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ORIGINAL PAPER

Waste deposition in leaf-cutting ants is guided by olfactory cues from waste

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Abstract
Social insects often use olfactory cues from their environment to coordinate colony tasks. We investigated whether leaf-cutting ants use volatiles as cues to guide the deposition of their copious amounts of colony refuse. In the laboratory, we quantified the relocation of a small pile of colony waste by workers of *Atta laevigata* towards volatiles offered at each side of the pile as a binary choice, consisting of either waste volatiles, fungus volatiles, or no volatiles. Fungus volatiles alone did not evoke relocation of waste. Waste volatiles alone, by contrast, led to a strong relocation of waste particles towards them. When fungus and waste volatiles were tested against each other, waste particles were also relocated towards waste volatiles, and in a high percentage of assays completely moved away from the source of fungus volatiles as compared to the previous series. We suggest that deposition and accumulation of large amounts of refuse in single external heaps or a few huge underground waste chambers of *Atta* nests is due to both olfactory preferences and stigmergic responses towards waste volatiles by waste-carrying workers.

Keywords Volatiles · Waste chamber · Stigmergy · Nest hygiene · Orientation · *Atta laevigata*

Introduction

Animals often use odors as orientation cues, and insects in particular possess an extraordinarily developed olfactory system (Sachse and Krieger 2011). In social insects, olfactory cues help to coordinate the behavior of thousands or even millions of colony members, for example, during foraging (Roces 1990; Arenas et al. 2007; Provecho and Josens 2009; Arenas and Roces 2018).

Living in large social groups poses challenges, such as higher sanitary risks (Côté and Poulin 1995; Rifkin et al. 2012) and management of large amounts of waste. In social insects, waste management is a response that ensures nest hygiene and controls the spread of pathogens originating from the waste (Wilson-Rich et al. 2009). Waste management in leaf-cutting ants (LCAs) supports not only the health of the individual (Bot et al. 2001), but also the survival of a fungal symbiont threatened by its own specific pathogens (Currie et al. 1999).

In a LCA colony, most waste originates from the fungus chamber. Here, workers weed and groom the fungus garden, i.e., pick-up exhausted substrate and pathogen-infected fungus (Currie and Stuart 2001), and relocate large quantities of waste to aboveground heaps, as in most *Acromyrmex* and a few *Atta* species, or to voluminous underground chambers as in most *Atta* species (Stahel and Geijskes 1939; Jonkman 1980; Hart and Ratnieks 2002; Bollazzi et al. 2012; Farji-Brener et al. 2016). These are usually excavated in deep soil layers below the fungus-garden zone. *Atta* workers use environmental cues to decide where to deposit waste particles and establish waste chambers, such as low air humidity (Ribeiro and Navas 2007), temperature, but not underground CO_2 levels (unpublished results). LCA are highly sensitive to odorants (Andryszak et al. 1990; Kleineidam et al. 2007; Kelber et al. 2009), and have been shown to recognize their waste based on chemosensory signals (Jaffé 1982). We asked whether LCAs use volatiles as spatial cues that guide waste accumulation inside the nest. We therefore investigated the relocation of a small waste pile towards volatiles originating from either the

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colony waste or the fungus, offered to workers as a binary choice in a laboratory setup, to draw conclusions about the involvement of olfactory responses during waste management.

Methods

The experiments were performed with two mature laboratory colonies of *Atta laevigata* (from Botucatu, Brazil), which deposit their waste in underground waste chambers (personal observations). Colonies consisted of 15 boxes (19 × 19 × 9 cm) filled with fungus gardens, and containers for feeding and colony waste. They were reared in a climate chamber under a 12:12 L/D cycle, 25 °C, and 50% air humidity, and supplied ad libitum with *Rubus fruticosus* leaves, diluted honey, and water.

The rationale of the experimental design was to offer fungus and waste volatiles side by side in one small chamber containing a waste heap above the two volatile sources, and quantify the relocation of waste particles towards them. The small dimensions of the experimental setup, as described below, allowed workers to perceive the different volatiles over short distances without leaving the arena. For each assay, a box containing a fungus garden was disconnected from the main colony and connected to the experimental setup (via a rubber tube, length 20 cm, diameter 2 cm; Fig. 1a). The setup consisted of an experimental tower with three levels separated by a fine plastic mesh (clear plastic, diameter 7 cm, height of each level, from top to bottom, 3, 2, and 2 cm). The upper level was the experimental arena (Fig. 1b), closed at the top by a glass plate, and acted as a passive two-field olfactometer. A line drawn on the mesh floor divided the arena visually into two equal semicircles. With the help of a metal ring (diameter 2 cm), 0.5 g of colony waste was placed in the center of the arena as a heap with a circular base, so that an equal amount of waste was located above each semicircular container (Fig. 1c and d; middle level) that contained the odor cues. The middle level of the tower was divided by a plastic wall to form these two semicircular containers, which either remained empty or contained colony waste or fungus as volatile sources. Fresh colony waste (identifiable by its lighter brown color) was collected from the top of the colony's waste pile and used as source for waste volatiles. Fresh fungus from the top of a fungus garden was collected as source for fungus volatiles. Fungus and waste (2 g each) were cleared of workers or brood before being filled into the containers. The mesh allowed for the perception of waste or fungus volatiles in the experimental arena. Should the clearing of fungus and waste have resulted in any disturbance and consequently a release of alarm pheromones by the ants, these should have vanished by the time the assays were started, approximately 1 h later. The ground level of the arena, also composed of two semicircular containers, contained water to humidify the setup and prevent the desiccation of the symbiotic fungus. The separated containers were needed to prevent mixing of the water sources, which may contain very small waste or fungus particles that passed through the mesh from the upper level.

Each assay was initiated by opening a sliding gate between the fungus-garden box and the experimental arena. Ants immediately entered the arena and had 4 h to relocate waste particles from the pile and to deposit additional waste brought from the fungus garden, which also occasionally occurred. The observed behaviors clearly indicate that ants were in the context of waste management, and no avoidance behaviors towards waste or its volatiles were observed as reported for workers in other contexts (Ballari and Farji-Brener 2006). Afterwards, the assay was ended and all workers were removed from the arena. As the waste was usually piled in a single heap, we separated the amount present on each side of the arena with the help of a metal spatula, bisecting the heap at the dividing line drawn on the arena floor. The amount of waste on each side was then collected, dried for 24 h at 50 °C, and weighed to the nearest 0.1 mg. The fungus-garden box was reconnected to the main colony. Each box was only used once in a given experimental series.

Four series were performed: (I) control—no odor cues on both sides (empty containers); (II) fungus volatiles vs. no odor cue; (III) waste volatiles vs. no odor cue; and (IV) waste volatiles vs. fungus volatiles. Each series consisted of 24 replicates, and the sides of odor cues were alternated between them to control for side biases.

Normality of the data was analyzed using the Shapiro-Wilk test. A Wilcoxon matched-pair test was used to evaluate differences in waste deposition between both sides of the arena (raw data, supplementary material, Data File S1).

Results and discussion

Overall, the amount of waste on each arena side did not differ in the control series without odor cues (Fig. 2a; $T = 143$, $Z = 0.2$, $n = 24$, $P = 0.84$). However, relocation of the waste did occur in 79.2% of all control assays. In 57.9% of these, there was a preference towards the left side, and in 42.1% towards the right side of the arena. In a single trial, a preference towards one side was defined as more than 60% of the waste heap being located on that side.

In the second and third series, a single volatile source, either fungus or waste, was tested against no odor. When fungus volatiles were presented against no odors (series II), the location of the waste pile was independent of the presence of fungus volatiles (Fig. 2b; $T = 114$, $Z = 1.03$, $n = 24$, $P = 0.3$). However, a particular arena side was preferred in 79.2% of the assays. In 52.6% of these, more waste was present at the side without odors, and in 47.4% at the side with

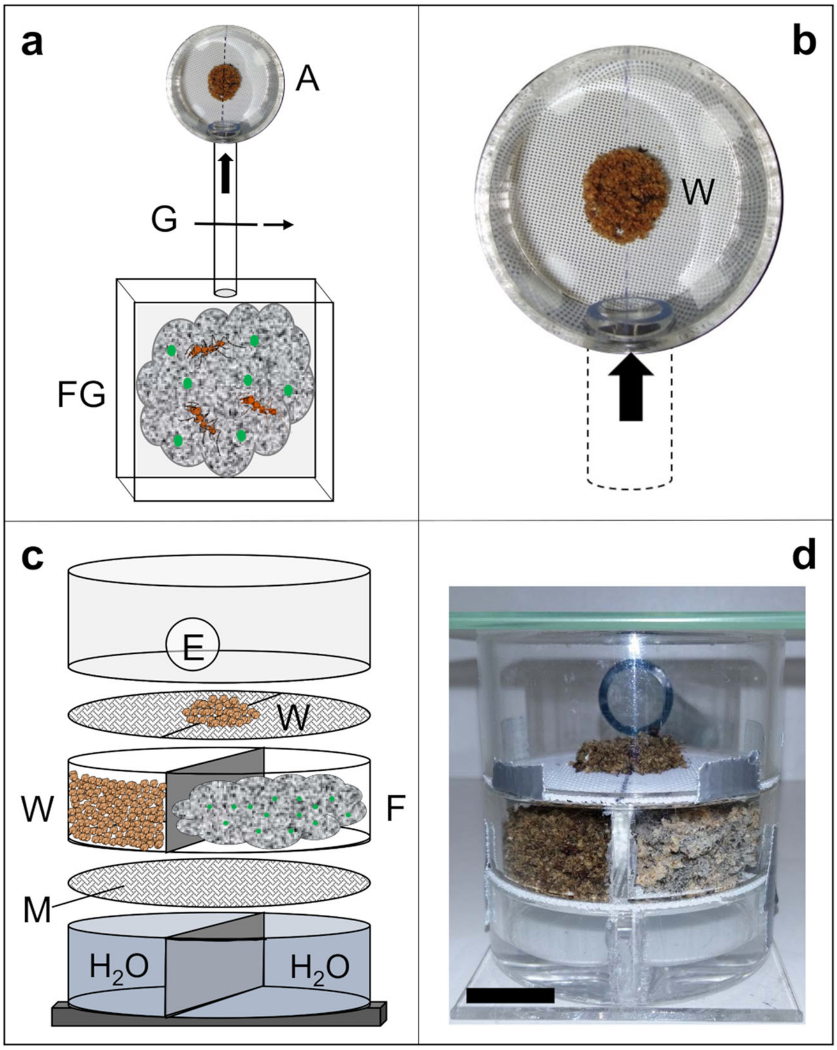

Fig. 1 Experimental setup **a** setup with subcolony (top view): a fungus garden (FG) was connected to the experimental arena (A), separated by a sliding gate (G). **b** Close-up of experimental arena: a small waste pile (0.5 g) was placed in the middle **c** + **d** experimental tower (side view, schematic, and photo); upper level, experimental arena; middle level, containers for waste and fungus material; bottom level, containers for water to humidify the setup. M, fine mesh; E, entrance to arena; W, colony waste; F, symbiotic fungus, black scale bar = 2 cm

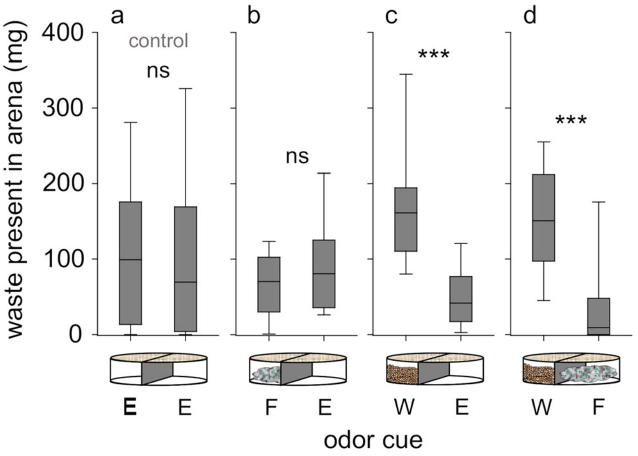

Fig. 2 Amount of waste (mg) present on each side of the experimental arena at the end of the assays **a** control: no odor cues on both sides (E, empty). **b** Fungus volatiles (F) vs. no odor cue (E). **c** Waste volatiles (W) vs. no odor cue (E). **d** Waste volatiles (W) vs. fungus volatiles (F); line, median; box, 25–75% percentiles; whiskers, min-max values; ns, not significant; ***$P < 0.001$

fungus volatiles. LCA are known to rearrange the waste particles in the colony dumps (Bot et al. 2001), probably to aerate the pile and control the spread of pathogens. Workers appeared to pick up waste particles and to relocate them without being guided by fungus volatiles, leading to an overall similar distribution of waste on both arena sides as in the first series without any volatiles. This pattern changed when workers were offered waste odor against no odor as choice (series III). Here, significantly more waste was located on the side of the arena releasing waste volatiles (Fig. 2c; $T = 5$, $Z = 4.14$, $n = 24$, $P < 0.0001$).

When offering both odor cues simultaneously (series IV), as would occur in a natural fungus chamber of the nest where the waste particles originate, workers showed a significant preference for the side releasing waste volatiles (Fig. 2d; $T = 32$, $Z = 3.37$, $n = 24$, $P = 0.0007$). In addition, a complete removal of the waste initially located above the fungus volatiles was observed in a higher percentage of assays (33.3%), as compared to the situation when no fungus volatiles were present as choice (4.2%, series III). This reaction shows that

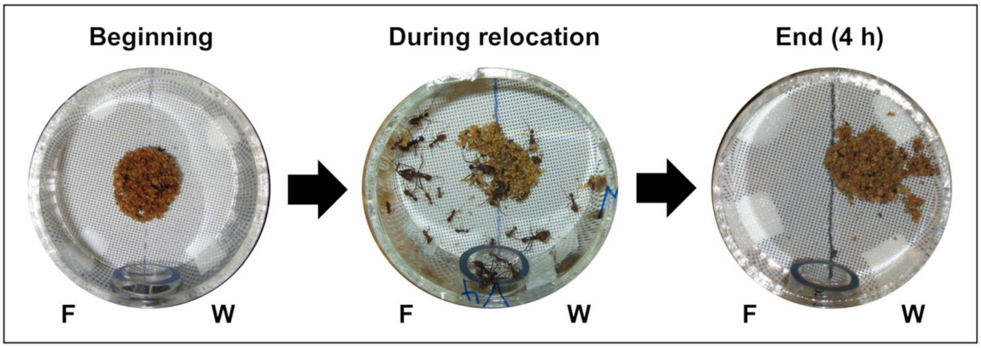

Fig. 3 Exemplary photos of the experimental arena at the beginning of an experiment, during the relocation process, and after removal of the ants at the end of the experiment. F, fungus volatiles; W, waste volatiles

workers do perceive the fungus volatiles, but only relocate the waste particles away from them if a source of waste volatiles is present (as compared to the results of series I).

Observations indicate that workers did not just randomly drop the waste particles on the arena side that releases waste volatiles. Rather, most ants picked up waste particles from the periphery of the waste pile, from the side not releasing waste volatiles, and placed them on the opposite side of the waste pile located above the waste odors (Fig. 3). In this way, the whole heap appeared to "wander" towards the side of the waste volatiles (time-lapse video, Online Resource 1). On the pile, ants "worked" these particles with their front legs into the waste heap, together with additional waste particles brought from the adjacent fungus garden (video, Online Resource 2 + 3), similar to their building behavior with soil pellets (personal observation).

The present results show that *Atta laevigata* workers prefer waste volatiles and use them to decide where to place waste particles. Waste volatiles appear to be stigmergic cues (Grassé 1959), by relaying indirect information to workers about where others have already deposited their waste, leading to accumulation of more particles at the site. LCA also use stigmergic cues to coordinate other behaviors, for example the accumulation of excavated soil pellets during nest excavation (Pielström and Roces 2013).

As waste particles originate in the fungus chamber, workers might react to the presence of waste particles with pick-up and random relocation behavior, if no other cue to guide their deposition is perceived, and the location of the waste dump is unknown. When both odor cues are present in the same chamber and there is an established dump site, the presence of fungus volatiles appear to trigger the pick-up of waste particles and their removal, and waste volatiles spatially guide the relocation towards the dump, likely aided by chemical markings along the way from walking ants or transported waste (Heyman et al. 2017). Using volatiles as cues to make decisions during waste management is not only limited to LCA, as volatiles released from ant corpses, i.e., necrophoric volatiles, can trigger their removal out of the nest in *Pogonomyrmex badius* and *Solenopsis invicta* (Wilson et al. 1958; Howard and Tschinkel 1976). Odor cues perceived in the waste dump can even be a source of information for LCA, since volatiles from harvested yet unsuitable plants for the symbiotic fungus that are dumped in the waste chamber are known to influence foraging decisions (Arenas and Roces 2016, 2017). *Atta* nests can consist of thousands of fungus chambers (Moreira et al. 2004), yet only a few underground waste chambers or single external heaps (Stahel and Geijskes 1939; Hart and Ratnieks 2002; Forti et al. 2017). The attractiveness of waste volatiles for waste-carrying workers should lead to the deposition and concentration of colony refuse at a few sites. This would benefit the colony in two ways. First, concentrating waste at a few sites should aid nest hygiene and keep potentially pathogenic material from spreading across a wide area, limiting contact with the colony members and reducing pathogen threat to fungus gardens and workers. Second, it would ensure that workers, including foragers, have access to information about unsuitable plants workers harvested and then disposed of (Arenas and Roces 2016, 2017, 2018) at a spatially restricted place, and not spread across many different sites.

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Data availability The dataset of this study is available in the supplementary material.

Compliance with ethical standards

All international, national, and institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare that they have no conflict of interest.

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