

## RESEARCH ARTICLE

## Learning through the waste: olfactory cues from the colony refuse influence plant preferences in foraging leaf-cutting ants

Andrés Arenas\* and Flavio Roces

## ABSTRACT

Leaf-cutting ants learn to avoid plants initially harvested if they prove to be harmful for their symbiotic fungus once incorporated into the nest. At this point, waste particles removed from the fungus garden are likely to contain cues originating from both the unsuitable plant and the damaged fungus. We investigated whether leaf-cutting ant foragers learn to avoid unsuitable plants solely through the colony waste. We fed subcolonies of *Acromyrmex ambiguus* privet leaves treated with a fungicide undetectable to the ants, then collected the produced waste, and placed it into the fungus chamber of naive subcolonies. In individual choice tests, naive foragers preferred privet leaves before waste was put into the fungus chamber, but avoided them afterwards. Evidence on the influence of olfactory cues from the waste on decision making by foragers was obtained by scenting and transferring waste particles from subcolonies that had been fed either fungicide-treated or untreated leaves. In choice experiments, foragers from subcolonies given scented waste originating from fungicide-treated leaves collected fewer sugared paper discs with that scent compared with foragers from subcolonies given scented waste from untreated leaves. The results indicate that foragers learn to avoid plants unsuitable for the fungus by associating plant odours and cues from the damaged fungus that are present in waste particles. It is argued that waste particles may contribute to spread information about noxious plants for the fungus within the colony.

**KEY WORDS:** Colony waste, Delayed avoidance, *Acromyrmex ambiguus*, Odour learning, Plant rejection, Symbiotic fungus

## INTRODUCTION

Insect societies show responses at the colony level that result from the decisions made by each individual worker. Even though it is tempting to consider a social insect colony as a unit that collectively decides about, for instance, the selection of a given food source, decisions ultimately arise from individuals responding to local information. To generate a coordinated collective pattern, information from thousands of nestmates and from the environment is integrated at the colony level (Deneubourg and Goss, 1989; Seeley and Buhman, 1999; Camazine et al., 2001; Conradt and Roper, 2005; Gordon, 2010). How information then spreads within a colony of social insects is of great interest to understand the organization of collective responses. Studying the mechanisms underlying information transfer and colony organization is especially challenging in leaf-cutting ants (genus *Atta* and *Acromyrmex*, Hymenoptera: Formicidae), as colony

success depends not only on sharing information among nestmates but also on gathering information about the requirements of the fungus that lives in symbiosis inside their nest (Stahel, 1943; Weber, 1972).

In leaf-cutting ants, foraging decisions are particularly complex because most of the resources they collect are not for their own consumption but for their symbiotic fungus (Roces and Bollazzi, 2009). Plant selection is therefore expected to be partially driven by the requirements of the fungus (Ridley et al., 1996; North et al., 1999; Herz et al., 2008; Saverschek and Roces, 2011). Leaf-cutting ants usually forage several plant species around their nest (Cherrett, 1989; Wirth et al., 2003). Selection of their host plants is based both on innate preferences and on the foragers' previous experience with the plants (Cherrett and Seaforth, 1970; Hubbell et al., 1983; Wirth et al., 2003; Camargo et al., 2003; Saverschek et al., 2010). A further level of quality control for the harvested leaves is mediated by the symbiotic fungus (*Leucocoprinus gongylophorus*, Lepiotaceae, Basidiomycota). If the incorporated leaves contain chemical compounds that impair the fungus, foragers can stop harvesting that plant even if it is harmless for them (Ridley et al., 1996; North et al., 1999; Herz et al., 2008). Acceptance of a host plant can turn into avoidance if cycloheximide (CHX), a compound with fungicidal effects, infiltrates the leaf tissue, which maintains the attractiveness of the leaves for the ants but makes them no longer suitable for the fungus (Ridley et al., 1996; North et al., 1999). As CHX cannot be directly detected by the ants, foragers discontinue harvesting of the initially accepted leaves by reacting to putative, unknown changes in the state of the fungus after processing of the leaves. This phenomenon is called delayed avoidance, as plant rejection does not occur immediately but several hours after the incorporation of the fungicide-treated leaves into the fungus garden (Herz et al., 2008), and it involves robust long-term memories (Saverschek et al., 2010). Delayed avoidance lasts over several weeks and is mediated by olfactory memories that ants establish when they associate unknown cues from the damaged fungus with odours from the plant (Saverschek and Roces, 2011; Falibene et al., 2015), although the involvement of other learning modalities (e.g. gustatory, tactile, etc.) cannot be ruled out. Delayed avoidance has also been documented towards plants with induced chemical defences probably acting on the fungus (Thiele et al., 2014).

Fungus cultivation produces large quantities of waste, mostly composed of decaying fungus and plant material, and also dead ants (Fowler and Louzada, 1996; Bot et al., 2001). Several weeks after the incorporation of leaf fragments into the fungus garden, and after both hyphae and gongylidia were harvested by the ants (Weber, 1972), decaying fungus is removed as waste to avoid its accumulation inside the fungus chamber, thus reducing the risk of infections (Bot et al., 2001; Hart and Ratnieks, 2002). Leaf-cutting ants actively transport pieces of exhausted garden from the fungus chambers, where they originate, to specific subterranean chambers or external deposits, according to the species (Jonkman, 1980; Herz

Department of Behavioural Physiology and Sociobiology, Biozentrum, University of Würzburg, 97070 Würzburg, Germany.

\*Author for correspondence (aarenas@bg.fcen.uba.ar)

 A.A., 0000-0003-2199-0827

Received 25 February 2016; Accepted 3 June 2016

et al., 2007). We recently observed that colonies of the leaf-cutting ant *Acromyrmex ambiguus* fed fungicide-treated leaves disposed of not only exhausted particles of fungus garden but also partially processed fragments of the unsuitable leaves (i.e. fragments partially chewed and covered with fungus tufts), and even unprocessed leaf fragments (Arenas and Roces, 2016). Disposal of recently incorporated plant material has not yet been reported for colonies fed suitable plants, thus suggesting that harmful, fungicide-treated leaves were readily detected as noxious and removed from the fungus chamber. Although waste disposal activity is displayed around the clock with little variation over time (Herz et al., 2007), waste disposal rates may significantly increase within hours of colonies foraging on unsuitable leaves because of the removal of both damaged fungus garden and fragments of unsuitable leaves. Waste particles originating from the damaged garden may contain cues from both the unsuitable plant and the impaired fungus. Under this scenario, we reason that foragers could learn to avoid specific host plants directly through information contingent in the waste particles.

In this study, we investigated whether foraging workers of the leaf-cutting ant *Acromyrmex ambiguus* learn to avoid plants unsuitable for the symbiotic fungus through the colony waste. We first quantified the dynamics of waste production to characterize possible transient increases in the rate of waste removal after colonies were fed fungicide-treated leaves. Subsequently, we performed two different experiments aimed at evaluating the influence of waste from unsuitable leaves on decision making by foraging ants. In the first experiment, we asked whether foragers from subcolonies exposed to waste originating from fungicide-treated leaves learn to avoid the plant species from which the waste originated. To this end, we placed waste particles produced by subcolonies fed fungicide-treated leaves of privet into the fungus chamber of naive subcolonies. Plant preferences of naive foraging ants were tested before and after waste was placed in the fungus chamber, by offering single foragers a binary choice between leaf discs of privet and firethorn, both untreated. In the second experiment, we investigated the extent to which plant avoidance by foragers relies upon olfactory cues present in the waste particles. For this purpose, waste particles from either untreated or fungicide-treated leaves were scented with apple or mandarin odours. In independent series, scented waste from one of the four possible waste–odour combinations was placed into the fungus chamber of naive subcolonies. Foraging preferences were evaluated in binary choice assays as in the previous experiment, by offering single foragers sugared paper discs scented either with the odour paired to the waste, or with an alternative odour.

## MATERIALS AND METHODS

### Preparation of subcolonies

Experiments were conducted during 2012 and 2013 at the Biocentre of the University of Würzburg, Germany, with queenless subcolonies built from six large queenright laboratory colonies of *Acromyrmex ambiguus* Gonçalves 1961 collected in Uruguay in 2002, and reared in a climatic chamber at 25°C, 50% air humidity and under a 12 h:12 h light:dark cycle. Each subcolony contained about 600 workers, brood at different developmental stages and 1000 cm<sup>3</sup> of fungus garden (i.e. fungus garden plus a large number of gardeners within the matrix). Subcolonies remained active in the laboratory for several weeks. The artificial nests housing each subcolony were organized into three compartments; namely, the foraging box, the fungus chamber and the waste chamber. The bottom of the fungus chamber was

covered with moistened expanded clay pebbles and remained closed with a sealed cover to keep humidity high and to prevent fungus desiccation. Compartments consisted of transparent plastic boxes (19×8.5×8.5 cm) connected to each other in a ‘T’ fashion by clear PVC tubes (15 cm long, 1.27 cm outside diameter) and a T-junction. Subcolonies received fresh blackberry leaves (*Rubus fruticosus*), diluted honey and water every day.

### Leaf suitability and delayed avoidance

Delayed avoidance of a plant harmful to the fungus was induced by lacing leaves with a CHX solution (0.03% w/w), using a well-established pressure-vacuum infiltration method previously employed in several studies (Herz et al., 2008; Saverschek et al., 2010; Saverschek and Roces, 2011; Falibene et al., 2015; Arenas and Roces, 2016). CHX (Sigma-Aldrich, Deisenhofen, Germany) is a compound with a fungicidal effect that impairs the ants’ symbiotic fungus a few hours after treated leaves have been incorporated (Ridley et al., 1996; North et al., 1999; Herz et al., 2008), but remains undetectable to the ants. Leaf discs (diameter 0.6 cm) were punched from freshly collected privet leaves (*Ligustrum* sp.) and treated with the CHX-solution. To induce delayed avoidance, subcolonies were fed 200 CHX-treated leaf discs. Leaf discs were given at the foraging box all at once.

### Dynamics of waste disposal

We expected an increase in the pruning and removal of the fungus garden soon after the incorporation of fungicide-treated leaves, and subsequently in the disposal of leaf fragments. Under this scenario, early pruning should lead to a transient increase of waste disposal soon after workers learn to avoid plants unsuitable for the fungus, which occurs as early as 10 h after leaf collection (Herz et al., 2008). We quantified the dynamics of waste disposal before and after feeding subcolonies fungicide-treated leaves by counting the number of ants carrying waste particles per unit time at the entrance of the waste chamber. For that, an area of 2 cm<sup>2</sup> was recorded with a video camera hooked up to a monitor. Five-minute samples were taken every 4 h for 64 consecutive hours. In order to determine variations from the basal activity, samplings were compared with counts at 0 h, when fungicide-treated discs of privet were initially offered, using the Wilcoxon test.

### Effects of waste from unsuitable leaves on plant choice by foragers

Pieces of impaired garden removed early as waste might contain both cues originating from the unsuitable plant and cues from the damaged fungus. In this first experiment, we asked whether ants from subcolonies exposed to waste originating from unsuitable, fungicide-treated leaves learn to avoid the plant species from which the waste originated. We hypothesized that ants would establish memories whilst they disposed of or handled the waste particles, which may be recalled in the foraging context to recognize and avoid specific plants. Preliminary observations indicated that small amounts of waste particles directly placed into the fungus chamber were readily removed by workers and carried to the waste chamber, as expected. Taking advantage of this fact, the rationale of the present experiment was to place a small amount of waste particles (4 cm<sup>3</sup>) produced in subcolonies fed fungicide-treated privet (henceforth, ‘donor subcolonies’) into the fungus chamber of naive subcolonies (henceforth, ‘receiver subcolonies’). Thereafter, plant preferences were evaluated by offering foragers a choice between privet (*Ligustrum* sp.) and a novel plant, firethorn (*Pyracantha* sp.), before and after the waste was given into the chamber.

To ensure that donor subcolonies learned to avoid privet leaves initially treated with CHX as a fungicide, which would lead to production of waste that contained cues from both the plant and the damaged fungus, foragers' preferences were quantified in individual tests 4 h before and 22 h after the subcolonies were fed CHX-treated privet leaves. Each test extended for 2 h, in which choices made by an average of  $20.2 \pm 2.1$  foragers per subcolony were quantified for seven different subcolonies (total number of choices recorded:  $N=262$ ). During tests, foragers were allowed to choose between untreated leaf discs of privet and firethorn as a novel alternative. For the choice experiments, the foraging box of the subcolony was connected to a foraging arena via a 1 m-long wooden bridge. To guarantee a well-established foraging column, workers foraged on the arena for at least 1 h before the choice assays. Thereafter, single workers were allowed to reach a small platform ( $3 \times 3$  cm) located at one side of the bridge, on which they could come into direct contact with the leaf discs of the two alternatives offered, and the collection of one was recorded as a choice. Once a single worker picked a disc up, it was removed from the subcolony on its way back to the nest and returned to the colony after the end of the tests. The intake of privet discs over the total intake of the two kinds of discs was used as an index to quantify the standardized acceptance of privet, which ranged from 0.0 to 1.0, with a value of 0.5 indicating equal acceptance of the two offered plant species. Standardized acceptance in donor colonies before and after feeding CHX-treated privet leaves was compared using the Wilcoxon test.

Leaf acceptance by foragers from the receiver subcolonies was evaluated in the same way, i.e. foragers were allowed to choose between untreated leaf discs of privet and firethorn, but choice tests were carried out 4 h before and 3 h after waste from donor subcolonies was placed into the fungus chamber. Decisions made by an average of  $19.9 \pm 0.8$  foragers per subcolony were recorded for seven subcolonies (total number of choices recorded:  $N=259$ ), and compared using standardized acceptance of privet as indicated above. Waste samples were directly collected from the previously emptied waste chambers of donor subcolonies 24 h after the feeding event, to ensure collection of freshly produced waste. Because colony odours mediate nestmate recognition in ants (e.g. Jutsum et al., 1979), and waste from donor subcolonies might be recognized as foreign material by ants from receiver subcolonies, transfer of waste was always carried out using donor and receiver subcolonies built from the same large queenright laboratory colonies (henceforth, sister subcolonies). Waste samples were offered in square dishes ( $3 \times 3 \times 0.7$  cm) placed just next to the fungus garden. Depending on the receiver subcolonies, it took workers between 2 and 3 h for the complete removal of the waste particles towards the waste chamber. If waste was not removed after 3 h, no tests were performed and the sister subcolonies were excluded from the experiment. This occurred twice out of a total of nine subcolonies. Standardized acceptance in receiver colonies before and after waste was introduced into the fungus chamber was compared using the Wilcoxon test.

### Effects of scented waste from unsuitable and suitable leaves on olfactory-based foraging choices

In the second experiment, we investigated the extent to which plant acceptance relies upon olfactory cues present in the waste particles. In particular, we asked whether olfactory cues from waste originating from unsuitable leaves will suffice to determine foraging preferences. We hypothesized that odours scenting the waste from fungicide-treated leaves should be less preferred by

foragers than those scenting the waste from untreated leaves. To that aim, receiver subcolonies were given waste samples from one of the following waste–odour combinations: waste originating from either CHX-treated leaves or untreated leaves, scented with either apple or mandarin odours (Duftöl, Germany).

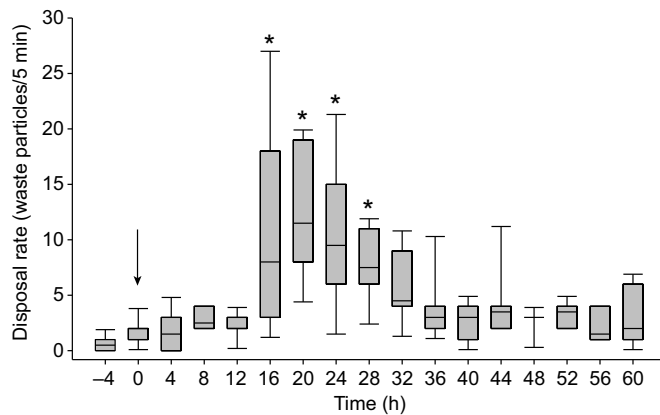
Preferences were quantified by offering foraging ants a binary choice between two sugared paper discs using a pick-up assay (Roces, 1993), each impregnated with one of the two odours. To scent the sugared paper discs, they were enclosed overnight inside a dish (diameter 9 cm) containing a cup with 2 ml of the liquid scent. Assays were performed 3 h after scented waste from donor subcolonies was introduced into the fungus chamber. When apple odour was used to scent the waste, mandarin acted as the novel alternative, and vice versa. The collection of apple- or mandarin-scented discs over the total number of discs collected was used as an index to calculate the standardized acceptance of apple- or mandarin-scented discs, respectively. Standardized acceptance of the receiver subcolonies that were given scented waste from treated or from untreated leaves was compared using Mann–Whitney *U*-tests. When apple was used to scent the waste, seven subcolonies were supplied with scented waste from untreated leaves, and an average of  $16.6 \pm 2.1$  foragers were tested per subcolony (total number of choices recorded:  $N=116$ ); another seven subcolonies received scented waste from treated leaves, and  $20.4 \pm 2.6$  foragers were tested per subcolony ( $N=143$ ). When mandarin was used to scent the waste, seven subcolonies were supplied with scented waste from untreated leaves, and  $16.0 \pm 1.5$  foragers were tested per subcolony ( $N=112$ ); another seven subcolonies received scented waste from treated leaves, and  $21.9 \pm 2.6$  foragers were tested per subcolony ( $N=153$ ).

In this experiment, waste particles from treated and untreated leaves were not obtained from the colonies' waste chambers, but produced by removing and drying pieces of fungus garden after the incorporation of the leaves. Thus, we controlled for the presence of colony debris other than the fungus garden (e.g. dead ants), which may influence the results if artificially scented. To this end, we harvested about  $10 \text{ cm}^3$  of the fungus garden 24 h after the incorporation of either untreated or treated blackberry leaves. We exposed the garden sample to room air for at least 4 h until desiccation. Once dried, the fungus was crushed into small particles that were used as waste. Waste made from a single donor subcolony was split in two samples, and each one was scented with either apple or mandarin odour. To scent the waste at saturation, waste samples of  $4 \text{ cm}^3$  were enclosed inside a dish (diameter 9 cm) containing a cup loaded with 2 ml of the liquid scent for 4 h. They were afterwards placed into the fungus chamber of two different receiver subcolonies. All three subcolonies used in a single replicate were sisters.

## RESULTS

### Dynamics of waste disposal

Waste disposal rates varied over time. Before the offering of fungicide-treated leaves and up to 12 h thereafter, the disposal rate remained constant and at low levels with less than one particle being disposed of per minute (Fig. 1). The disposal rate abruptly increased after 12 h, showing a peak 20 h after the incorporation of fungicide-treated leaves. Comparisons revealed statistical differences between the onset of the offering of treated leaves at 0 h and at 16, 20, 24 and 28 h thereafter (Wilcoxon matched pairs test: 0 versus 16 h:  $Z=1.991$ ,  $P=0.046$ ; 0 versus 20 h:  $Z=2.201$ ,  $P=0.027$ ; 0 versus 24 h:  $Z=2.022$ ,  $P=0.043$ ; 0 versus 28 h:  $Z=2.201$ ,  $P=0.027$ ;  $N=6$ ; Fig. 1). The rate of waste disposal

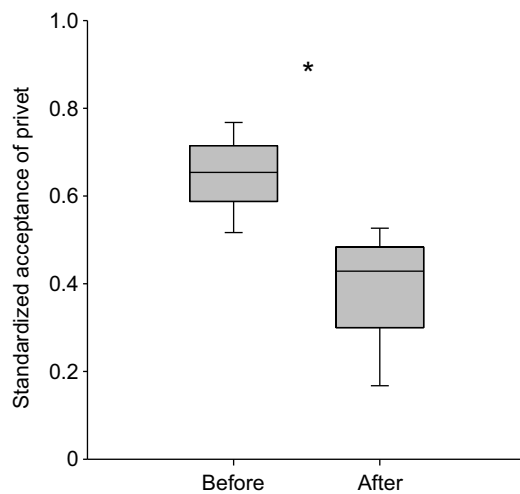


**Fig. 1. Disposal rate of waste particles before and after the colony was fed fungicide-treated leaves.** The disposal rate was calculated as the number of waste particles carried into the waste chamber per unit time. Subcolonies were fed fungicide-treated leaves 4 h after the onset of the recordings (arrow), at the time indicated as 0 h. The box plots show medians, quartiles and 5th and 95th percentiles from six subcolonies. Significant differences are reported between any particular sampling event and the event at 0 h, when fungicide-treated leaves were initially offered ( $*P < 0.05$  after Wilcoxon test).

decreased 32 h after the offering of unsuitable leaves, returning to its initial levels about 36 h later.

#### Effects of waste from unsuitable leaves on plant choice by foragers

Workers from donor subcolonies changed their foraging preferences after the subcolony incorporated fungicide-treated leaves, as expected from previous studies (Herz et al., 2008; Saverscek and Roces, 2011). Standardized acceptance of privet significantly decreased from 0.65 4 h before to 0.39 22 h after the incorporation of fungicide-treated leaves (Wilcoxon matched pairs test:  $Z = 2.366$ ,  $P = 0.017$ ,  $N = 7$ ; Fig. 2). The observed delayed avoidance of previously accepted fungicide-treated privet leaves allowed the collection of freshly produced waste particles, which probably contained cues from both the privet leaves and the damaged fungus, 24 h after the feeding event.



**Fig. 2. Acceptance of leaves by foragers from donor subcolonies.** Standardized acceptance of privet (intake of privet leaves/total intake) was recorded in individual binary choice assays 4 h before and 22 h after subcolonies were fed fungicide-treated privet leaves. The box plots show medians, quartiles and 5th and 95th percentiles from seven subcolonies. The asterisk indicates a statistical difference ( $*P < 0.05$  after Wilcoxon test).

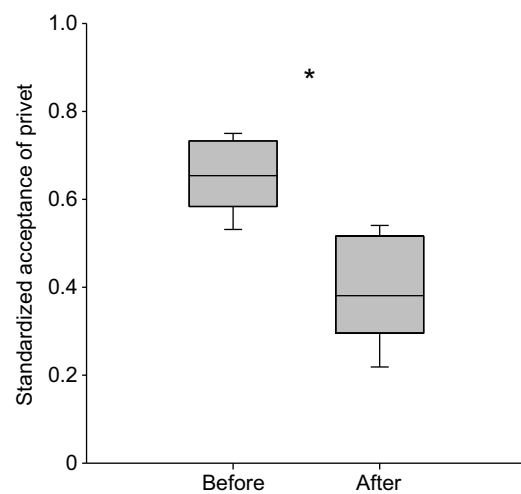
Foragers from receiver subcolonies, i.e. those that were given waste from fungicide-treated leaves, significantly changed their plant preferences after waste was introduced into the fungus chamber (Fig. 3). They slightly preferred privet to firethorn 4 h before waste was introduced into the chamber. This preference significantly changed 3 h after the addition of waste, as privet became less likely to be chosen than firethorn (Wilcoxon matched pairs test:  $Z = 2.267$ ,  $P = 0.023$ ,  $N = 7$ ; Fig. 3). These results indicate that ants learned to avoid unsuitable plants solely through the waste particles, without having experienced the plants' noxious effect on the fungus by themselves.

#### Effects of scented waste from unsuitable and suitable leaves on olfactory-based foraging choices

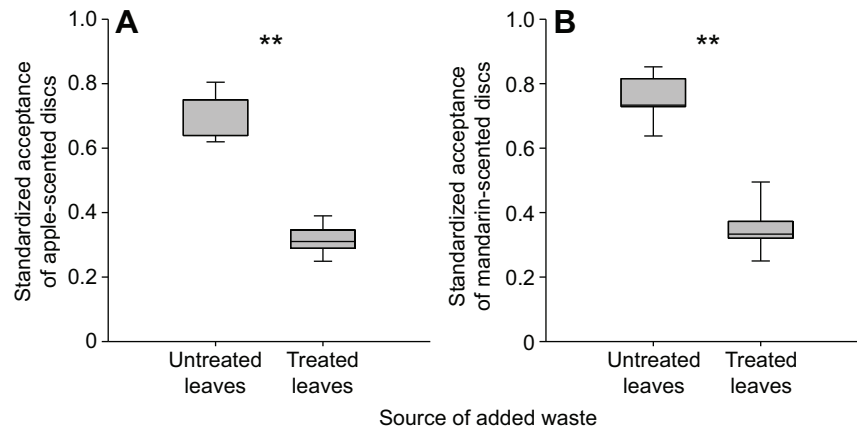
Three hours after scented waste was given to receiver subcolonies, foragers' choices were markedly different depending on the source of the scented waste. If the scented waste originated from subcolonies fed untreated leaves, foragers slightly preferred paper discs scented with the odour used to scent the waste (standardized acceptance higher than 0.5). However, this preference was significantly reduced and changed to avoidance if the scented waste originated from treated leaves (standardized acceptance lower than 0.5; Fig. 4A). The observed pattern was independent of the odour initially used to scent the waste (apple odour: Mann–Whitney  $U$ -test:  $Z = 3.130$ ,  $P = 0.001$ ,  $N = 7$ ; Fig. 4A; mandarin odour: Mann–Whitney  $U$ -test:  $Z = 3.130$ ,  $P = 0.001$ ,  $N = 7$ ; Fig. 4B).

#### DISCUSSION

How do leaf-cutting ant workers recognize which plants should be avoided during foraging? As the effects of unsuitable plants on the fungus are expected to start soon after plant fragments have been incorporated into the fungus garden, the most likely way to learn about plant suitability would be through direct contact with the impaired fungus. The present results, however, indicate that even foragers from subcolonies with an undamaged fungus garden exhibit species-specific plant avoidance if exposed to the waste produced in subcolonies fed fungicide-treated, unsuitable leaves. This suggests that information about plant unsuitability spreads from the fungus to



**Fig. 3. Acceptance of leaves by foragers from receiver subcolonies.** Standardized acceptance of privet (intake of privet leaves/total intake) was recorded in individual binary choice assays 4 h before and 3 h after subcolonies were given waste produced by donor subcolonies. The box plots show medians, quartiles and 5th and 95th percentiles from seven subcolonies. The asterisk indicates a statistical difference ( $*P < 0.05$  after Wilcoxon test).



**Fig. 4. Acceptance of scented paper discs by foragers from receiver subcolonies, measured 3 h after scented waste from untreated or treated leaves was placed into the fungus chamber.** (A) Standardized acceptance of apple-scented discs (intake of apple-scented discs/total intake) was recorded in foragers from naive subcolonies that were given apple-scented waste produced in donor subcolonies fed untreated or treated leaves. (B) Similarly, standardized acceptance of mandarin-scented discs (intake of mandarin-scented discs/total intake) was recorded in foragers from naive subcolonies that were given mandarin-scented waste produced in donor subcolonies fed untreated or treated leaves. The box plots show medians, quartiles and 5th and 95th percentiles. Asterisks indicate statistical differences (\*\* $P < 0.01$  after Mann–Whitney  $U$ -test). Twenty-eight subcolonies were used in the experiment, seven in each group.

the ants via the waste particles produced shortly after the incorporation of unsuitable plant material. We speculate that ants were able to identify plants and to recognize the state of the fungus based on both plant cues and putative fungus metabolites contingent in the waste particles. In particular, we observed that volatiles of the waste mediated the identification of host plants, as the recall of olfactory memories formed inside the nest, probably while interacting with the waste particles, was sufficient for workers to recognize and to avoid the unsuitable plant in the foraging context.

We recently showed that *A. ambiguus* leaf-cutting ants use different behavioural strategies inside and outside the nest to prevent the negative effects that leaves with fungicide compounds have on the symbiotic fungus (Arenas and Roces, 2016). Foragers, gardeners and midden workers (i.e. ants working in the waste chamber) learned to avoid plants unsuitable for the fungus at their specific worksites. Foragers avoided the collection of leaves previously experienced as unsuitable for the fungus, a phenomenon earlier reported for other leaf-cutting ant species (Ridley et al., 1996; North et al., 1999; Herz et al., 2008). Gardeners discontinued the processing of leaves previously experienced as unsuitable inside the fungus chambers. And finally, midden workers disposed of recently incorporated and even fresh leaves if they were experienced as being unsuitable for the fungus. Even though clear-cut evidence is still lacking, it is likely that gardeners could learn plant-related cues associated with the impaired fungus through direct contact with the garden, as suggested by North et al. (1999). Furthermore, foragers might also learn about plant suitability through direct interactions with the garden or even with informed nestmates (N. Saverschek, The influence of the symbiotic fungus on foraging decisions in leaf-cutting ants – Individual behavior and collective patterns, PhD Thesis, University of Würzburg, Germany, p. 127, 2010), for instance when they stay inside the fungus chambers in between their foraging trips. Our results go beyond this by showing that information about plant suitability can additionally be learned solely through interactions with waste particles.

We observed that disposal of waste drastically increased after subcolonies were fed fungicide-treated leaves. Approximately 80% of the waste particles disposed of looked like pieces of exhausted fungus garden. Although the turnover of the whole fungus garden in

leaf-cutting ant colonies has been estimated to take about 7 weeks (Weber, 1972), our measurements showed that ants started pruning and removing the fungus growing on fungicide-treated leaves much earlier. The increase in disposal activity started about 16 h after the incorporation of unsuitable leaves, which is close to the onset of the foragers' learned avoidance response in *Acromyrmex lundii*, which occurs 10 h after leaf intake (Herz et al., 2008).

The observation that workers learned about the suitability of the host plants solely through the waste suggests that cues that enable plant identification and cues or signals that inform about the state of the fungus are contingent in waste particles. Information about plant unsuitability could therefore be transferred as a by-product of the removal of inappropriate substrates and damaged fungus from impaired areas of the garden (Arenas and Roces, 2016). Furthermore, waste produced in larger quantities might increase the demands for hygienic tasks, thus influencing the distribution of workers allocated to different colony tasks, as recently described for the leaf-cutting ant *Atta sexdens* (Lacerda et al., 2013). If the demands for garden pruning and waste removal increase after the incorporation of unsuitable leaves, additional workers allocated to these tasks may have the opportunity to learn and memorize information directly through interactions with the waste particles. We speculate that foragers are likely to change tasks and participate in removal and transport of waste particles after a transient increase in waste production. In this scenario, memories established while managing the waste might influence individual decisions as soon as workers resume foraging once the demands for waste-related tasks decrease again.

Given the importance of olfactory cues for food location in social insects, it is not surprising that odours incorporated into the fungus garden strongly influenced plant acceptance. It is already known that odours of the harvested plants can serve either as an orientation cue attracting leaf-cutting ants to the host plants (Littleddyke and Cherrett, 1978; Therrien and McNeil, 1990), or as a learned cue used during decision making by foraging workers (Roces, 1990, 1994; Saverschek and Roces, 2011; Falibene et al., 2015). During a foraging process, *Acromyrmex lundii* recruits are influenced in their choices by the odour of the loads carried by successful scouts returning to the nest (Roces, 1990, 1994). Conditioning to odours of food items carried by nestmates has also been observed among

foragers of *Atta colombica* (Howard et al., 1996) and *Atta cephalotes* (Farji-Brener et al., 2010). In nectar feeders, olfactory information about the food sources is propagated during the distribution of the nectar within the colony (in honey bees: Pankiw et al., 2004; Grüter et al., 2006; in *Camponotus* ants: Provecho and Josens, 2009). In honey bees, social sharing of scented food allows other foragers and even workers not directly involved in foraging tasks to obtain information from individuals that actively participate in resource exploitation (Arenas et al., 2007, 2008). Here, we demonstrated that odours of host plants learned through the waste can also be used by foragers when making decisions outside of the nest.

In our experiments with artificially scented waste particles, standardized acceptance of sugared paper discs with the same scent as that added to waste from untreated leaves was surprisingly high, i.e. 0.71 for apple-scented waste and 0.75 for mandarin-scented waste. It is unclear whether such preferences represent an innate reaction, or a primed response resulting from exposure to an odour that scented the environment of the fungus chamber. Although this question remains to be answered, the results are consistent with the hypothesis that odours in the fungus chamber associated with waste from suitable plants may positively influence plant acceptance by workers in the foraging context.

The presence of waste inside the fungus chambers of leaf-cutting ant colonies implies a risk of infection for the fungus, i.e. the presence of fungal competitors, parasites and pathogenic microorganisms, as characterized in the colonies' waste deposits (Fisher et al., 1996; Scott et al., 2010). The transport, manipulation and disposal of refuse in the waste chamber are thought to be adaptive responses that minimize the spread of pathogens (Bot et al., 2001; Hart and Ratnieks, 2002). Besides the undesirable effect of waste, we demonstrated here that waste particles carry information that foraging ants use outside the nest when selecting plants for their fungus. We assume that foragers do not need to visit the waste chambers to learn to avoid plants through cues in the waste. Because waste particles originate inside the fungus garden and are transported through nest tunnels or trails to waste deposits, it is an open question whether learning occurs inside the fungus chamber, during waste transport, or after the final waste disposal into the dumps. We argue that different mechanisms, such as interactions with waste-carrying workers along tunnels or trails, worker–worker interactions during sequential transport of waste particles (Hart et al., 2002; and F.R., personal observations for *A. ambiguus*), and accumulation of plant-related cues in the waste chamber, might all contribute to propagate relevant information originating in the fungus chamber all across the nest. A number of questions arise from our results. For instance, can naive foragers learn to reject a plant if the information about suitability is no longer retrievable from the fungus but solely from the waste particles disposed of in the waste chamber? If yes, do foragers visit the waste chambers or receive the information via interactions with midden workers? Do waste particles accumulated in the waste chamber act as a source of information? If yes, for how long is the information about unsuitable plants available? Can newly emerged ants establish memories at the waste chamber for unsuitable plants they have never experienced on the fungus garden? What are the cues from the damaged fungus that enable avoidance learning?

Although there is a report on the opportunistic use of dry waste particles disposed of outside the nest as building materials (Farji-Brener and Tadey, 2012), research on waste management in leaf-cutting ants has so far solely focused on hygienic strategies (Bot et al., 2001; Lacerda et al., 2006; Ballari et al., 2007; Waddington

and Hughes, 2010). Our results offer a new perspective on the importance of waste as an information source, and highlight the relevance of learning and memory in the context of waste management.

#### Acknowledgements

We thank A. Falibene for fruitful comments and discussions at the early project stage and O. Geissler for logistical support for the experimental work.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

A.A. and F.R. conceived and designed the experiments. A.A. performed the experiments. A.A. performed data analysis. A.A. and F.R. drafted the manuscript.

#### Funding

This study was supported by a German Academic Exchange Service (Deutscher Akademischer Austauschdienst, DAAD) fellowship to A.A. (ref. no.: A/11/81698) and by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG, SFB 1047 TP C1).

#### Data availability

#### References

- Arenas, A., Fernández, V. M. and Farina, W. M. (2007). Floral odor learning within the hive affects honeybees' foraging decisions. *Naturwissenschaften* **94**, 218–222.
- Arenas, A., Fernández, V. and Farina, W. M. (2008). Floral scents experienced within the colony affect long-term foraging preferences in honeybees. *Apidologie* **39**, 714–722.
- Arenas, A. and Rocas, F. (2016). Gardeners and midden workers in leaf-cutting ants learn to avoid plants unsuitable for the fungus at their worksites. *Anim. Behav.* **115**, 167–174.
- Ballari, S., Farji-Brener, A. G. and Tadey, M. (2007). Waste management in the leaf-cutting ant *Acromyrmex lobicornis*: division of labour, aggressive behaviour, and location of external refuse dumps. *J. Insect Behav.* **20**, 87–98.
- Bot, A. N. M., Currie, C. R., Hart, A. G. and Boomsma, J. J. (2001). Waste management in leaf-cutting ants. *Ethol. Ecol. Evol.* **13**, 225–237.
- Camargo, R. S., Forti, L. C., de Matos, C. A. O., Lopes, J. F., de Andrade, A. P. P. and Ramos, V. M. (2003). Post-selection and return of foraged material by *Acromyrmex subterranean brunneus* (Hymenoptera: Formicidae). *Sociobiology* **42**, 93–102.
- Camazine, S., Deneubourg, J. L., Franks, N. R., Sneyd, J., Theraulaz, G. and Bonabeau, E. (2001). *Self-Organization in Biological Systems*. Princeton, N.J.: Princeton University Press.
- Cherrett, J. M. (1989). Leaf-cutting ants. In *Ecosystems of the World 14B - Tropical Rain Forest Ecosystems* (ed. H. Lieth and A. M. J. Werger), pp. 473–488. Amsterdam; Oxford; New York; Tokyo: Elsevier.
- Cherrett, J. and Seaforth, C. (1970). Phytochemical arrestants for the leaf-cutting ants, *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich), with some notes on the ants' response. *Bull. Ent. Res.* **59**, 615–625.
- Conradt, L. and Roper, T. J. (2005). Consensus decision making in animals. *Trends Ecol. Evol.* **20**, 449–456.
- Deneubourg, J. L. and Goss, S. (1989). Collective patterns and decision-making. *Ethol. Ecol. Evol.* **1**, 295–311.
- Falibene, A., Rocas, F. and 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.
- Farji-Brener, A. G. and Tadey, M. (2012). Trash to treasure: leaf-cutting ants repair nest-mound damage by recycling refuse dump materials. *Behav. Ecol.* **23**, 1195–1202.
- Farji-Brener, A. G., Amador-Vargas, S., Chinchilla, F., Escobar, S., Cabrera, S., Herrera, M. I. and Sandoval, C. (2010). Information transfer in head-on encounters between leaf-cutting ant workers: food, trail condition or orientation cues? *Anim. Behav.* **79**, 343–349.
- Fisher, P. J., Stradling, D. J., Sutton, B. C. and Petrini, L. E. (1996). Microfungi in the fungus gardens of the leaf-cutting ant *Atta cephalotes*: a preliminary study. *Mycol. Res.* **100**, 541–546.
- Fowler, H. G. and Louzada, J. (1996). Refuse production in leaf-cutting ants (Hymenoptera: Formicidae) and its relationship with leaf harvest. *Biol. Rhythm Res.* **27**, 148–152.
- Gordon, D. M. (2010). *Ant Encounters - Interaction Networks and Colony Behavior*. Princeton: Princeton University Press.
- Grüter, C., Acosta, L. E. and Farina, W. M. (2006). Propagation of olfactory information within the honeybee hive. *Behav. Ecol. Sociobiol.* **60**, 707–715.

- Hart, A. G. and Ratnieks, F. L. W. (2002). Waste management in the leaf-cutting ant *Atta colombica*. *Behav. Ecol.* **13**, 224-231.
- Hart, A. G., Anderson, C. and Ratnieks, F. L. W. (2002). Task partitioning in leaf-cutting ants. *Acta Ethol.* **5**, 1-11.
- Herz, H., Beyschlag, W. and 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. and Roces, F. (2008). Delayed rejection in a leaf-cutting ant after foraging on plants unsuitable for the symbiotic fungus. *Behav. Ecol.* **19**, 575-582.
- Howard, J. J., Henneman, M. L., Cronin, G., Fox, J. A. and Hormiga, G. (1996). Conditioning of scouts and recruits during foraging by a leaf-cutting ant, *Atta colombica*. *Anim. Behav.* **52**, 299-306.
- Hubbell, S. P., Wiemer, D. F. and Adejare, A. (1983). An antifungal terpenoid defends a neotropical tree (*Hymenaea*) 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). Part II. The internal nest structure and growth". *Z. ang. Ent.* **89**, 217-246.
- Jutsum, A. R., Saunders, T. S. and Cherrett, J. M. (1979). Intraspecific aggression in the leaf-cutting ant *Acromyrmex octospinosus*. *Anim. Behav.* **27**, 839-844.
- Lacerda, F. G., Della Lucia, T. M. C., Lima, E. R. and Campos, L. A. O. (2006). Waste management by workers of *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) in colonies supplied with different substrates. *Sociobiology* **48**, 165-173.
- Lacerda, F. G., Della Lucia, T. M. C., De Souza, O., de Souza, L. M. and de Souza, D. J. (2013). Task performance of midden workers of *Atta sexdens rubropilosa* Forel (Hymenoptera: Formicidae). *J. Insect Behav.* **26**, 873-880.
- Littledyke, M. and 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.
- North, R. D., Jackson, C. W. and 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. and 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.
- Provecho, Y. and Josens, R. (2009). Olfactory memory established during trophallaxis affects food search behaviour in ants. *J. Exp. Biol.* **212**, 3221-3227.
- Ridley, P., Howse, P. E. and 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. (1993). Both evaluation of resource quality and speed of recruited leaf-cutting ants (*Acromyrmex lundii*) depend on their motivational state. *Behav. Ecol. Sociobiol.* **33**, 183-189.
- Roces, F. (1994). Odour learning and decision-making during food collection in the leaf-cutting ant *Acromyrmex lundii*. *Insectes Soc.* **41**, 235-239.
- Roces, F. and Bollazzi, M. (2009). Information transfer and the organization of foraging in grass- and leaf-cutting ants. In "Food Exploitation by Social Insects: Ecological, Behavioral, and Theoretical Approaches" (ed. S. Jarau and M. Hrncir), pp. 261-275. Boca Raton, USA: CRC Press: Contemporary topics in entomology series.
- Saverschek, N. and Roces, F. (2011). Foraging leafcutter ants: olfactory memory underlies delayed avoidance of plants unsuitable for the symbiotic fungus. *Anim. Behav.* **82**, 453-458.
- Saverschek, N., Herz, H., Wagner, M. and Roces, F. (2010). Avoiding plants unsuitable for the symbiotic fungus: learning and long-term memory in leaf-cutting ants. *Anim. Behav.* **79**, 689-698.
- Scott, J. J., Budsberg, K. J., Suen, G., Wixon, D. L., Balsler, T. C. and Currie, C. R. (2010). Microbial community structure of leaf-cutter ant fungus gardens and refuse dumps. *PLoS ONE* **5**, e9922.
- Seeley, T. D. and Buhrman, S. C. (1999). Group decision making in swarms of honey bees. *Behav. Ecol. Sociobiol.* **45**, 19-31.
- Stahel, G. (1943). The fungus gardens of the leaf-cutting ants. *J. N. Y. Bot. Gard.* **44**, 245-253.
- Therrien, P. and McNeil, J. N. (1990). The discovery of new resources and subsequent trail formation by *Acromyrmex octospinosus* in Guadeloupe. In *Applied Myrmecology: A World Perspective* (ed. R. K. Vander Meer and K. Jaffé), pp. 373-381. Boulder, Colorado: Westview Press.
- Thiele, T., Kost, C., Roces, F. and 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.
- Waddington, S. J. and Hughes, W. O. H. (2010). Waste management in the leaf-cutting ant *Acromyrmex echinator*: the role of worker size, age and plasticity. *Behav. Ecol. Sociobiol.* **64**, 1219-1228.
- Weber, N. A. (1972). In *Gardening Ants: the Attines*, p. 146. Philadelphia: The American Philosophical Society.
- Wirth, R., Herz, H., Ryel, R., Beyschlag, W. and Hölldobler, B. (2003). Herbivory of leaf-cutting ants: a case study on *Atta colombica* in the tropical rainforest of Panama. *Springer, Berlin.* **164**, 195-233.