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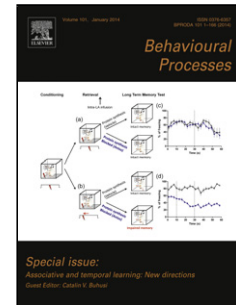
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PLANT ODORS TRIGGER CLEARING BEHAVIOR IN FORAGING TRAILS- DO THEY REPRESENT OLFACTORY OBSTACLES?

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Highlights

- Essential oils from native and exotic plants disrupted foraging behavior of leaf cutting ants *Acromyrmex lobicornis* when placed on foraging trails and triggered clearing behavior even when they did not constitute a physical impediment.
- Ant flux was reduced by 15-28% and treated pieces of paper were removed from the trail in most cases.

- Small pieces of filter paper which did not constitute a physical impediment triggered clearing behaviors in a similar fashion as physical obstacles and essential oils unless they contained trail odour.
- Ants tapped the tip of their gaster against the ground around papers treated with essential oil odour. The number of gaster tappings as well as the time delay between placement of the paper and its removal increased with plant odor concentration.
- Clearing ants were smaller than forager ants, suggesting there is caste differentiation in clearing behavior on the trail.

Abstract

Foraging trails of leaf-cutting ants may be exposed to plant material that interferes with foragers' flux either by physically blocking it or due to secondary metabolites which affect insect behavior. We hypothesized that plant secondary metabolites such as plant volatiles may interfere with pheromone communication, triggering clearing behavior. We impregnated small pieces of paper with different plant odors from native and exotic species and placed them in the middle of foraging trails of the leaf-cutting ant *Acromyrmex lobicornis*. As a control, we used papers impregnated with trail odor. The paper used as substrate for the odors did not constitute a physical obstacle based on its small surface area. Papers treated with trail odor did not interfere with ant flux and were not removed from the

trail. However, when papers were treated with plant odors, they were removed from the trail in most of the cases and ant flux was reduced significantly by 15-28%. We found that ants tapped the tip of their gaster against the ground around the treated papers only when they were impregnated with foreign odors. The number of gaster tappings as well as the time between the placement of the paper and its removal increased with plant odor concentration. However, the decision to remove the paper was not correlated with the number of gaster tappings. Interestingly, clearer ants were smaller than forager ants, suggesting there is morphological differentiation in clearing behavior of the trail. Results from the current study also suggest that odors trigger clearing behavior on foraging trails and affect trail marking behavior. Our results provide information about the potential for plant compounds to constitute obstacles, even when they do not physically obstruct the trail. We conclude that odors may trigger clearing behavior by interfering with pheromone communication.

Key words: *Acromyrmex lobicornis*, clearing behavior, communication, interference, plant secondary metabolites, plant volatiles.

1. Introduction

The ecological success of ants is founded on cooperative behavior and an efficient communication system involving visual and chemical signals (Hölldobler and Wilson, 1990). The use of chemical signals allow ants to gather information about the quality of resources (Hubbell et al., 1984, Littleddyke and Cherrett, 1978), to distinguish nest-mates (Lenoir et al., 1999), as well as to communicate fast and effectively about potential dangers, triggering a defense response (Detrain and Deneubourg, 1997; Lalor and Hughes, 2011; Pearce-Duvert and Feener Jr., 2010). One of the most elaborate forms of chemical communication involves the use of trail pheromones, used by many species of ants to guide workers, regulate colony foraging, and recruit nest-mates (Czaczkes et al., 2015).

Most ant species rely on pheromone trails to find and exploit resources (Hölldobler and Wilson, 1990, Czaczkes et al., 2015), and a number of ant species build trails cleared of debris and plant material. For example, species of genus *Atta*, *Formica*, *Lasius*, *Pogonomyrmex*, *Messor*, *Camponotus*, *Iridomyrmex*, and *Acromyrmex* have been reported to build and maintain cleared trails (Acosta et al., 1993, Gamboa, 1975, Greaves and Hughes, 1974, Hölldobler and Wilson, 1990, Plowes et al., 2013, Wetterer, 1995). The most prominent and well-studied species among trail clearers are the leaf-cutting ants (*Atta* and *Acromyrmex*). Their trail system may include up to seven individual trails and each trail usually extends for more than 100 meters (Lugo et al., 1973, Shepherd, 1982). Workers travel along these from the nest to feeding sites to retrieve plant material as substrate for their symbiotic fungus (Hölldobler and Wilson, 1990). The trail system of leaf-cutting ants includes permanent trunk trails which may be used for months or even years as well as ephemeral trails which branch from them (Kost et al., 2005).

There are several advantages to building and maintaining cleared trails, well marked and free of debris. Trails lead foragers to known resources, reducing traveling time and increasing the resource discovery rate (Rockwood and Hubbell, 1987, Shepherd, 1982). In addition, trails allow ants to transfer leaf resources (Bruce and Burd, 2012, Röschard and Roces, 2011, 2003), to recruit defenders (Powell and Clark, 2004) and to exchange information (Bollazzi & Roces, 2011, Farji-Brener et al., 2010). However, maintaining a large and permanent trail system free of debris requires time and energy. For example, a single colony of *Atta colombica* can maintain up to 2730 m of trails each year (Howard, 2001), for which they need to remove different types of plant material (leaves, flowers and twigs) that naturally fall over the trails (estimate of annual rates of leaf fall range between 441 and 1050 g/m²) (Haines and Foster, 1977, Howard, 2001, Sampaio et al., 1993).

Costs and benefits of leaf-cutting ants trail construction and maintenance have been recently studied by several authors who have investigated the factors that determine ants to clear trails (Alma et al., 2019, Bochynek et al., 2017, Bruce et al., 2017, Cevallos Dupuis and Harrison, 2016, Farji-Brener et al., 2015). For example, for *Atta colombica* colonies, trail clearing required around 11,000 ant-days of effort (i.e. an annual energetic cost approximately equivalent to the intake of 8000 leaf fragments), which is relatively inexpensive (Howard, 2001) considering that colonies often field more than 10,000 foragers at a given time (Bruce and Burd, 2012). Conversely, Bochynek et al (2017) argued that this study has overlooked the cost of providing a standby clearing workforce, or distinct caste, which responds to sudden and unpredictable obstructions (Bochynek et al., 2017). However, the mentioned studies have ignored the potential for chemical compounds such as plant odors to become an obstacle. Plant material that falls on the foraging trails may physically block ant flux but also contain chemicals (e.g. secondary metabolites) that may interfere with social

communication. Some studies have shown that certain volatile compounds can interfere with pheromone communication by negatively affecting ants and modifying their behavior. For example, trail following behavior of the Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae), can be disrupted by a high concentration of (*Z*) 9-hexadecenal, a compound which was found to be attractive to ants in certain situations, although it is not part of the pheromone system (Choe et al., 2012, Suckling et al., 2010, Sunamura et al., 2011). In addition a plant secondary metabolite from leaves of *Eucalyptus maculate* Hook (Myrtaceae), β -eudesmol, interferes with kin recognition, inducing aggressiveness towards nest-mates in *Atta laevigata* and *A. sexdens rubropilosa* ants (Marinho et al., 2005).

We hypothesized that plant volatile compounds may interfere with pheromone communication when present on foraging trails, and that when plant material falls onto foraging trails, the decision to remove it or not will be in part related to its chemical composition. To test this, we measured the effect of plant odors on ant traffic and clearing behavior. We evaluated several essential oils derived from plant species occurring in the foraging area of the leaf-cutting ant *Acromyrmex lobicornis* and which are known to be collected by ants. Specifically, our aims were to determine whether plant compounds interfere with ant flow on the foraging trail. Based on perceptual differences in trail-following by leaf-cutting ants related to body size (Kleineidam et al., 2007), we also investigated the size of ants manipulating the essential oils placed on the trails. Our results provide basic information about the potential for plant odors to be perceived as obstacles when present on the foraging trails, and to trigger clearing behavior.

2. Materials and Methods

2.1. Study species

We worked with the leaf-cutting ant *Acromyrmex lobicornis*. It has a trail system of up to 7 individual trails, 5 cm in width and 50 m in length (Alma et al., 2017). Sampling was carried out during the summer of 2017 and 2019 in Dina Huapi, Río Negro, Argentina (41°04'S y 71°09'O) in an herbaceous/shrub steppe vegetation. The dominant vegetation of the foraging area of *Acromyrmex lobicornis* in the study area is a mix of native species typical of Patagonian steppes (e.g. *Stipa speciosa*, *Mulinum spinosum*, *Imperata condensata*, *Dysphania ambrosioides*, *Fabiana imbricata* and *Baccharis pingraea*), and exotic species (e.g. *Bromus tectorum*, *Marrubium vulgare*, *Carduus nutans*, *Verbascum thapsus* and *Pseudotsuga menziesii*) (supplementary material, Fig. S1) (Franzel and Farji-Brener, 2000). We worked with 29 nests of similar size, based on visual estimation of dome size, which ranged from 60 to 80 cm in diameter and 30 to 40 cm in height. At each nest, we worked on trails of 6 ± 1.77 cm in width (mean \pm SD).

2.2. Essential oils

We worked with the essential oils derived from *Fabiana imbricata* (hereafter pichi) and *Dysphania ambrosioides* (hereafter paico) both native plants, and *Pinus ponderosa* (hereafter pine) and *Pseudotsuga menziesii* (hereafter oregon) both exotic plants in Patagonia. These plants are present in the foraging area of *A. lobicornis* and are known to be foraged upon by them (Franzel and Farji-Brener, 2000, Pérez et al., 2011). Thus, the ants might encounter these odors either when foraging or through contact with leaves, twigs or flowers which might fall on foraging trails. Considering that along the distribution range of *A. lobicornis* plants may contain between 0.7 and 18% of essential oils in their leaves (Laciar

et al., 2009, Lima et al., 2011, Soberón et al., 2007, Zavala and Ravetta, 2002), we diluted the essential oils with dichloromethane at 0.1 and 1%. Pichi, pine and oregon essential oils were obtained from Hierbas Patagonica SRL (Esquel, Chubut, Argentina). Paico essential oil was obtained from Esquel, Universidad San Juan Bosco, Chubut, as described by (Yossen et al., 2019). Biochemical characteristics are explained in the supplementary material Table S1.

2.3. Bioassays

To evaluate whether odors trigger clearing behavior, we used small semicircular pieces (2.5 mm in radius) of filter paper (1) impregnated with trail odor; (2) untreated; (3) treated with the solvent used to dilute the plant compounds tested (i.e. dichloromethane); and (4) treated with the plant compounds. The size of the papers used ensured that they did not constitute a physical obstacle for ants (i.e. pieces block physically ~ 4% of trail width, considering their radius and trail width of 6 cm; Fig. 1). To impregnate papers with trail odor, we placed a filter paper (5 × 8 cm) at the center of foraging trails and allowed ants to walk over it for 24 hours. After 24 h, the filter paper was cut to a semicircle of 2.5 mm radius and immediately placed in the middle of foraging trails. Each event consisted of placing one piece of treated paper in the middle of a foraging trail and observing the behavior of trail ants. We used different trails or trail sectors from 29 selected nests, sequentially and randomly at intervals of one hour among them obtaining a total of 262 events as detailed in Table 1. From the selected nests 14 of them were assigned all treatments, while the rest of the nests were only assigned some of the treatments, totaling 20 or 22 nests per treatment. Each event lasted until the ants deposited the removed filter papers on the ground or after a maximum of 30 minutes had passed if there was no removal.

To determine any potential interference with ant flux on the trail, we measured ant traffic on the trail for one minute before and immediately after placing the piece of paper (ant flux before and after, respectively). To measure ant traffic, we counted the number of ants (loaded and unloaded, inbound and outbound) crossing a given point on the trail during 1 min. With these data we estimated a proportional ant flux change (PAFC) due to papers presence as:

$$PAFC = \frac{(Ant\ flux\ before - Ant\ flux\ after)}{Ant\ flux\ before} \quad (1)$$

PAFC equal to 0 corresponds to no change in ant flux after we placed the piece of paper, higher than 0 there was a reduction, and lower than 0 indicated an increment. We also recorded whether ants removed papers or not, time of removal initiation after paper placement (removal delay), time spent manipulating the papers once they started removing (handling time), distance between the place where ants left the removed papers and the trail (removal distance) and if the papers were removed in direction toward or away from the nest. We observed that workers tapped the tip of their gaster against the ground, so we measured the number of times we observed this behavior in 8 nests at least (trail odor in 8 nests, untreated in 11 nests, solvent in 10 nests, pichi 0.1% in 13 nests, pichi 1% in 14 nests, paico 0.1% in 10 nests, paico 1% in 11 nests, pine 0.1% in 14 nests, pine 1% in 13 nests, oregon 0.1% in 15 nests and oregon 1% in 14 nests) (Video 1). Finally, 93 clearing ants from 14 nests were collected and measured to determine whether there was a difference in the size of foragers versus clearers (removers). We measured their body length from the head to the tip of the gaster in a stereoscopic microscope and compared it with the size of foragers obtained from the same nests as those from which we collected the clearers (34 forager ants).

2.4. Data analysis

To evaluate whether there was an ant flux change induced by treatments and there were differences in ant flux change among treatments, we compared the proportional ant flux change (PAFC) with a linear mixed model (LMM), where the response variable was the PAFC with normal distribution, the predictive variable was the treatment (as a categorical variable) and nest as random factor (we had repeated measurements in each nest). To accomplish homogeneity assumptions, we used a variance structure for treatments (varIdent function). We performed two different post-tests. On one hand, we compared the PAFC of each treatment level against an intercept of 0 by using the test() function of the emmeans package (and setting null=0). On the other hand, we compared the PAFC among treatments using the same package. To evaluate treatment effects on removal decision, we used a generalized linear mixed models (GLMM) where the response variable was the removal decision as a binary variable (1 when ants removed papers and 0 when they left them on the trail) with binomial distribution, the predictive variable was the treatment and the random factor as the nest. The removal delay, the handling time, and the removal distance were compared among treatments with a LMM with normal distribution, treatment as fixed factor and nest as random factor. These variables were log- transformed to meet the assumption of normal distribution. We corrected the significance level with a Bonferroni correction for multiple comparisons (i.e. $\alpha/11 = 0.004$). To evaluate the effect of treatment concentration (i.e. essential oils at 0, 0.1 and 1%) on the number of times ants tapped their gaster, we compared treatments with a GLMM with negative binomial distribution and treatment as fixed factor (essential oils at 0, 0.1 and 1%). We did not include the trail odor treatment in this analysis because ants never tapped their gaster around it. We also analyzed the effect of

the number of gaster tappings on the removal decision with a GLMM, where the response variable was the removal decision with binomial distribution, the predictive variable was the number of gaster tappings and the random factor was the nest. We analyzed the effect of the number of gaster tappings on removal delay with a LMM where the response variable was the removal delay log-transformed to meet the assumption of normal distribution, the predictive variables were the number of gaster tappings and the treatments and the random factor was the nest. When necessary, normal and homoscedasticity assumptions were tested graphically by qqplot function and plotting residuals against fitted values. Finally, we compared the distributions of body length for clearers and foragers with a Kolmogorov-Smirnov test. Analyses were performed in the R version 3.5.0 (R Development Core Team, 2013) with packages nlme (Pinheiro et al., 2017) and lme4 (Bates et al., 2015). Models were tested with a likelihood ratio test.

3. Results

We demonstrated that the pieces of paper used did not constitute a physical obstacle, based on the fact that they did not reduce ant flux (comparisons of PAFC each treatment level against an intercept of 0, Bonferroni correction: trail odor: $t = 0.54$, d.f. = 28, $P = 1.00$, untreated: $t = 0.23$, d.f. = 28, $P = 1.00$; solvent: $t = 2.22$, d.f. = 28, $P = 0.38$; Fig 2A). On the contrary, plant odor treatments reduced ant flux significantly (pichi 0.1%: $t = 7.30$, d.f. = 28, $P < 0.0001$; pichi 1%: $t = 4.59$, d.f. = 28, $P = 0.0009$; paico 0.1%: $t = 3.74$, d.f. = 28, $P = 0.009$; paico 1%: $t = 6.86$, d.f. = 28, $P < 0.0001$; pine 0.1%: $t = 5.63$, d.f. = 28, $P = 0.0001$; pine 1%: $t = 4.12$, d.f. = 28, $P = 0.003$; oregon 0.1%: $t = 4.70$, d.f. = 28, $P = 0.0007$; and with oregon 1%: $t = 6.08$, d.f. = 28, $P < 0.0001$). Comparison of the ant flux change among treatments (calculated using formula 1) showed that there were no differences in flux change

between untreated, solvent and trail odor treatments. However, plant odors induced a greater reduction in ant flux than treatment with trail odor (GLMM, $\chi^2 = 164.23$, d.f. = 10, $P < 0.0001$, $N = 262$; comparisons between trail odor and pichi 0.1%: $t = 6.50$, $p < 0.0001$, pichi 1%: $t = 4.11$, $p = 0.003$, paico 0.1%: $t = 3.43$, $p = 0.03$, paico 1%: $t = 6.12$, $p < 0.0001$, pine 0.1%: $t = 5.11$, $p < 0.0001$, pine 1%: $t = 3.50$, $p = 0.02$, oregon 0.1%: $t = 3.98$, $p = 0.004$, and oregon 1%: $t = 5.31$, $p < 0.0001$; Fig. 2A and Table S2).

Treatments affected the removal behavior of ants (GLMM, $\chi^2 = 31.22$, d.f. = 10, $P = 0.0005$, $N = 262$; Fig. 2B and Table S3). Papers impregnated with trail odors were not removed (one removal event out of 28 observations), while the control papers (untreated and treated with solvent) induced removal in many cases, in a similar way as the essential oil treatments. Ants removed untreated papers and those treated with solvent in 57% and 73% of the cases, respectively, while papers impregnated with plant odors were removed in 75-95% of the cases. Interestingly, removals were always performed by one worker.

For those papers that were removed, the time between treatment placement and removal was similar among treatments except for those papers treated with pichi at 0.1% which were removed faster than untreated papers (LMM, $\chi^2 = 20.89$, d.f. = 9, $p = 0.01$, $N = 185$; Fig. 3A and Table S4). Handling time was also similar among treatments (GLMM, $\chi^2 = 15.02$, d.f. = 9, $p = 0.09$, $N = 185$; Fig. 3B). Once an ant removed a filter paper from the trail, it placed it on the ground outside of the trail, at a short distance from it. The distance from the trail at which those obstacles were placed after removal, did not vary among treatments (GLMM, $\chi^2 = 11.63$, d.f. = 9, $p = 0.24$, $N = 185$; Fig. 3C).

With regards to the direction in which the obstacles were removed, ants typically carried the obstacles in a direction which was opposite to the nest entrance before placing them on the ground. This was the case both for untreated (70%) as well as treated papers (73-94%) placed on the trails.

We observed that ants tap the tip of their gaster against the ground around all the papers, including untreated ones, but not around papers impregnated with trail odor (Fig. 4A). Furthermore, the highest essential oil concentrations increased by 2.56 times the number of gaster tappings than the lowest concentrations (GLMM, $\chi^2 = 9.81$, d.f. = 1, $p = 0.002$, $N = 114$). There was no relation between the number of tappings and the decision to remove (GLMM, $\chi^2 = 0.89$, d.f. = 1, $p = 0.40$, $N = 133$), but we found a positive relation between the number of tappings and the removal delay: ants took longer to remove pieces of paper that were marked more (LMM, $\chi^2 = 6.71$, d.f. = 1, $p = 0.009$, $N = 133$; Fig. 4B).

Finally, clearer ants were smaller than forager ants (Kolmogorov-Smirnov test, $D = 0.78$, $p < 0.0001$, $N_{\text{foragers}} = 34$, $N_{\text{clearer}} = 93$; Fig. 5). Forager ants had a mean body length of 5.77 ± 0.39 mm while clearer ants had a mean of 4.38 ± 0.99 mm (mean \pm SD).

4. Discussion

This is the first study demonstrating that odors interfere with trail maintenance behavior, even when they do not represent a physical obstacle. Previous studies on foraging behavior with leaf-cutting ants had demonstrated that unsuitable plant resources are removed from the foraging trails (Roces, 1994, 1990, Saverschek et al., 2010, Saverschek and Roces, 2011). We observed that essential oils from plants that are typically used as food resources by ants triggered clearing behavior and were removed from the trail in the same way as obstacles.

Thus, we showed that odors alone, even when they are not part of a potentially unsuitable plant resource, and which do not constitute a physical obstacle either, were removed from the trail unless they were impregnated with the trail odor. Interestingly, we observed a similar behavior towards the odors from native and exotic plants.

We observed two different behaviors in relation to the obstacles we placed on the trails. Firstly, ants removed small pieces of paper treated with essential odor from the trail in a direction away from the nest entrance, as observed by Cevallos et al. (2016) for obstacles consisting of leaf fragments. Secondly, they tapped their gaster on the ground around those pieces of paper. In leaf-cutting ants as in other myrmicine ants, the venom gland is the source of pheromone trails (Morgan, 2009), and workers bring the tip of the gaster into contact with the ground to deposit pheromones. Thus, we might interpret this behavior as an indication of marking with trail pheromone. In agreement with our hypothesis that plant odor might interfere with trail communication, we found that ants tapped the tip of their gaster against the ground around all the pieces of paper we placed on the trail, except for those impregnated with trail odors. The highest concentration of essential oil elicited more gaster tappings. Moreover, those papers around which ants tapped the tip of the gaster more frequently remained on the trails longer than those with fewer tappings. Assuming this species lays pheromones when its ants tap their gaster, a plausible explanation for these results may be that ants mark the obstacles with trail pheromone to mask any foreign odors, strengthen the trail pheromone, and keep a clear trail that is easier to follow. In turn, this marking would make the papers less disruptive for forager ants, as it would not impact traffic, and also less obvious as an obstacle in need to be removed.

In addition, our results showed that pieces of paper treated with essential oils, reduced ant flux in similar ways as physical obstacles (Alma et al., 2019). Control pieces of paper (untreated and treated with solvent) and those treated with the trail odor did not reduce ant flux, while those treated with plant essential oils had a cost in terms of ant traffic reduction, which was not related to the size of the obstacle but to the odor. Even though pieces of paper in the current study blocked only ~ 4% of trail width, when they were impregnated with plant compounds they reduced ant flux in the same proportion as leaves blocking trails by 50% (Alma et al., 2019). A reduction in ant flux might be caused by a reduction in the number of ants on the trail or a reduction in the speed of ants on the trail. Considering that we measured the ant flux just before placing the filter papers and that there was no flux reduction in the control, the most plausible explanation is that the odors tested reduced the speed of ants. In accordance, we observed foraging ants stopped when first approaching the treated papers. Therefore, if not removed from the trail, plant compounds may decrease the foraging rate of ants consequently affecting colony fitness.

Trail maintenance is vital for leaf-cutting ants since trails connect the nest with resources. Therefore, they invest workforce, time and energy in maintaining their trails free of debris. Several studies have focused on the costs and benefits of physical obstacles and how ants maintain their trail system (Bochynek et al., 2017, Farji-Brener et al., 2015, Howard, 2001). For example, in *Atta* spp. leaf-cutting ants, leaf fragments in trails were considered as physical obstacles because they reduced ant flux, and the decision of removing the obstacle was correlated to the reduction in traffic, which in turn was correlated with obstacle size (Alma et al., 2019, Bruce et al., 2017). However, this is the first study showing that plant compounds might interfere with trail following behavior affecting the clearing

behavior of ants. We found that the only treatment that did not elicit removal was the trail odor, suggesting that foreign odors determine the removal decision possibly due to interference with trail odors (e.g. trail pheromones) or because they are recognized as foreign to the trail. Moreover, untreated filter papers were removed from the trail in 57% of the cases even when they did not reduce ant flux. Thus, traffic reduction would not be the only variable affecting decision making of clearing a trail. When a leaf, twig or flower fall over the trail, ants should decide whether to remove it or not according to their size (Alma et al., 2019), but also to their chemical composition.

We found that clearer ants were smaller than forager ants by ~ 32%. Very small ants (< 5.5 mm) did not deliver resources to the nest but cleared trails. Division of labor on foraging trails has been previously observed, where smaller ants present on the trails that are not carrying leaves are assumed to engage in trail maintenance (Evison et al., 2008, Lugo et al., 1973). Although a previous study found that larger ants of *Atta colombica* were primarily engaged in trail clearing (Howard, 2001), the study included only three nests. Here, we sampled more than 20 nests and found similar results to those obtained for *Atta sexdens* (Alma et al., 2019) where clearer ants were smaller than foragers by ~ 25%. On the other hand, Cevallos et al. (2016) observed all sizes of ants to be involved in obstacle removal in *Atta cephalotes*. These discrepancies suggest that morphological caste differentiation is variable among species of leaf-cutting ants. Large workers are assumed to detect trail odor at lower concentration than small workers because they have a larger number of sensilla and olfactory receptor neurons on their antennae (Kleineidam et al., 2007). Hence, the interference of trail pheromones with a plant's odor would have a greater impact on smaller ants who then decide to remove the papers. Future studies should evaluate mechanisms

explaining why small ants accomplish the clearing task and if this is a consequence of their chemical sensitivity.

This work provides novel information about how plant material present in foraging trails may disrupt foraging, delaying the input of plant material into the nest, irrespective of physical characteristics of size and shape. Given that pieces of paper containing trail odor did not reduce ant flux and were not removed from the trail, the mechanism by which ants decide to remove obstacles may be related to the odor as well as to the size of the objects. We demonstrated that even small objects that do not represent physical obstacles for walking ants are removed unless they are impregnated with the trail odor. This may be the reason why ants remove small obstacles that not necessarily obstruct ant flux. Thus, odors alone appear to trigger clearing behavior on the trails. Additional studies could analyze in more detail how size and odors of obstacles interact and shape trail maintenance. Also, future studies should determine the role of gaster tapping, and identify any pheromones released to understand the mechanisms behind this behavior. The role of plant secondary metabolites in plant insect interactions is typically studied in host search and foraging contexts. Our work suggests that these plant compounds are also used as cues, in conjunction with physical characteristics, to assess the state of foraging trails and to make decisions as to trail maintenance.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

AUTHOR CONTRIBUTION

AMA and MB conceived the idea, designed the sample, took the data and analyzed them.

Both authors participated in writing the manuscript.

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FIGURE LEGENDS

Fig 1. Photo showing a piece of filter paper placed in a foraging trail.

Arrow indicate the position and size of the paper piece in relation to the trail. Credit photo: AMA.

Fig 2. Effect of treatments on ant flux and removal decision.

(A) Proportional ant flux change (mean \pm SE) and (B) percentage of events of removal and non-removal for papers impregnated with the trail odor, untreated, treated with solvent alone, pichi, paico, pine and oregon at 0.1 and 1%. Different letters indicate significant differences among treatments ($\alpha = 0.004$).

Fig 3. Removal behavior of ants related to treatments.

(A) Removal delay, (B) handling time and (C) removal distance from the trail (mean \pm SE) for untreated papers, papers treated with solvent alone or impregnated with plant odor at 0.1% and 1%. Different letters indicate significant differences among treatments ($\alpha = 0.004$).

Figure 4. Number of gaster tappings.

(A) Number of gaster tappings per treatment (trail odor, solvent, essential oils at 0.1% and 1%) and (B) relationship between removal delay and number of gaster tappings. Dotted lines indicate upper and lower confident intervals for the LMM (LMM, $\chi^2 = 10.23$, d.f. = 1, $p < 0.001$)

Figure 5. Morphological difference between clearer and forager ants.

Absolute frequency of ant body length (mm) for cleaner and forager ants (Kolmogorov-Smirnov test, $D = 0.89$, $P < 0.0001$). Different letters indicate significant differences among treatments ($\alpha = 0.05$).

Table 1. Detail of the number of events per treatment and nests sampled. Each event consisted of placing one piece of treated paper in the middle of a foraging trail and observing the response of foraging ants. We used different trails or trail sectors from a total of 29 nests.

Treatment	Number of events observed	Number of nests observed
Trail odor	28	20
Untreated	40	22
Solvent	30	20
Pichi 0.1%	20	20
Pichi 1%	20	20
Paico 0.1%	21	20
Paico 1%	22	20
Pine 0.1%	20	20
Pine 1%	21	20
Total	262	29