a few fragments were still carried to the fungus garden on the following days, and were usually unloaded onto the fungus. Soon after their intake, we observed that these items were removed from the surface of the garden and often accumulated in a corner of the fungus box. This sorting of leaf fragments suggests that ants inside the nest recognized that the leaf disks as unsuitable, and handled them as fresh, inappropriate material that needed to be disposed of. Such behaviour became more evident on the second and third days of the experiment, likely as a result of the increasing proportion of gardeners that experienced the noxious effect of the unsuitable plant on the fungus. Although this is the first time such 'sorting' behaviour has been described inside the nest, the idea that inappropriate substrates initially carried into the nest are later removed from the fungus is not new. Camargo et al. (2003) described that some inert materials (e.g. polystyrene, plastic or clay) initially incorporated by workers of *Acromyrmex subterranean brunneus* were later discarded around the nest entrance and inside the waste chamber. A similar behaviour was described for *Atta sexdens rubropilosa* (Verza, Forti, Lopes, Camargo, & Matos, 2007). In agreement with this, we found that some of the leaf disks that had been deposited on the edge of the fungus chamber were later moved to the waste chamber. Interestingly, some of the leaf disks were later recovered from the dump pile and brought back to the garden, usually 24–36 h after the incorporation of fungicide-

treated leaves, suggesting that a number of workers still remained naïve regarding substrate suitability by this time.

Detailed observations of disposal activity indicated that midden workers not only disposed of unprocessed leaf fragments, but also disposed of pieces of leaves undergoing decomposition. Although the disposal dynamic of processed and unprocessed leaf fragments was not studied in control subcolonies, two lines of indirect evidence suggest that the transport of fresh plant material to the dump was triggered only by the noxious effect of the fungicide-treated leaves on the fungus. First, in subcolonies fed water-treated leaves (control group), the disposal index was zero (no disposal at all) over the 3-day period, showing that none of 300 disks offered was disposed of. Second, in subcolonies fed fungicide-treated leaves, disposal of unprocessed and partially decomposed leaf fragments only occurred 12 h after the initial collection of unsuitable leaves, a time very close to the onset of rejection behaviours by foragers of *Acromyrmex lundii* investigated using a similar experimental paradigm (Herz et al., 2008). The observed nonrandom disposal rates, with a peak of activity 12–28 h after the incorporation of fungicide-treated leaves, are consistent with the expected time required for a large proportion of ants to experience the effects of unsuitable leaves on the fungus.

Yet, whether the disposal of fresh, unprocessed leaf fragments should be considered a learned avoidance response or not remains elusive. On the one hand, rejected fragments could have been actively transported to the dump because ants recognized them as harmful for the fungus based on their previous experience with them. On the other hand, fragments could have been disposed of to prevent waste accumulation inside the fungus chamber because they were not appropriate as substrate for the fungus anymore, for instance because of desiccation or decay after a number of days inside the fungus chamber without processing. More experiments are needed to bring light into this matter.

Propagation of Information about Plants Unsuitable for the Fungus

Delayed avoidance of plants unsuitable for the fungus is assumed to be triggered by transient changes in the fungus' state or by the release of semiochemicals by the fungus itself that could be detected by ants (Herz, et al., 2008; Knapp et al., 1990; North et al., 1999; Ridley et al., 1996). Because of their intimate association with the fungus, gardeners are highly prone to perceive such putative cues or signals immediately after they appear. However, little is known about the information that predicts the deleterious effect of the harvested plants on the fungus and how it propagates from the garden to the rest of the nest (North et al., 1999). We speculate that foragers and midden workers visiting or staying on the fungus garden would be able to quickly associate cues or signals related to the state of the fungus with cues from the plant material embedded in it.

Other workers inside the nest, not directly involved in fungus tending, might also learn the noxious effect of plants indirectly through interactions with informed ants. The observation that unprocessed and partially decomposed leaf fragments are taken from the garden to the waste chamber opens the question of whether or not plant fragments disposed of as waste may be involved in the communication about plant suitability among workers. The presence of fresh leaf fragments inside the waste chamber might therefore provide ants with information about plant cues, likely olfactory, that should be avoided inside and outside the nest. In addition, if waste particles contain both plant-related cues and cues from damaged fungus, naïve workers might be able to learn about the suitability of a harvested plant through the waste (e.g. while it is being transported through the nest tunnels and/or as it accumulates in waste deposits). Under this hypothetical scenario, naïve ants might be able to associate, during

waste disposal, chemical features of the plant being disposed of with the detrimental effects of such a plant on the fungus. It is therefore tempting to speculate that colony waste originating from plants harmful for the fungus may influence plant acceptance by workers, thus representing a delayed, negative feedback loop that controls the behaviour of the ants through their symbiotic fungus.

Acknowledgments

We are deeply grateful to N. Adler for performing experiments in the framework of a practical course on behavioural physiology. We also thank A. Falibene for fruitful comments at the early project stage, O. Geissler for logistical support of the experimental work and Erik Frak for the photos in Fig. 1. This work was supported by a German Academic Exchange Service (DAAD) fellowship to A. Arenas (Ref. number: A/11/81698) and by the German Research Foundation (DFG, SFB 1047 TP C1).

References

- Bass, M., & Cherrett, J. M. (1995). Fungal hyphae as a source of nutrients for the leaf-cutting ant *Atta sexdens*. *Physiological Entomology*, *20*(1), 1–6.
- Bot, A., Currie, C. R., Hart, A. G., & Boomsma, J. J. (2001). Waste management in leaf-cutting ants. *Ethology Ecology and Evolution*, *13*, 225–237.
- Camargo, R. S., Forti, L. C., de Matos, C. A. O., Lopes, J. F., de Andrade, A. P. P., & Ramos, V. M. (2003). Post-selection and return of foraged material by *Acromyrmex subterranean brunneus* (Hymenoptera: Formicidae). *Sociobiology*, *42*, 93–102.
- Cherrett, J. M. (1989). Leaf-cutting ants. In H. Lieth, & M. J. Werger (Eds.), *Ecosystems of the world 14B: Tropical rain forest ecosystems* (pp. 473–488). Amsterdam, The Netherlands: Elsevier.
- Cherrett, J. M., & Seaforth, C. E. (1970). Phytochemical arrestants for the leaf-cutting ants, *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich), with some notes on the ants' response. *Bulletin of Entomological Research*, *59*(4), 615–625.
- Deneubourg, J. L., & Goss, S. (1989). Collective patterns and decision-making. *Ethology Ecology and Evolution*, *1*, 295–311.
- Falibene, A., Roces, F., & Rössler, W. (2015). Long-term avoidance memory formation is associated with a transient increase in mushroom body synaptic complexes in leaf-cutting ants. *Frontiers in Behavioral Neuroscience*, *9*, 84. <http://dx.doi.org/10.3389/fnbeh.2015.00084>.
- Fernandez-Marin, H., Zimmerman, J. K., & Wislco, W. T. (2003). Nest-founding in *Acromyrmex octospinosus* (Hymenoptera, Formicidae, Attini): demography and putative prophylactic behaviors. *Insectes Sociaux*, *50*, 304–308.
- Fowler, H. G., & Louzada, J. (1996). Refuse production in leaf-cutting ants (Hymenoptera: Formicidae) and its relationship with leaf harvest. *Biological Rhythm Research*, *27*(2), 148–152.
- Gordon, D. M. (1996). The organization of work in social insect colonies. *Nature*, *380*, 121–124.
- Hart, A. G., & Ratnieks, F. L. W. (2001). Task partitioning, division of labour and nest compartmentalisation collectively isolate hazardous waste in the leafcutting ant *Atta cephalotes*. *Behavioral Ecology and Sociobiology*, *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*(4), 476–481.
- Herz, H., Hölldobler, B., & Roces, F. (2008). Delayed rejection in a leaf-cutting ant after foraging on plants unsuitable for the symbiotic fungus. *Behavioral Ecology*, *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*. *Animal Behaviour*, *52*, 299–306.
- Hubbell, S. P., Wiemer, D. F., & Adejare, A. (1983). An antifungal terpenoid defends a neotropical tree (*Hymnaea*) against attack by fungus-growing ants (*Atta*). *Oecologia*, *60*, 321–327.
- 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). *Zeitschrift für angewandte Entomologie*, *89*(1–5), 158–173.
- Knapp, J. J., Howse, P. E., & Kermarrec, A. (1990). Factors controlling foraging patterns in the leaf-cutting ant *Acromyrmex octospinosus* (Reich). In R. K. Vander Meer, & K. Jaffé (Eds.), *Applied myrmecology: A world perspective* (pp. 382–409). Boulder, CO: Westview Press.
- Lacerda, F. G., Della Lucia, T. M. C., DeSouza, O., de Souza, L. M., & de Souza, D. J. (2013). Task performance of midden workers of *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae). *Journal of Insect Behavior*, *26*, 873–880.
- Mangone, D., & Currie, C. (2007). Garden substrate preparation behaviours in fungus-growing ants. *Entomological Society of Canada*, *849*, 841–849.
- Menzel, R. (1999). Memory dynamics in the honeybee. *Journal of Comparative Physiology A*, *185*, 323–340.
- 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. *Physiological Entomology*, *24*, 127–133.
- Pavlov, I. P. (1927/2010). Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. *Annals of Neurosciences*, *17*(3), 136–141.
- Quinlan, R., & Cherrett, J. (1977). Role of substrate preparation in symbiosis between leaf-cutting ant *Acromyrmex octospinosus* (Reich) and its food fungus. *Ecological Entomology*, *2*, 161–170.
- Quinn, G., & Keough, M. (2002). *Experimental design and data analysis for biologists*. Cambridge, U.K.: Cambridge University Press.
- Rahbé, Y., Febvay, G., & Kermarrec, A. (1988). Foraging activity of the attine ant *Acromyrmex octospinosus* (Reich) (Hymenoptera: Formicidae) on resistant and susceptible yam varieties. *Bulletin of Entomological Research*, *78*(2), 329–337.
- 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.
- Roces, F. (1990). Olfactory conditioning during the recruitment process in a leaf-cutting ant. *Oecologia*, *83*, 261–262.
- Roces, F. (1994). Odour learning and decision-marking during food collection in the leaf-cutting ant *Acromyrmex lundi*. *Insectes Sociaux*, *41*, 235–239.
- Roces, F. (2002). Individual complexity and self-organization in foraging by leaf-cutting ants. *Biological Bulletin*, *202*, 306–313.
- Saverschek, N., Herz, H., Wagner, M., & Roces, F. (2010). Avoiding plants unsuitable for the symbiotic fungus: learning and long-term memory in leaf-cutting ants. *Animal Behaviour*, *79*, 689–698.
- Saverschek, N., & Roces, F. (2011). Foraging leafcutter ants: olfactory memory underlies delayed avoidance of plants unsuitable for the symbiotic fungus. *Animal Behaviour*, *82*, 453–458.
- Stahel, G. (1943). The fungus gardens of the leaf-cutting ants. *Journal of the New York Botanical Garden*, *44*(527), 245–253.
- 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. *Journal of Chemical Ecology*, *40*, 617–620.
- Verza, S. S., Forti, L. C., Lopes, J. F. S., Camargo, R. S., & Matos, C. A. O. (2007). Influence of physical and chemical factors during foraging and culture of the symbiont fungus in *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). *Insect Science*, *14*, 295–300.
- Waddington, S. J., & Hughes, W. O. H. (2010). Waste management in the leaf-cutting ant *Acromyrmex echinator*: the role of worker size, age and plasticity. *Behavioral Ecology and Sociobiology*, *64*, 1219–1228.
- Weber, N. (1972). *Gardening ants: The attines*. Philadelphia, PA: American Philosophical Society.
- Wilson, E. O. (1980). Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). I. The overall pattern in *A. sexdens*. *Behavioral Ecology and Sociobiology*, *7*, 143–156.
- Wirth, R., Herz, H., Ryel, R., Beyschlag, W., & Hölldobler, B. (2003). *Herbivory of leaf-cutting ants: A case study on Atta colombica in the tropical rainforest of Panama*. Berlin, Germany: Springer.