



# The role of chemical signalling in maintenance of the fungus garden by leaf-cutting ants

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## Abstract

How foraging trails are formed and the chemical communication between individual ants is well known. However, communication between partners in mutualistic relationships, such as the leaf-cutting ants (LCA) and their symbiotic fungus, is less studied. There is a feedback mechanism that operates in LCA colonies, with the fungus garden communicating its condition to the ants, most probably using chemicals. We discuss the literature on the chemistry of the LCA–forage–fungus system starting from selection of plants and its effect on the fungus garden. We suggest, using chemical examples, how the fungus might communicate with attendant ants and suggest areas for future research into this fascinating and complex system.

**Keywords** Attini · Ant foraging · Communication · Fungal chemistry · Mutualism

## Introduction

The success of ant societies depends on the foraging workers selecting the most appropriate foods that are nutritive and non-toxic (Hölldobler and Wilson 1990). Many species of ants collect food and feed it directly to conspecifics and brood, with minimal processing, for example, the prey of army ants (Gotwald Jr. 1995), aphid honeydew gathered by mutualist species (Fischer et al. 2001) or detritus collected by omnivorous ants (McGlynn et al. 2009). Some ants collect seeds, which are propagated in nutrient-rich soils in so-called “ant gardens”, with ants feeding on the plant tissue (Davidson 1988; Chomicki and Renner 2016). Other ants, such as fungus farming ants from the sub-tribe Attina (tribe Attini), use fungal symbionts to digest plant material and the ants then feed on the fungal structures, rather than the foraged material: in essence the insects are “farming” the fungus (Mehdiabadi and Schultz 2010; Hölldobler and Wilson 2011).

The most derived form of fungus farming by ants involves the cultivation of *Leucoagaricus gongylophorus* (Singer 1986) by leaf-cutting ants (LCA) within the genera *Atta* (Fabricius 1804) (*At.*) and *Acromyrmex* (Mayr 1865) (*Ac.*) (Mehdiabadi and Schultz 2010). There is a great deal of evidence that this system is co-ordinated by chemical signals, but in comparison with other aspects of ant biology there is a lot more to discover. This ant–fungus chemical interaction is likely to involve a range of chemical signals—semiochemicals—including allomones, kairomones and synomones (see Beck et al. 2017). It is known that compounds produced by *L. gongylophorus* can cause growth of pathogens towards the fungus (Folgarait et al. 2011; Masiulionis et al. 2015; Birnbaum and Gerardo 2016). We focus on the chemical communications between the foraged plant material, the LCAs and the fungus garden highlighting what is known and where there are gaps in knowledge.

## Leaf-cutting ant biology and essential conditions for the fungus garden

The LCAs consist of ca. 40 described species and are major defoliating herbivores in the New World impacting upon plant community structure (Hölldobler and Wilson 1990; Costa et al. 2008; Mehdiabadi and Schultz 2010; Leal et al. 2014). Fungus-growing ants evolved approximately 60 MYA, with recent evidence suggesting that extant LCAs

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evolved around 19 MYA in dry habitats in Central America (Branstetter et al. 2017). The key to the success of LCA is the highly evolved mutualistic association between the ants and their fungal symbiont, *L. gongylophorus* (Ridley et al. 1996; North et al. 1997; Schultz and Brady 2008). The fungus is cultivated on pulped leaf material supplemented by faeces (Hölldobler and Wilson 1990), to form the so-called “fungus gardens”. Swollen hyphal tips—gongylidia—are a unique fungal adaptation and provide the sole food source for the ants and their developing brood (Hölldobler and Wilson 2011).

Complex assemblages of beneficial microorganisms contribute to the health of the colony with actinomycetous bacteria, e.g. *Pseudonocardia*, used as a source of antibiotics by LCAs (Poulsen et al. 2007; Sen et al. 2009) while other bacteria fix nitrogen, increasing the productivity of the colony (Pinto-Tomás et al. 2009). Yeasts, while not generally regarded as essential, appear to contribute to degradation of plant materials and detoxification of potentially harmful compounds, such as galacturonic acid (Mendes et al. 2012). The fungal garden is propagated and maintained by hygienic behaviour (Fernandez-Marin et al. 2006; Mangone and Currie 2007; Della Lucia et al. 2014). For example, the metapleural gland secretion from ants contains antimicrobial compounds, such as phenyl acetic acid (Fernández-Marín et al. 2015), which restrict the growth of fungal and bacterial pathogens (Ortius-Lechner et al. 2000), such as the microfungus *Escovopsis* (Ascomycota: Hypocreales) (Currie et al. 1999; Haeder et al. 2009). The ants’ hygienic behaviour is very effective as the fungal garden does not persist if the ants are removed (Weber 1966; Mueller et al. 2005).

Maintenance of the microbiome is important for the health of the fungal garden but the primary factor affecting the growth of the gongylidia is the collection and preparation of a suitable substrate by foragers. Foraging trails can be highly adaptable to make use of the most nutritious and least toxic plant material within constantly changing environments (Silva et al. 2013). Some LCAs are specialist foragers on either mono- or dicotyledonous plant species (e.g. Pereira et al. 2016), although within this range of acceptability suitable plant material is collected based upon its physical (Cherrett 1972; Nichols-Orians and Schultz 1989; Bollazzi et al. 2011) and chemical properties (Hubbell et al. 1984; Howard 1987, 1988).

## Factors governing selection of plants

### Nutrients

The acceptance of plant material can be affected by leaf nutrients, and young leaves with higher concentrations of phosphorous, potassium or nitrogen are collected by *At.*

*cephalotes* (L.) (Linnaeus 1758) (Berish 1986) and *At. laevigata* (Smith 1858) (Mundim et al. 2009), whereas *At. colombica* (Guérin-Méneville 1844) selects drought-stressed leaves, that contain a greater concentration of carbohydrates and proline (Meyer et al. 2006). Production of cellulolytic and carbohydrate-active enzymes by the microorganisms associated with fungus gardens varies between *Atta* and *Acromyrmex*, and this difference could be an adaptation to decomposition of the plant material foraged (Suen et al. 2010; Kooij et al. 2014, 2015).

### Secondary compounds

Within plant tissues, the mixture of either constitutive or induced secondary compounds can be a reliable predictor of plant utilization by LCA (Howard 1987, 1988, 1990; Howard et al. 1989). In general, compounds or mixtures of compounds from plants can attract or repel insects, acting as “push–pull” stimuli (Cook et al. 2007). In relation to the “pull” effect, some volatile compounds can attract LCAs in laboratory studies (Perri et al. 2017) but it is unclear how these compounds act in natural situations. There is more information on repellent and toxic compounds. Some examples, mainly from laboratory bioassays, have shown that LCAs can be repelled by plant chemical defences induced by mechanical damage or by hormones, such as jasmonic acid (Kost et al. 2011). Constitutive plant compounds prevent collection of leaves since *At. sexdens rubropilosa* (Forel 1908) does not feed on leaves of *Ricinis communis* L. (Euphorbiaceae) under natural conditions, which is probably due to a mixture of fatty acids and the presence of an ant toxin, ricinine (Bigi et al. 2004) and *At. cephalotes* will avoid plant leaves containing high levels of toxic saponins (Folgarait et al. 1996).

It has been suggested that *At. cephalotes* are more common where young or “pioneer” plants predominate, rather than in more mature or established neotropical forest, because young and pioneer plants have lower levels of protective—toxic or repellent—chemicals (Farji-Brener 2001). These findings are supported by laboratory bioassays, where discs cut from young leaves were selected in preference to old leaves both by *At. cephalotes* and *Ac. octospinosus* (Reich) (Littlelyke and Cherrett 1975, 1978).

### Endophytes and plant fungal pathogens

Plants produce their own chemical defences, but this can be modulated by the presence of benign endophytes that do not cause disease but alter plant physiology so that asymptomatic cucumber leaves inoculated with a single species of endophyte emit compounds associated with

damage, such as  $\beta$ -ocimene and 4,8-dimethyl-1,3,7-nonatriene which can repel ants (Estrada et al. 2013). It is also possible that LCAs select plant materials based upon the compatibility of the endophyte community with the fungal garden (Van Bael et al. 2011; Estrada et al. 2014, 2015).

Pathogenic fungi also induce chemical changes in plants. As a selected cohort of 42 plants in the dry forest of Costa Rica became more infected with fungal pathogens in the wet season, they produced more phytoalexins which repelled *At. cephalotes* foragers (Hubbell et al. 1984). Among these compounds, there were sesquiterpenes and triterpenes in the leaves of *Cordia alliodora* (Ruiz and Pav) Oken (Boraginaceae) and *Verbesina gigantea* Jacq. (Asteraceae), which were found to be repellent at naturally occurring concentrations (Chen et al. 1983; Hubbell and Wiemer 1983; Hubbell et al. 1984).

### Imperfect foraging and the need for feedback

Free-living ants self-select forage in an attempt to optimize its preparation for growth of fungal monocultures (De Fine Licht and Boomsma 2010), but if ants have not encountered and foraged a plant before, collection and transport back to the fungal garden is driven by acceptability of the material to the ants (Rockwood and Hubbell 1987). Foragers are able to detect and rapidly reject plants that contain compounds toxic or repellent to the ants (Seal and Tschinkel 2007). Some plants that repel ants also retard growth of the fungus (Diaz Napal et al. 2015) and individual compounds (e.g. caffeine) inhibit the growth of fungi and can explain why *At. sexdens rubropilosa* forages low-caffeine varieties of *Coffea* sp. (Miyashira et al. 2012). Other plants with mycostatic effects *in vitro* are those that are not foraged upon (Lapointe et al. 1996). For example, *Virola sebifera* Aubl. (Myristicaceae) is not collected by *At. sexdens rubropilosa* and this may be due to lignans, which show fungistatic effects in the laboratory (Pagnocca et al. 1996).

Although leaf-cutting ants assess the suitability of plant material, we know that foraging is imperfect as what LCAs select and incorporate into the fungal garden may not always be optimal for the growth of the fungus (Herz et al. 2008). Some extracts of plants, plant compounds and synthetic molecules can be toxic to the fungus, but neither toxic nor repellent to the ants (Ambrozin et al. 2006; Bigi et al. 2004; Bueno et al. 2005; Howard et al. 1988; Pagnocca et al. 1990, 1996, 2006; Victor et al. 2001). So, survival of the colony in circumstances where ants make a wrong decision then depends on effective communication between the fungal garden and the attendant ants.

### Delayed responses and learning

Once plant material is taken back to the nest, the response of the ants to mycotoxic compounds in particular can be delayed (Saverschek et al. 2010; Saverschek and Roces 2011) termed “delayed rejection” (Ridley et al. 1996). For example, there are some plants in the habitat of *Ac. ambiguus* (Emery 1888) that are not collected as they are unsuitable for the fungus garden (Saverschek et al. 2010). However, colonies of *Ac. ambiguus* that had not previously been exposed to these plants did not reject immediately (Saverschek and Roces 2011). When *Ac. ambiguus* were provided with plant material treated with undetectable fungicide, they learned to associate plant odours and cues from damaged fungi with the foraged leaves, which caused rejection in behavioural experiments (Arenas and Roces 2016a, b). This rejection is thought to be due to volatile signals, re-enforced by close contact with leaf surfaces (Saverschek et al. 2010; Saverschek and Roces 2011). Under no-choice laboratory conditions *At. sexdens* (Linnaeus 1758) foragers will collect leaves of *Sesamum indicum* L. (Pedaliaceae) and although the leaves eventually repel ants the colony cannot recover (Bueno et al. 1995), due at least in part to a mixture of fatty acids which are toxic to the fungus when combined (Ribeiro et al. 1998). This suggests that there is some chemical change in the fungus as it utilizes the resource due to plant toxins and which is perceived by the ants within the fungal garden (Herz et al. 2008; Thiele et al. 2014). Chemicals are transferred between *At. sexdens rubropilosa* and the fungus colony by direct contact (North et al. 1999), which may take the form of chemically marking acceptable food (Bradshaw et al. 1986) or by antennal contact between workers (Lenoir 1982).

### Detection of changes in the fungal garden

We argue that based upon the perception of chemicals emitted by fungal gardens LCAs are able to distinguish between healthy and disturbed or degrading colonies, based upon characteristics of the strain of fungus that they cultivate and the chemical compounds produced by other organisms in the microbiome, and are able to detect perturbations in the chemical profile. Production of primordia by the fungal garden would reduce somatic growth and the nutrients available to the colony and be perceived by ants as a change in the chemistry of the fungus. Reproduction of the fungus is suppressed by the activities of ants (Pagnocca et al. 2001) and the presence of these mushrooms is usually a symptom that the colony is in decline

(Fisher et al. 1994a, b). Of more immediate significance for the survival of the colony is fungal distress or death due to toxins or pathogens. North et al. (1999) suggest that as the fungus dies it produces breakdown products and these compounds act as semiochemicals. Ants that self-select and take many different types of forage back to the nest create a patchwork of different plant materials in the fungal garden. In nests of *At. sexdens rubropilosa*, this is manifested as unequal production of staphylae—clusters of gongyliidia—by the fungal garden (Camargo et al. 2008) and may be what the ants detect. In a healthy colony, a first response of LCAs is to secrete antimicrobial molecules to prevent or halt the spread of pathogens (Fernandez-Marin et al. 2006, 2015) and pathogens are excised by *At. colombica* (Mighell and van Bael 2016). This ability to discriminate pathogens and mutualists presents the possibility that the pathogenic species causes chemical changes in the fungal garden as it produces enzymes or physical structures that disrupt the gongyliidia (Marfetán et al. 2015). The fungal garden that is damaged either by toxic forage or by pathogens is disposed of at the waste dump and there is some research to show that the process of transferring damaged fungi, together with toxic forage to the waste dump, is an important step in the associative learning process, especially for naive worker ants (Arenas and Roces 2016a, b, 2017; Scott et al. 2010).

## Fungal compounds governing ant behaviour

As saprotrophs, fungi cause chemical changes in substrates, and this activity can be detected as changes in the chemicals surrounding the fungus, especially as volatiles produced by primary or secondary metabolism (Morath et al. 2012). There is significant evidence that fungi produce semiochemicals which affect physiology, survival and behaviour in many insect taxa (e.g. Davis et al. 2013), including ants (Holighaus and Rohlf 2016). Mueller et al. (2017) have recently highlighted the diversity of fungal genotypes cultivated by the extant *Atta* and *Acromyrmex*, and this is likely to have an effect on the metabolism of the fungi and inter alia the compounds involved in communication with LCAs. In turn, the range of acceptable plant materials and, therefore, the diet breadth of individual colonies, could be driven by the different strains of fungus, as Mueller et al. (2017) suggest. We speculate that these differences, over evolutionary time, may result in greater degrees of fidelity between LCAs and their fungal strains. Colony-specific chemical profiles emitted by the different genotypes of fungi—in particular aldehydes, amides and their methyl esters—contribute to nestmate recognition in *Ac. octospinosus* nests (Hernandez et al.

2006; Sainz-Borgo et al. 2013). Ants that fed on fungi from colonies other than their own are accepted more readily into the foreign colony (Richard et al. 2007).

So, it is probable that different strains of *L. gongylophorus* each produce a chemical profile, or signature, which helps to perpetuate associations across lineages of ants. A healthy fungus would not have to invest significant metabolic resources for the maintenance of this chemical signature as it is a consequence of primary metabolism. Induction of stress in the fungus, due to sub-optimal forage or pathogens is likely to alter this chemical signature. Since the fungus would incur a metabolic cost by allocating resources to the production of secondary compounds (Böllmann et al. 2010), we believe that the semiochemicals perceived by ants would be existing metabolites that are up-regulated or that undergo minor structural modification. We further suggest that these compounds would be relatively simple metabolites. For example, the eight carbon oxylipins, derived from peroxidation of lipids constitute a structurally diverse group of fungal compounds with a wide range of ecological functions (Brodhun and Feussner 2011), while in fungi they are involved in growth, development (Tsitsigiannis 2005; Tsitsigiannis and Keller 2006, 2007; Brodhun and Feussner 2011) and as signalling molecules between pathogenic fungi and their plant hosts (Tsitsigiannis and Keller 2007). Furthermore, other molecules of C<sub>7</sub> and greater, such as alkanes, aliphatic alcohols, acids and ketones initiate the alarm response of leaf-cutting ants, but occur in varying proportions between *Atta* and *Acromyrmex* species (Norman et al. 2017). It would be interesting to investigate if there is some similarity between the blends of molecules that govern recognition of nestmates or that signal alarm in LCAs and stress in fungi.

## Conclusions

Plant chemistry determines whether plants are collected by LCAs and, in turn, the effects of this material on the fungal garden modify behaviour of LCAs. Within the nest chemical treatment with secreted molecules, removal of damaged areas and disposal on waste dumps are key behaviours; the process of removal and disposal seems to drive colony learning. Exactly how the fungus communicates either distress as it is damaged or a change in reproductive state and which compounds are involved and the genetic basis of their production are not yet known. Collection, extraction, analysis and bioassay of fungal compounds associated with *Atta* and *Acromyrmex* would start to piece together their role and how they convey information.

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