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

Andrés Arenas*, Flavio Roces

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

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

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

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

* Correspondence: A. Arenas, Laboratorio de Estudio de Insectos Sociales, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, IFIBYNE-CONICET, Universidad de Buenos Aires, Intendente Güiraldes 2160, Ciudad Universitaria, Buenos Aires, C1428EHA, Argentina.
E-mail address: aarenas@bg.fcen.uba.ar (A. Arenas).

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

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

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

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

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

METHODS

Ant Subcolonies and Leaf Suitability

For the experiments, performed during 2012 and 2013 at the Biocenter of the University of Würzburg, Germany, we built queenless, functional subcolonies containing about 600 workers, brood at different developmental stages and 1000 cm^3 of fungus garden (i.e. fungus plus gardeners within the matrix). Subcolonies remained active and showed intense foraging activity for up to 8 weeks. Eighteen subcolonies were obtained from six large queenright colonies of *Acromyrmex ambiguus* (three subcolonies per colony) collected in Uruguay in 2002, and reared in a climatic chamber at 25 °C and 50% relative humidity under a 12:12 h light:dark cycle. A single subcolony was organized in three transparent compartments: the foraging box, the fungus chamber and the waste chamber. The boxes containing the fungus and waste ($19 \times 8.5 \times 8.5$ cm) remained closed with a sealed cover. The bottom of the fungus box was covered with moistened expanded clay pebbles to maintain high humidity and prevent desiccation of the fungus. The foraging box ($19 \times 19 \times 8.5$ cm) remained open. Paraffin oil was applied to the walls to prevent the ants from