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Symbiotic partnerships and their chemical interactions in the leafcutter ants (Hymenoptera: Formicidae)

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Abstract

Leafcutter ants are indigenous to Central and South America and the southern US and are noticeable for their active herbivorous behaviours, collecting mostly fresh plant parts to manure underground gardens of their *Leucoagaricus gongylophorus* fungal cultivars. These gardens contain a single clone of the cultivar but are also susceptible to pathogens, most notably the specialised mycopathogen *Escovopsis*. Pathogen pressure led to the evolution of intensive grooming behaviours assisted by antimicrobials produced in endocrine glands and by domesticated antibiotic-producing actinobacteria grown on the integument of workers. The most notable of these are *Pseudonocardia* species that are abundant in *Acromyrmex* but have been lost in *Atta* leafcutter ants. The leafcutter ant symbiosis represents a fascinating example of chemical warfare of which the details are becoming increasingly known. Here, we review recent progress in understanding the complex interactions that take place between the mutualistic and parasitic symbionts, particularly between the ants, their mutualistic fungal cultivars and cuticular actinobacteria, and their *Escovopsis* parasites.

Key words: Leafcutter ants, antibiotics, *Pseudonocardia*, *Escovopsis*, *Streptomyces*, review.

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Introduction

A leafcutter ant colony represents a remarkable example of multipartite symbiosis, including a mutualism between an ant colony, a clonal cultivated food fungus and, in the genus *Acromyrmex*, a predominantly single strain of *Pseudonocardia* actinobacteria (Fig. 1, Box 1) (Weber 1966, Hölldobler & Wilson 1990, Currie 2001, Andersen & al. 2013). The food fungus provides the ants with the ability to break down fresh plant material, which opened up a food source that was inaccessible to ancestral attine ants, a development that significantly enhanced larval provisioning so that colonies could become large (Mueller & al. 1998, Schultz & Brady 2008, De Fine Licht & al. 2010, Aylward & al. 2013). There is also a parasitic symbiont, the specialised mycoparasite *Escovopsis*, which kills and feeds on the hyphae of the *Leucoagaricus gongylophorus* cultivar (Currie & al. 1999a, Yek & al. 2012, de Man & al. 2016). The *Pseudonocardia* bacteria maintained by *Acromyrmex* leafcutter ants have a positive effect on the fitness of the ant colonies by producing antifungal compounds that are antagonistic towards *Escovopsis* (Samuels & al. 2013). While it is generally agreed upon that the cultivars of the higher attine ants, including the leafcutters, evolved the special hyphal tips called gongylidia that the farming ants feed on in response to full domestication, the extent to which mutualistic and antagonistic coevolution has shaped the details of extant interactions between the higher attine and leafcutter ants and their fungal and bacterial symbionts has remained remarkably controversial.

Attine ants are found throughout Central and South America with around 250 species belonging to 15 recognised genera. 95% of these are located in South and Central America and 5% are from the nearctic region (Mayhé-Nunes & Jaffé 1998, Branstetter & al. 2017). Across the genera, there are five grades of farming: lower agriculture, coral fungus agriculture, yeast agriculture, higher agriculture, and leafcutter agriculture (reviewed in Schultz & Brady 2008). Here, we focus on the positive and negative interactions between mutualists and parasites in the leafcutter ants (*Atta* and *Acromyrmex*) as these are the most conspicuous and evolutionarily derived genera and have been studied most intensively. *Atta* leafcutter ants develop large colonies capable of collecting enough leaf material to equal consumption of

Box 1: Important players in Attini-symbiont relationships.

Leafcutter ants (Order Hymenoptera: Family Formicidae) comprise the genera *Atta* and *Acromyrmex*. They actively cut leaves to manure gardens of their mutualist food fungus *Leucoagaricus*, which they groom and weed particularly to control the spread of *Escovopsis* mycopathogens.

Leucoagaricus gongylophorus (Order Agaricales: Family Agaricaceae) is a vertically transmitted obligate fungal cultivar of *Atta* and *Acromyrmex* species. The ants house and feed this fungus and in return it provides nutrients in the form of hyphal tips called gongylidia that are rich in lipids and sugars.

Pseudonocardia spp. (Order Actinomycetales: Family Pseudonocardiaceae) is a vertically transmitted bacterial mutualist that grows on the cuticles of *Acromyrmex*, but not *Atta* workers. It provides antifungal compounds used by the ants to control *Escovopsis* and antibacterials that prevent most other bacteria from invading its niche.

Escovopsis spp. (Order Hypocreales: Family Hypocreaceae) are co-evolved parasites of leafcutter ants that feed on the *Leucoagaricus* fungus and can cause colony collapse.
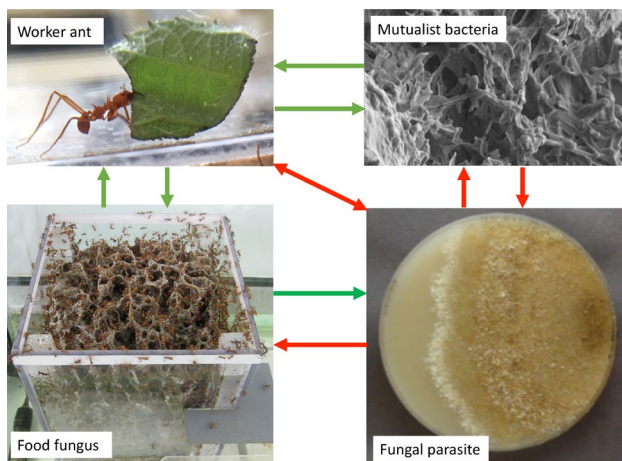

Fig. 1: Summary of the symbiotic relationships between *Acromyrmex* leafcutter ants and their partners. The ants provide freshly cut leaves to their clonal food cultivar *Leucoagaricus gongylophorus*, which in return is the sole food source for the ant larvae. Parasitic mycopathogens of the genus *Escovopsis* can infect the *Leucoagaricus* food fungus, an antagonism that the farming ants can control by weeding and grooming infected fungus-garden patches. *Acromyrmex* ants also have a mutualism with filamentous actinomycete bacteria of the genus *Pseudonocardia* that grow on their cuticle and have been inferred to be maintained via secretions of tiny subcuticular glands. These bacteria provide antifungal compounds to control *Escovopsis* and antibacterials to monopolise their cuticular niches. However, the mycopathogens have also evolved antibacterials to neutralise *Pseudonocardia* defences and neurotoxins to induce incoherent behaviour and enhanced mortality in the farming ants.

a large terrestrial mammal (HÖLLDOBLER & WILSON 1990, HERZ & al. 2007). Colonies of *Acromyrmex* can number up to 50,000 workers and *Atta* up to 5 million. These ants are thus a considerable pest to farmers whose crops they consume (HÖLLDOBLER & WILSON 1990).

Attines first began to farm fungus from a single origin in the Neotropics approximately 55 - 60 million years ago (CURRIE 2001, SCHULTZ & BRADY 2008, DE FINE LICHT & al. 2010, NYGAARD & al. 2016). At the outset of fungus farming, the ants appear to have farmed a variety of fungal cultivars, all belonging to a paraphyletic clade within the Leucoco-

prineae, which may have been regularly acquired *de novo* from their environment (MUELLER & al. 1998, CURRIE 2001, DE FINE LICHT & al. 2010). The emergence of the obligately dependent, "higher attine" lineages occurred approximately 30 million years ago and coincided with a shift in garden substrate, from the debris collected by lower attines to supplementation with other plant material, and with a shift to drier habitats (SCHULTZ & BRADY 2008, BRANSTETTER & al. 2017). This trajectory culminated in the evolution of the leafcutter ant genera (consisting of *Atta* and *Acromyrmex* species) approximately 15 million years ago (SCHULTZ & BRADY 2008, NYGAARD & al. 2016, BRANSTETTER & al. 2017). These genera almost exclusively collect fresh plant substrates for their fungal gardens that only ever consist of related clones of the fungus *Leucoagaricus gongylophorus* (SCHULTZ & BRADY 2008, DE FINE LICHT & al. 2014). Recently, it has been suggested that the fungal cultivar of leafcutters can be divided into two clades: one comprising *L. gongylophorus* which is often grown using dicotyledonous plants (though it has also been observed to be cultivated on grasses) found distributed in North America, Central America and South America, and another which is often grown using grasses found only in South America (MUELLER & al. 2017).

Processing plant material: fungal enzymes

The primary importance of the attine fungal cultivar is to convert otherwise indigestible foraged plant material into nutrients that can be consumed by the ants, thus allowing them to make use of a food source that is not available to other ant species (DE FINE LICHT & al. 2010). These nutrients are available to the ants in the form of lipid and carbohydrate rich hyphal swellings, called gongylidia (DE FINE LICHT & al. 2014). These specialised feeding structures first evolved in the fungus when it became an obligate symbiont of the higher attines, approximately 30 million years ago (SCHULTZ & BRADY 2008, DE FINE LICHT & al. 2014). Gongylidia cluster together to form structures known as staphylae that are the primary food source for the colony, particularly the larvae, which exclusively feed on the fungus (CURRIE 2001, DE FINE LICHT & al. 2014).

The necessity to break down plant substrates in order to fuel fungal growth and supply important nutrients to their ant symbionts, has resulted in substantial changes to the metabolic and enzymatic capabilities of the fungal cultivar over evolutionary time (DE FINE LICHT & al. 2010). The fungal cultivars of lower attine species, which receive predominantly dead plant material and debris, are known

to have a similar enzymatic profile to those of free-living saprotrophic fungi which break down plant material by attacking the complex polysaccharide components of the cell wall such as hemicellulose (cross-linking glycans) and pectin, before fully degrading other cellular components such as cellulose and intracellular starch (DE FINE LICHT & al. 2010). However, an investigation of the *Leucoagaricus gongylophorus* fungal cultivar associated with leafcutting *Atta* and *Acromyrmex* species has revealed several differences in its metabolic profile (DE FINE LICHT & al. 2010, 2014). The *L. gongylophorus* genome encodes approximately 145 lignocellulase enzymes, including many pectinases, xylanases and amylase enzymes (AYLWARD & al. 2013). Comprehensive microarray polymer profiling of the garden strata of *Acromyrmex echinatior* colonies has suggested a rapid degradation of pectin in the upper layers of the garden where fresh leaf substrate is integrated by the ants (MOLLER & al. 2011). This initial disassociation of the pectin cell wall matrix causes disruption of plant cell walls and potentially allows the fungus better access to intracellular resources such as starch and protein (DE FINE LICHT & al. 2010, SCHIØTT & al. 2010, MOLLER & al. 2011, AYLWARD & al. 2013, KOOIJ & al. 2014). Metaproteomic data and expression analysis also suggest a much higher level of endo-protease and alpha-amylase activity in the fungus compared to free-living counterparts and lower attines (DE FINE LICHT & al. 2010, AYLWARD & al. 2013). Cellulose and some xylans, on the other hand, are still found in high abundance in the lower garden strata as well as the waste dump material (MOLLER & al. 2011) and cellulases are expressed at much lower levels than in free-living fungi, suggesting that this is only partially degraded by the leafcutter crop fungus (DE FINE LICHT & al. 2010, KOOIJ & al. 2014). In support of this, expression analysis has suggested that cellulose conversion primarily occurs only towards the end of the plant decomposition process (at the bottom of the fungus garden) and may be more important in supporting the continued growth of the fungus and avoiding the build up of decaying mycelia in this region, rather than for providing nutrients to the ants (GRELL & al. 2013). Together, these results suggest that *L. gongylophorus* has evolved to target superior plant cell resources, such as protein and starch, for the generation of fungal biomass and gongylidia, the primary nutrients source for the ants, rather than recalcitrant cell wall polysaccharides such as cellulose (DE FINE LICHT & al. 2010, MOLLER & al. 2011). This increasingly targeted use of specific plant cell material by *L. gonglyophorus* is thought to be a logical corollary of the much higher nutritious quality of the garden substrate in leafcutter ants (KOOIJ & al. 2014), because fresh plant material contains higher levels of protein than dead leaf litter, and increasing protein availability to the ants is desirable as this is normally a major factor limiting insect growth (GRELL & al. 2013, KOOIJ & al. 2014). Down-prioritizing recalcitrant polysaccharides may also explain why increasingly larger quantities of leaf material are often collected by the more derived leafcutter genera since only a fraction of the molecular components are actually used by the fungus for biomass generation and the remainder is deposited as waste (MOLLER & al. 2011).

Fecal droplet enzymes

An additional layer of complexity in the degradation of plant material and further evidence of the emerging complementarity between the ants and their fungal symbiont is the finding that leafcutter ants are also able to concentrate key plant degrading enzymes in their faecal droplets, including cell wall degrading pectinases, proteases and laccases (MARTIN 1970, SCHIØTT & al. 2010, DE FINE LICHT & al. 2014, KOOIJ & al. 2014). Laccases are thought to be involved in the degradation of lignin as well as the detoxification of plant secondary metabolites (DE FINE LICHT & al. 2014). When adding new material to the fungus garden the ants first masticate the plant material into tiny fragments to enhance fungal entry into the plant cell matrix as hyphae exclusively colonise the cut leaf edges (ERTHAL & al. 2009). The ants then apply the enzyme-containing fecal droplets to the newly inserted leaf fragments before inoculating them with fungal hyphae (CURRIE 2001, ERTHAL & al. 2009). However, although these enzymes are applied by the ant, studies have revealed that they initially derive from the gongylidia of the fungal cultivar (MARTIN 1970, MARTIN & MARTIN 1971, SCHIØTT & al. 2010, KOOIJ & al. 2014). Substituting the ants' fungal diet with glucose eliminates enzymatic activity in fecal droplets (SCHIØTT & al. 2010, KOOIJ & al. 2014) and several of the genes encoding the production of fecal fluid enzymes are upregulated in the gongylidia (SCHIØTT & al. 2010, DE FINE LICHT & al. 2014, KOOIJ & al. 2014). In fact, of the seven protease enzymes identified in the fecal droplets of *Acromyrmex echinatior,* only a single one is produced by the ants (KOOIJ & al. 2014). These findings imply that coevolution between leafcutter ants and their fungal cultivar has enabled the ants to vector crucial enzymes from the more prolific central parts of the garden where there is an abundance of gongylidia, to the newly established and fast growing peripheral parts where no gongylidia are as yet produced (KOOIJ & al. 2014). At the garden periphery (usually mostly the top layer), these enzymes primarily aid in the initial breakdown of plant material (SCHIØTT & al. 2010, DE FINE LICHT & al. 2014, KOOIJ & al. 2014). Evidence from the sequences of these fungus-derived enzymes also suggested signatures of positive selection, possibly to allow passage and survival through the ant gut (DE FINE LICHT & al. 2014). This, along with reciprocal benefits provided by the ants, may explain the evolution of gongylidia which represent a major metabolic investment by the mutualistic fungal cultivar (DE FINE LICHT & al. 2014).

Division of labour: the production of essential amino acids

The fungal cultivar plays a key role in providing ants access to carbohydrate and protein resources, allowing them to fill a niche not occupied by non-herbivorous species. However, another common outcome of an obligate mutualistic symbiosis is a close dependency of partner organisms upon one another due to the production and exchange of essential amino acids (FISHER & al. 2017). This can subsequently result in the reduction of the genomes of symbiotic partners (MARTINEZ-CANO & al. 2014). A study of the *Acromyrmex echinatior* genome has revealed that this species lacks two key genes involved in arginine biosynthesis (NYGAARD & al. 2011, 2016), similar to *Atta* (SUEN & al. 2011). It is postulated that the ants receive all of their arginine from the fungal cultivar, since *Leucoagaricus gonglyophorus* encodes and expresses the full set of genes for arginine biosynthesis in its gongylidia (DE FINE LICHT & al. 2014). Genes for phenylalanine and tyrosine are also upregulated here, both of which are essential amino acids that are required in high abundance for cuticle production and growth of immature

insects (DE FINE LICHT & al. 2014). Phenylalanine and tyrosine are amongst the most expensive amino acids to produce in terms of ATP requirements (BARTON & al. 2010), implying a division of labour between the ants and their cultivar (DE FINE LICHT & al. 2014).

Growing a fungal monoculture: ant and fungal defence strategies

Attine ant colonies and their fungal cultivars have a tight dependency upon one another in terms of accessing and exchanging essential nutrients and enzymatic compounds. However, it is vital that processes are in place to ensure the fungal cultivar is maintained free of parasitic microorganisms, and has preferential access to the leaf material supplied by the ants. Numerous studies of the makeup of ant fungus gardens have shown that, although the gardens are predominantly made up of the fungal cultivar, a diversity of other microorganisms can also be observed in the fungus-garden habitat. For example, several species of microfungi and yeast have been identified (RODRIGUES & al. 2005, 2008a, 2009 & 2011) as well as a low diversity core group of bacteria, particularly consisting of gamma proteobacteria (SCOTT & al. 2010, SUEN & al. 2010, AYLWARD & al. 2012a). Since the fungus is the sole food source of the ant brood it is expected that there will be a strong selective pressure to maintain the purity of the fungal cultivar. This has resulted in a suite of defensive and prophylactic adaptations in both the ants and the garden cultivars, many of which have attracted interest given that this fungal farming symbiosis appears to have suffered little from emerging resistance problems against its defences to pathogens and parasites (POULSEN & al. 2010). Monocultures of clonal fungal cultivars appear to be actively maintained, because chimeric fungus gardens have not been observed in field colonies (GREEN & al. 2002, POULSEN & BOOMSMA 2005, DENTINGER & al. 2009, MUELLER & al. 2010b, MEHDIABADI & al. 2012, KOOIJ & al. 2015), which appears to be mediated by incompatibility compounds in the mycelia and cultivar-derived fecal droplets that tend to eliminate newly introduced cultivars from sympatric colonies, particularly in *Acromyrmex* (POULSEN & BOOMSMA 2005, KOOIJ & al. 2015).

Prophylactic ant behaviour

In *Atta* and *Acromyrmex* species, waste is carried away to underground compost chambers of spatially separate waste piles downstream in order to minimise the spread of infection (BOT & al. 2001, HART & RATNIEKS 2002). The ants also try to minimise the introduction of foreign microbes into fungal gardens through a behaviour called "licking", which implies processing all freshly cut substrates through a filtering device within their oral cavity, known as the infrabuccal pocket, which acts to selectively remove microbes and hazardous debris from the newly collected forage-material (EISNER & HAPP 1962, CURRIE & STUART 2001). These are later expelled as compressed pellets onto the waste dump (EISNER & HAPP 1962, CURRIE & STUART 2001, LITTLE & al. 2006). The ants also carry out "grooming" behaviours by pulling pieces of the growing fungus though their mouth parts to remove foreign microbes and their spores (CURRIE & STUART 2001). Studies of the composition of infrabuccal pellets show that they contain non-viable fungal spores and tissue implicating the role of the pocket in detoxification of foreign material (LITTLE & al. 2006). Several viable actinomycete bacterial species

can also be isolated from pellets, particularly in colonies infected with the specialised fungal parasite *Escovopsis* (see LITTLE & al. 2006). Bioassays have shown that these bacteria can inhibit *Escovopsis in vitro* suggesting that antibiotics produced by these bacteria may be partially responsible for the detoxification of fungal spores within the infrabuccal pockets (LITTLE & al. 2006). It remains unclear whether these actinobacteria are permanently housed within the pocket or continuously acquired from the ants' cuticle (discussed later) in response to infections (LITTLE & al. 2006). All of these protective behaviours increase upon the introduction of invasive spores to ant sub-colonies (CURRIE & STUART 2001). These behaviours are even more intense when the cultivar is infected with *Escovopsis* but are not observed when irradiated non-viable *Escovopsis* spores are applied to the nest. This suggests that ants have the ability to detect foreign fungal infections, an observation which is supported by the fact that workers rapidly move into infected parts of the garden, possibly in response to chemicals signals (CURRIE & STUART 2001, UGELVIG & CREMER 2007). Attine ants regularly carry out "weeding" of the fungus garden which can include the removal of infected areas of the cultivar (CURRIE & STUART 2001).

Chemical defences of the ants

Leafcutter ants have a number of chemical adaptations to protect their fungal cultivar from aggressive microbes. One of these, the metapleural glands (MGs), is located on the posterior lateral end of the metathorax and continuously secrete a mixture of chemicals onto the surface of ants (YEK & MUELLER 2011, VIEIRA & al. 2012). Such secretions are used by almost all major lineages of ants to ward off infection, but leafcutter ants groom their MG openings using specific foreleg movements and then spread the secretions to their cultivar (FERNANDEZ-MARIN & al. 2006, AYLWARD & al. 2012b, VIEIRA & al. 2012). MG secretions contain an abundance of hydroxyacids, including indolacetic acid and myrmicacin, which negatively influence bacterial and fungal growth and spore germination (DO NASCIMENTO & al. 1996, ORTIUS-LECHNER & al. 2000, BOT & al. 2002). In addition, a study of the MG secretions of *A. octospinosus* identified 20 other compounds including several fatty acids and alcohols, many of which have general antimicrobial properties against an array of bacteria and fungi (ORTIUS-LECHNER & al. 2000, BOT & al. 2002). One of the most abundant secretions from the MGs of *Atta* is phenylacetic acid (PAA), which is absent in *Acromyrmex* species (VIEIRA & al. 2012, FERNANDEZ-MARIN & al. 2015). It has been demonstrated that this compound can act to inhibit mitosis and also the germination of fungal spores (VIEIRA & al. 2012, FERNANDEZ-MARIN & al. 2015). In addition to the direct antimicrobial functions seen *in vitro* for many MG compounds, the acidic nature of the combined set of secreted compounds is thought to play a primary role in maintaining a low pH in the fungal garden (ORTIUS-LECHNER & al. 2000, BOT & al. 2002). This is likely to maintain an optimal pH for the growth of the *Leucoagaricus gongylophorus* cultivar, and has been hypothesised to inhibit the spread of unwanted microbes in the garden – for example, the growth of several pathogenic bacteria is detrimentally affected by low pH (ORTIUS-LECHNER & al. 2000, BOT & al. 2002).

Beyond bioassays of individual MG compounds, several attempts have been made to determine the extent to which the secretions have hygienic functions *in vivo*. Some authors

have suggested that the greater number of MG cells per unit biomass and the greater relative size of MGs of minor garden workers, which are more important in the maintenance of the crop fungus, is circumstantial evidence for their explicit role in the protection of the fungus garden (Poulsen & al. 2003, Vieira & al. 2012). Poulsen & al. (2003) used a more direct approach and showed that, although the secretions impose substantial metabolic costs on the ant, they are continuously secreted under natural conditions. Active secretion was also important in protecting the ants against an entomopathogenic fungus *Metarhizium anisopliae.* When glands were experimentally closed there was a much greater level of mortality caused by these infections, suggesting that the antifungal activity of MG secretions was important for the protection of the ants themselves (Poulsen & al. 2003). Another study showed that the observed level of MG grooming and the transfer of compounds to the fungus significantly increased when *Atta* colonies were exposed to fungal infection (Fernandez-Marin & al. 2015). PAA could be detected in these gardens, as well as on the forelegs of ants, but only in infected fungal gardens (Fernandez-Marin & al. 2015). This selective and pointed application on demand, rather than continuous prophylactic use, of PAA has been suggested to be a key factor in reducing the evolution of resistance to metapleural gland secretions in the ant-fungus system (Fernandez-Marin & al. 2015). Other authors have also suggested that ants may be able to alter the composition and relative concentrations of their MG secretions to specifically target individual pathogenic species, increasing the specificity of this process and minimising unnecessary exposure to preserve the efficacy of these antimicrobials (Bot & al. 2002, Fernandez-Marin & al. 2006, Yek & Mueller 2011, Fernandez-Marin & al. 2015).

In addition to MG secretions, mandibular secretions and compounds in the fecal fluid of *Atta* ants also reduce spore germination of particular species of microfungi found in their fungal gardens (Rodrigues & al. 2008b). Together, these chemical adaptations act in a synergistic fashion with protective ant behaviours and likely reinforce protection provided by symbiotic actinobacteria in *Acromyrmex* species. Such a multifaceted and adaptable set of defences is believed to have been a key factor underlying the success of leafcutter ants, and likely fungus-growing ants in general, in preventing the overgrowth of fungal cultivars and the evolution of resistance against defensive compounds. The prudency of operating with multiple lines of defence that can each be applied specifically rather than prophylactically is now beginning to take hold in human medical endeavours (Fortman & Mukhopadhyay 2016).

The evolutionary dynamics of ant defences against disease

In general, ants are remarkably efficient in disease defence, which has meant that very few specialised diseases of ants are known (Cremer & al. 2018). This appears to be mediated by extremely efficient combinations of individual and social immune defences that have remained robust over evolutionary time. With that perspective it is perhaps not surprising that the leafcutter ants were able to both maintain these general defences, and to extend them to include active control of diseases in their fungus gardens, where as far as known their only specialised and co-evolving diseases occur. More efficient defence against disease pressure has likely been one of the selective forces shaping the evolution of obligate multiple insemination of queens in the *Atta* and *Acromyrmex* leafcutter ants (Villesen & al. 2002). Multiple queen mating implies that colonies become "chimeric" in the sense that workers are an assembly of full-sisters (patrilines) that are half-sisters to each other. The ensuing higher genetic diversity of colony workers as collective likely gave a series of advantages related to division of labour and disease defence in the leafcutter ants with their large and long-lived colonies, relative to the basal attine genera with smaller colonies that retained ancestral single insemination of queens (Boomsma & al. 2009). The leafcutter ants also evolved higher degrees of caste polymorphism, that is, distinct small and large workers in *Acromyrmex* and even higher worker caste diversity in *Atta*, which also evolved a specialised soldier caste. Several studies have shown that this enhanced social diversity boosted colony-level social immunity of *Acromyrmex* colonies (Hughes & Boomsma 2004, 2006, Hughes & al. 2008). Another study showed that there is significant genetic variation in the relative size of metapleural glands, particularly in small workers who are most active in disease defence. This suggests that some worker-patrilines specialise on specific defence activities not shared by other patrilines, consistent with the hypothesis that genetically diverse colonies are more robust in their collective social immune defences (Hughes & al. 2010).

Fungal defence compounds

Although the ants play an integral role in providing protection to their fungal cultivars, the crop-symbionts have also evolved their own set of defences that work in concert with the ants and respond to a variety of infections. Basidiomycete fungi are generally known to encode a diversity of secondary metabolites including several antimicrobial compounds (Aylward & al. 2012b). Despite this, relatively few studies have actually investigated the bioactive potential of the ant fungal cultivar and there have often been variable results. Several of the attempts to demonstrate antifungal activity of ant cultivar extracts have been negative (Martin & al. 1969, Weber 1972, Hervey & al. 1977) but two compounds, 7-Chloro-4,6-dimethoxy-1(3H)-iso-benzofuranone and basidalin, have been isolated from the fungal species *Leucoagaricus carneifolia* (a close relative of *L. gongylophorus*), which demonstrated weak to strong bioactivity against several bacterial and fungal species *in vitro* (Huff & al. 1994). Another study has shown that fungal cultivars isolated from the nests of a basal attine ant of the genus *Apterostigma* can, at least to some extent, suppress the growth of a variety of morphologically distinct *Escovopsis* lineages on agar plates, suggesting the cultivars may also have some of these defensive capacities *in vivo* (Gerardo & al. 2006a). A study of the uniquely unicellular yeast form of the cultivar maintained by *Cyphomyrmex minutus* ants, also showed that the fungus was capable of strong antifungal activity when grown on plates (Wang & al. 1999). Organic extracts from the fungus revealed three diketopiperazines which, when isolated, showed moderate inhibition of fungi in bioassays (Wang & al. 1999). It was hypothesised that this fungal cultivar may actually secrete a more potent mixture of these compounds *in vivo* leading to a greater degree of inhibition when live cultivars rather than extracts would be used. Diketopiperazine compounds have additionally been implicated to have antibacterial properties (Arnone & al. 1966). Leafcutter cultivars are also able to inhibit *Escovopsis* strains isolated from sym-
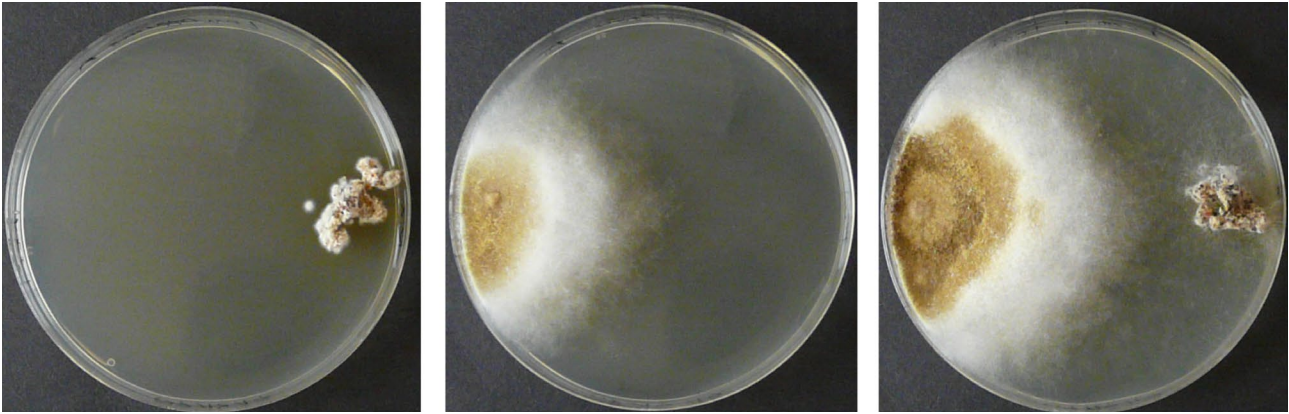
Fig. 2: *Escovopsis* fungi are attracted by and feed upon *Leucoagaricus gongylophorus* cultivars. *Escovopsis* grown on potato glucose agar plates with pieces of *Leucoagaricus gongylophorus* (right panel) grow faster than when grown alone (centre panel). The left panel displays a culture of *Leucoagaricus gongylophorus* growing alone.

patric colonies of phylogenetically more basal attine ants, especially those isolated from *Apterostigma* colonies with pterulaceous fungal cultivars (BIRNBAUM & GERARDO 2016). These small colonies are often found in considerable numbers in the close proximity of *Atta* and *Acromyrmex* colonies, so further work on the chemical interactions underlying these patterns of defence specificity would be of interest.

Diversity and virulence of *Escovopsis*

Parasitism of attine ant nests by *Escovopsis* fungi is an ancient symbiosis that evolved shortly after the ancestral attine ants adopted a farming lifestyle 55 - 60 million years ago (YEK & al. 2012, NYGAARD & al. 2016). *Escovopsis* belongs to the order Hypocreales, which comprises a number of mycoparasitic fungi, including the biocontrol agents *Trichoderma* and *Clonostachys* and the specialised *Tolypocladium* species that are insect pathogens and can also attack ectomycorrhizal truffle fruiting bodies (for a recent review of necrotrophic mycoparasites see KARLSSON & al. (2017). Traditionally there were just two species of *Escovopsis* recognised, *E. weberi* and *E. aspergilloides*, based on the formation of cylindrical or globose vesicles on their spore-bearing cells. However, a recent phylogenetic study suggests there are in fact nine lineages of *Escovopsis* belonging to five species (MEIRELLES & al. 2015). A sixth species, *E. trichodermoides*, was subsequently isolated from a nest of the lower attine ant, *Mycocepurus goeldii*, and does not have vesicles and phialides, but instead develops spores on a trichoderma-like conidiophore (GERARDO & al. 2006b, MASIULIONIS & al. 2015). Although *Escovopsis* evolution is heavily dependent on its ability to parasitise attine ant cultivars, the phylogeny of *Escovopsis* strains does not match the phylogeny of the attine ants they were isolated from (CURRIE & al. 2003b). This may be due to rare horizontal transfers of *Leucoagaricus* cultivars across attine ant genera or may suggest that closely related *Escovopsis* strains are capable of infecting a wide variety of attine ant cultivars (CURRIE & al. 1999a).

Escovopsis hyphae can be observed to grow towards *Leucoagaricus in vitro* (Fig. 2) and direct contact between their hyphae can be an important factor in causing the degradation of *Leucoagaricus* (MARFETÁN & al. 2015, VARANDA-HAIFIG & al. 2017). However, this process does not necessarily require contact between hyphae since

Escovopsis can also produce soluble factors, including a number of toxins and enzymes, which may diffuse towards *Leucoagaricus* from a distance and contribute to cultivar degradation (REYNOLDS & CURRIE 2004, MARFETÁN & al. 2015, VARANDA-HAIFIG & al. 2017). The 27 Mbp genome of *Atta*-derived *Escovopsis weberi* is small relative to its closest free-living relatives consistent with specialisation and gene loss in this parasite (DE MAN & al. 2016). This was further supported by sequencing of *Escovopsis* genomes from *Acromyrmex echinatior* (~30 Mbp) and additional *Atta* colonies (~27 Mbp) (HEINE & al. in press). Primary metabolism genes are still present in *E. weberi*, but the number of carbohydrate metabolism genes has been reduced and genes necessary for sexual reproduction have also been lost. *Escovopsis* has not been isolated from environments outside attine ant nests, consistent with this genus being fully specialised on its attine host cultivars and being unable to reproduce away from attine ant colonies (SEIFERT & al. 1995, CURRIE & al. 1999a).

Attine ants suppress *Escovopsis* using behavioural mechanisms, as well as bioactive molecules produced by their MGs and their bacterial mutualists. Anecdotal evidence suggests that when infections are severe the ants abandon their garden after which it is rapidly overgrown by *Escovopsis* (CURRIE & al. 1999a). This implies that weeding and grooming behaviours of worker ants are essential to keep *Escovopsis* at bay. *Escovopsis* is consistently present in the waste dumps of uninfected nests and spores can thus be picked up by worker ants (AUGUSTIN & al. 2017). A possible infection mechanism suggested by these observations is that spores of *Escovopsis* are washed from waste dumps of attine ants into the soil of nearby foraging tracks where they may be picked up by foraging workers and carried back to the nest. Unlike the mutualist cultivar fungus, *Escovopsis* is not vertically transmitted by founding queens and the claustral founding colonies of *Atta* do not contain *Escovopsis,* so that infections only emerge after the first foraging workers start to leave the nest (CURRIE & al. 1999a). More detailed studies on the spread of *Escovopsis* species between attine colonies are sadly lacking and should be the focus of future research.

***Escovopsis* compounds**

The first *Escovopsis weberi* genome sequence, from a strain isolated from an *Atta cephalotes* colony, revealed that it

Shearinine A: R = H
Shearinine D: R = OH
Shearinine E: R = OCH₃

Shearinine F

Shearinine J

Cycloarthropsone

Shearinine L: R = H
Shearinine M: R = OH

22, 23-dehydroshearinine A

Emodin

Melinacidin IV: x = 2 y = 2 R = OH
Melinacidin III: x = 2 y = 2 R = H
Chetracin B: x = 2 y = 3 R = OH
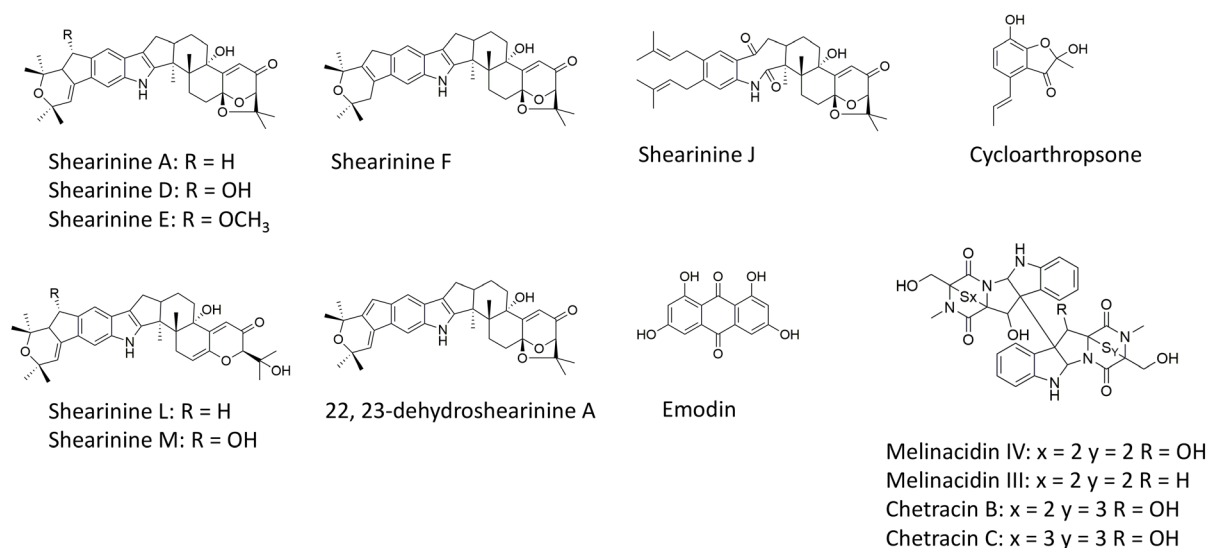Chetracin C: x = 3 y = 3 R = OH

Fig. 3: Bioactive compounds produced by *Escovopsis* fungi. *Escovopsis* mycopathogens synthesise triterpenic indole alkaloid shearinine compounds as well as emodin, cycloarthropsone, and epipolythiodiketopiperazine alkaloids of melinacidins and chetracins.

encodes a number of putative secondary metabolites (DE MAN & al. 2016). Furthermore, RNA sequencing of this *E. weberi* strain growing towards its host revealed that some of the genes for these compounds were upregulated, implying a role in pathogenesis. A separate study attempted to identify secondary metabolites produced by an *Escovopsis* species growing alongside an ant-associated *Streptomyces* strain using imaging mass spectrometry (BOYA & al. 2017). The *Escovopsis* species was not identified but the study showed that this isolate produced shearinines D, F and J (Fig. 3). The shearinines are triterpenic indole alkaloids with activity against calcium-activated potassium channels (XU & al. 2007) and were first isolated from an endophytic fungus *Penicillium janthinellum*. More recently, cultivation of an *E. weberi* strain on solid agar led to the detection of five shearinines (A, E, D, L & M), cycloarthropsone and emodin (Fig. 3) (DHODARY & al. 2018). These compounds were also detected on plates with *Leucoagaricus gongylophorus* and two of the shearinines, L and M, are novel compounds. The shearinines are part of a larger group of metabolites, the penitrems, which are mycotoxins associated with insecticidal and tremorgenic activity (STAUB & al. 1993). Similar fungal alkaloids are encoded by *Ophiocordyceps* fungi (DE BEKKER & al. 2015) and induce "zombie" behavior in infected ants (details below). Provision of oat flakes coated with shearinine L to subcolonies of *A. octospinosus* eventually led to them being rejected for incorporation into the fungus garden (DHODARY & al. 2018), presumably due to shearinine L being repellent as a substrate for *L. gongylophorus*. Shearinine L didn't directly lead to a zone of inhibition against *L. gongylophorus* whereas emodin and cycloarthropsone both displayed antifungal activity against the nest cultivar (DHODARY & al. 2018). Emodin has previously been known to have a number of activities including antibacterial (LEVIN & al. 1988), antifungal (IZHAKI 2002), antiviral (BARNARD & al. 1992), anticancer (LIU & al. 2011), anti-inflammatory (PARK & al. 2009) and antiulcerogenic (GOEL & al. 1991) effects. It is also known to be an insecticidal compound against mosquitoes (YANG

& al. 2003), caterpillar larvae (TRIAL & DIMOND 2012) and adults of the white fly *Bemisia tabaci* (GEORGES & al. 2008). Cycloarthropsone has been isolated previously as a fungal metabolite from *Arthropsis truncata* (AYER & CRAW 1992), but a functional role for this compound has not been determined. More recently, artificial infection of *Leucoagaricus* nest material with *E. weberi* led to the identification of two upregulated compounds; shearinine D and melinacidin IV (8.9- and 3.4-fold, respectively), when *E. weberi* was grown on *Leucoagaricus* (HEINE & al. 2018). However, monocultures of *E. weberi* also produced shearinine D, shearinine A, 22,23-dehydroshearinine A, melinacidin IV, melinacidin III, chetracin B and C and emodin (Fig. 3) (HEINE & al. 2018). Shearinine D was shown to reduce the mobility of *A. echinatior* worker ants, leading to uncoordinated behaviour, spasmodic leg movements, and ultimately death. These observations are consistent with the roles of shearinines as ion channel modulators and potential neurotoxins. Finally, shearinine D was also shown to be active against the two *Pseudonocardia* species associated with *A. echinatior ants*, *P. echinatior* and *P. octospinosus*, previously known as Ps1 and Ps2 (POULSEN & BOOMSMA 2005, ANDERSEN & al. 2013, HOLMES & al. 2016), suggesting that this might be used by *E. weberi* to antagonistically suppress the antifungal producing mutualist *Pseudonocardia* bacteria (HEINE & al. 2018). Melinacidins have antibacterial activity against Gram-positive bacteria (REUSSER 1968) and also inhibit the *Pseudonocardia* strains associated with *A. echinatior* ants, suggesting they also play a role in counteracting the inhibitory effects of *Pseudonocardia* on *Escovopsis* (HEINE & al. 2018). The chetracins are cytotoxic compounds, previously identified from the fungus *Oidiodendron truncatum* (LI & al. 2012), but their effects on the mutualists in the *A. echinatior* system have not been tested.

The *Pseudonocardia* mutualists

Many attine worker ants have a visible white bloom on their cuticles, often concentrated on their laterocervical plates (Fig. 4). This white covering was eventually identified
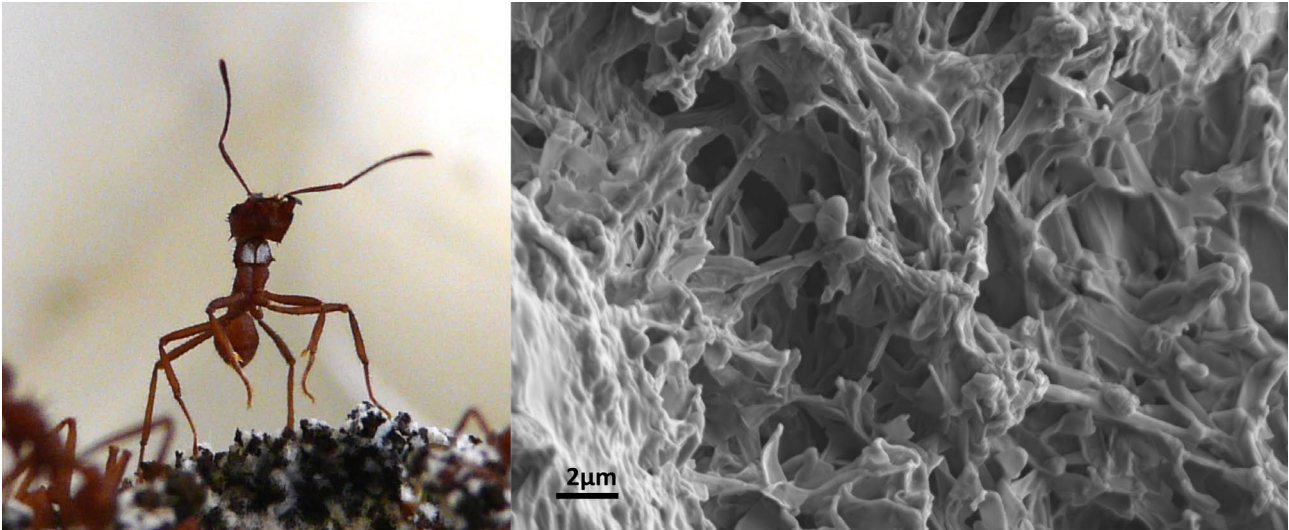
Fig. 4: Filamentous actinobacterial mutualists growing on *Acromyrmex echinatior* ants. *Pseudonocardia* bacteria can be seen growing on the integument of *Acromyrmex echinatior* ants (left panel) where they appear as a white covering on young callow ants or on the laterocervical plates of more mature workers. The filamentous growth of this actinobacterial mutualist can be readily observed using electron microscopy (right panel).

as a dense filamentous growth of actinomycete bacteria, mostly belonging to the genus *Pseudonocardia* which can be isolated from the cuticles of *Acromyrmex* but not *Atta* leafcutter ant workers (CURRIE & al. 1999b). On a smaller scale, these bacterial patches concentrate in species-specific cuticular regions where subcuticular exocrine glands are hypothesised to provision the bacteria (CURRIE & al. 2006). The bacteria were shown to produce antifungal antibiotics, presumably in return for ant resources, that inhibit the growth of *Escovopsis* (Fig. 5) (CURRIE & al. 1999b, CURRIE & al. 2003a). These defensive compounds, in addition to the ants' behavioral defences, are likely to be important in providing protection to the fungus gardens, which may be particularly prone to infection because they grow as monocultures and have low genetic diversity (KOST & al. 2007, MUELLER & al. 2010b). *Pseudonocardia* has since been found to be faithfully transmitted between generations within a colony with newly eclosed workers being inoculated by older workers within 24 hours after hatching (MARSH & al. 2014). Partner Fidelity Feedback (PFF) has therefore been suggested to help maintain this mutualism since the fitness interests of both partners remain aligned during the life of a colony; ant colony productivity increases due to the bacterially-derived antimicrobial substances and this, in turn, improves the likelihood of bacterial transmission to the next generation via dispersing virgin queens that found new colonies (FOSTER & WENSELEERS 2006, BARKE & al. 2011). A tight relationship between the *Acromyrmex* worker ants and their *Pseudonocardia* mutualist strains may also have facilitated the on-going dynamics of a co-evolutionary arms race between these bacterial symbionts and the *Escovopsis* pathogen, possibly helping to explain the apparently low levels of observed antimicrobial resistance (MUELLER & al. 2005, CURRIE & al. 2006). It is noteworthy that *Atta* leafcutter ants lack the cuticular crypts that maintain the *Pseudonocardia* symbionts in *Acromyrmex* and that isolation of *Pseudonocardia* in *Atta* yields negligibly low results which are comparable to levels expected for contaminations (MUELLER & al. 2008, MARSH & al. 2013).

The loss of *Pseudonocardia* might be attributed to the gain of PAA produced by the MG secretions, a powerful antifungal compound that may have made the antifungals produced by *Pseudonocardia* redundant (VIEIRA & al. 2012, FERNANDEZ-MARIN & al. 2015).

Strains of mutualistic *Pseudonocardia* produce antifungal antibiotics with novel structures, such as the cyclic depsipeptide dentigerumycin in the lower attine *Apterostigma*, and the polyene nystatin P1 (Fig. 5) in *Acromyrmex* (OH & al. 2009, BARKE & al. 2010). Further evidence of the interaction specificity between *Pseudonocardia* and leafcutter ants has emerged from the finding that colonies of *Acromyrmex echinatior* tend to maintain a single strain of *Pseudonocardia*, either Ps1 or Ps2 (POULSEN & al. 2005, ANDERSEN & al. 2013). Cross-fostering experiments have suggested some degree of co-adaption between each particular *Pseudonocardia* strain and the ants that vertically-transmit them (ANDERSEN & al. 2015). A population of each of the two *Pseudonocardia* strains was genome sequenced, which allowed them to be identified as two distinct species, named *P. octospinosus* (Ps1) and *P. echinatior* (Ps2) (HOLMES & al. 2016).

Construction of a wider attine ant derived *Pseudonocardia* phylogeny revealed that the predicted model of vertical transmission which would result in coevolution and codivergence does not fit observed taxonomic trees based on 16S rRNA gene sequencing (MUELLER & al. 2010a). This suggests that *Pseudonocardia* strains either occur in other microbiomes of the ants (e.g., the guts) or that they can be free-living in the environment before they are acquired as ant symbionts, possibly to replace an existing *Pseudonocardia* symbiont whose secondary metabolites *Escovopsis* has become resistant to (POULSEN & al. 2010). *Pseudonocardia* strains have also been predicted to set up a complex partner choice mechanism using the antibacterial antibiotics that they produce to defend their primacy of place in the ant cuticular biofilms (SCHEURING & YU 2012). That scenario implies that only other actinobacteria with similar antibiotics production can invade *Pseudonocardia*
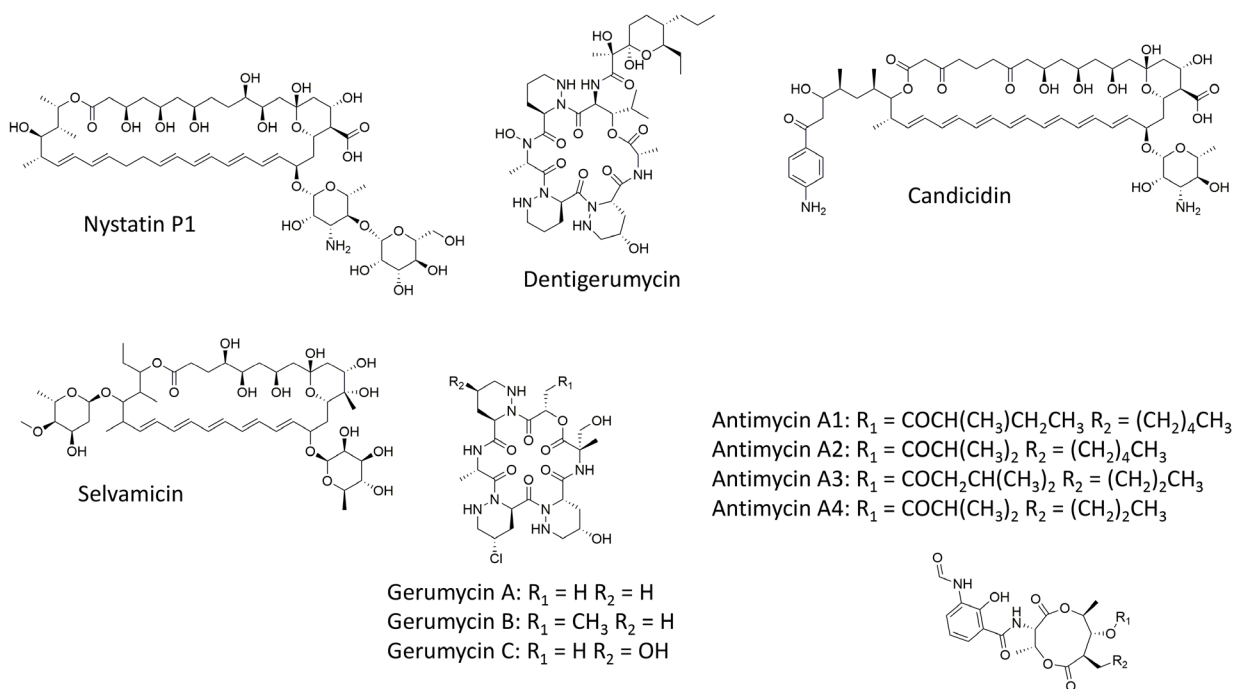
Fig. 5: Bioactive compounds produced by filamentous actinobacteria isolated from attine ants. *Pseudonocardia* isolated from various attine ants have been demonstrated to make antifungal polyenes such as nystatin P1 and selvamicin as well as the antifungal gerumycins. *Streptomyces* isolated from lab colonies of *Acromyrmex octospinosus* and *Trachymyrmex cornetzi* make the antifungals candicidin and the antimycins.

biofilms established by vertical transmission within colonies, because these producers are usually also resistant to multiple antibiotics (Barke & al. 2011).

Pseudonocardia-produced antifungal compounds

The first compound to be detected from a *Pseudonocardia* mutualist of attine ants was the antifungal dentigerumycin (Oh & al. 2009). Dentigerumycin is a cyclic depsipeptide made by a *Pseudonocardia* strain associated with the lower attine *Apterostigma dentigerum* in Panama. It is active against the nest pathogen *Escovopsis* and can be actively used by the ants to keep their fungus garden clear of *Escovopsis* infection (Oh & al. 2009). Dentigerumycin was later shown to be produced by another *Pseudonocardia* strain associated with Panamanian *Trachymyrmex cornetzi* ants (Sit & al. 2015), suggesting this molecule might also occur in fungus-farming ants with cuticular *Pseudonocardia*. Variants of dentigerumycins called gerumycins A-C are produced by another *Pseudonocardia* strain associated with *A. dentigerum* ants (Sit & al. 2015). Gerumycins A-C contain an almost identical depsipeptide to dentigerumycin but are distinguished by the absence of the PKS derived side chain (Fig. 5). The arrangement of genes in the biosynthetic gene clusters that encode these molecules show hallmarks of recent evolution (Sit & al. 2015). A full suite of PKS and NRPS genes are present in one chromosomal location for dentigerumycin but the PKS genes are absent and do not contribute to the final molecule for the gerumycins. Instead, the clusters are situated on mobile elements and are either spatially distributed on plasmids and split into pieces or located in so-called recently acquired "genomic islands" on the chromosome (Sit & al. 2015). The genes are likely under direct selective pressure to keep up in an arms race

with the *Escovopsis* strains that infect the fungus gardens that the ants maintain.

Pseudonocardia octospinosus (Ps1) isolated from a captive colony of *Acromyrmex octospinosus* ants was discovered to produce a nystatin-like polyene (Barke & al. 2010). Nystatin A1 was originally discovered from the terrestrial strain *Streptomyces noursei* and is widely used as an antifungal drug (Brautaset & al. 2000). *P. octospinosus* (Ps1) was demonstrated to make a similar molecule with an additional hexose sugar that was subsequently named nystatin P1 (Fig. 5). More recently, genome sequencing of *P. echinatior* and *P. octospinosus* populations associated with captive colonies of *A. echinatior* ants demonstrated they have matching nystatin polyene biosynthetic clusters (Holmes & al. 2016). *P. echinatior* strains also have nystatin-like biosynthetic gene clusters that do not resemble those encoding nystatin A1 or P1 biosynthesis and potentially encode as yet unidentified novel nystatin-like polyenes. Furthermore, a *Pseudonocardia* strain isolated from *Trachymyrmex cornetzi* also contained a biosynthetic gene cluster that encodes a polyene resembling nystatin P1 (Sit & al. 2015). A free-living terrestrial strain of *Pseudonocardia autotrophica* was also shown to make the polyene molecule NPP, which is similar to nystatin P1 (Kim & al. 2009), and it was shown that a similar additional hexose sugar made NPP 100x more soluble in water (Lee & al. 2012). Deletion of the additional glycosyl transferase gene in *Pseudonocardia autotrophica* and heterologous expression of the *nypY* glycosyl transferase gene from *Pseudonocardia* P1 resulted in addition of D-mannose which demonstrated lower antifungal activity than nystatin A1, but is presumably more soluble in aqueous solutions (Kim & al. 2017). This raises interesting questions as to the functional relevancy of these molecules

in different ecological settings (e.g., dry and wet habitats) that may be associated with different *Escovopsis* strains. Further evidence that polyenes are important antifungals in the attine fungus farming symbiosis comes from the discovery of the polyene selvamicin from a *Pseudonocardia* strain associated with *Apterostigma* ants (Van Arnam & al. 2016). Selvamicin is a structurally distinct (Fig. 5) and shorter polyene than nystatin A1, P1 or NPP. It has a 6-deoxymannose sugar added at the canonical glycosylation site, but it also has a distinctive 4-*O*-methyldigitoxose on the other side of the molecule. The genomes of *P. echinatior* and *P. octospinosus* strains also suggest evolutionary divergence in their nystatin-like biosynthetic gene clusters with a set of representative strains demonstrating subtle variations and individual clusters sometimes being split into separate loci (Holmes & al. 2016). The variety of polyenes and their encoding biosynthetic gene cluster arrangements are generally consistent with ongoing evolutionary selection pressure and may also suggest a certain degree of variation in the compounds that can be produced across different colonies of the same attine ant species.

Other ant-associated symbionts

A diversity of other antibiotic-producing bacterial species have been isolated from the cuticles of attine ants, some of which have also been shown to inhibit the growth of *Escovopis* and other pathogenic microbial species (Kost & al. 2007, Haeder & al. 2009, Sen & al. 2009, Barke & al. 2010, Schoenian & al. 2011). In particular, several members of the genus *Streptomyces*, which are common in soil, and produce ~55% of the antibiotics used in human medicine and husbandry, have been shown to associate with *Acromyrmex* leafcutter ants, although with a high level of variability across different colonies of the same ant species (Andersen & al. 2013). Such findings have led to the suggestion that leafcutter ants may be actively and dynamically recruiting other antibiotic-producing bacterial symbionts from their environment, following the initial colonisation of their cuticular chest plates with vertically acquired *Pseudonocardia* (Kost & al. 2007, Mueller & al. 2008, Barke & al. 2010, Andersen & al. 2013). The external morphology of the ant cuticular crypts may have evolved to facilitate this process (Mueller & al. 2008), but it remains to be seen how common such secondary acquisitions are under natural field conditions since most studies that have isolated sctinobacterial symbionts have been conducted using laboratory-maintained colonies of *Acromyrmex*. Secondary acquisitions of non-*Pseudonocardia* actinobacteria may give the ants access to a more diverse set of antimicrobial compounds, possibly enabling defence against a larger variety of pathogens (Barke & al. 2011), but it has also become clear that *P. echinatior* (Ps2) and *P. octospinosus* (Ps1) biofilms on the cuticles of Panamanian *Acromyrmex* workers differ in the degree to which they allow secondary invasion by other actinobacteria (Andersen & al. 2013).

It is not known how the ants accumulate, regulate and maintain their cuticular microbiome, allowing antibiotic producers to dominate, whilst preventing colonisation by non-producers. A recent theoretical model suggests that the cuticular microbiomes are regulated via a screening process, whereby the nutrient rich conditions surrounding the cuticular crypts on the surface of the ant sets up a highly competitive environment for colonising microorganisms

(Scheuring & Yu 2012). This would select for greater antibiotic production by both the native *Pseudonocardia* symbionts and any other bacteria able to invade. Since antibiotic producing bacteria also carry antibiotic resistance genes they are likely to preferentially survive in this demanding environment so the ants will always end up being covered with antibiotic-producing actinomycetes independent of their taxonomic identity (Scheuring & Yu 2012). Consistent with this, the *Pseudonocardia* symbionts have been shown to encode and produce a broad spectrum of antibacterial compounds (Holmes & al. 2016). *Streptomyces* found on leafcutter ants have been shown to make various antimicrobials including candicidin and antimycins (Fig. 5)(Haeder & al. 2009, Barke & al. 2010, Schoenian & al. 2011, Seipke & al. 2011), both of which have antifungal activity and are active against *Escovopsis*. A study using matrix-assisted laser desorption ionization (MALDI) on *A. echinatior* ants and their *Streptomyces* symbionts detected the production of antimycins, valinomycins and actinomycins (Schoenian & al. 2011). Valinomycin was also detected directly on the exterior of the ants, whereas valinomycin and actinomycins were identified from the waste dump. To our knowledge this is the only report showing antibiotics being produced *in situ* in a leafcutter ant colony, but whether *Streptomyces* species are associated with *Acromyrmex* colonies in the field remains to be confirmed.

In addition to cuticular symbionts, Panamanian *Acromyrmex* leafcutter ants also harbor a relatively simple community of gut bacteria. Four bacterial taxa (a *Wolbachia* species, a species from the order Rhizobiales and two Mollicutes species from the order Entomoplasmatales) dominate the gut microbiome (Sapountzis & al. 2015). *Wolbachia* bacteria appear to be obligate symbionts that are maintained across all developmental stages of *Acromyrmex* but not *Atta* leafcutter ants, and to be maternally transmitted because they could be retrieved from the eggs (Zhukova & al. 2017). They exist intracellularly, interacting closely with mitochondria in the ant cytoplasm (Zhukova & al. 2017), but can also be found extracellularly. Further work is required to clarify their functional significance. The functions of the Entomoplasmatales species are also unknown but they appear to be facultative symbionts as there is variation in their abundance across and within *Acromyrmex* colonies (Zhukova & al. 2017). Rhizobiales bacteria are confined to the gut lumen where they form biofilms along the hindgut cuticle (Sapountzis & al. 2015). These bacteria have been shown to produce bacterial NifH proteins that are normally associated with the fixation or preservation of nitrogen (Sapountzis & al. 2015). Although further confirmation is needed, it is hypothesised that these highly compartmentalised symbionts somehow help to alleviate the nutritional constraints that emerge from an exclusive fungal diet which, in turn, is provisioned solely with leaf material (Zhukova & al. 2017). Finally, the bacterial genus *Enterobacter* was shown to be abundant in larval guts of both *Acromyrmex* and *Atta* but to be absent in the guts of adult workers. It has been suggested that these may be involved in immune priming, reducing the susceptibility of larvae to pathogens that they may encounter later in life (Zhukova & al. 2017).

In addition to symbionts found on or within the ants, strains of antibiotic producing bacteria have also been isolated directly from the fungus gardens of leafcutter ants. For example, a member of the bacterial genus *Burkholderia* was isolated from the garden material of *Atta sexdens*

rubropilosa (Santos & al. 2004). Isolates of this species had potent anti-fungal activity against entomopathogenic fungi as well as the specialist pathogen *Escovopsis weberi* (Santos & al. 2004), while not inhibiting the growth of the mutualistic cultivar fungus (Santos & al. 2004). These results suggest that a broad consortium of bacteria may contribute to fungal garden defence including both those associated with the ants and the fungus gardens.

Other putative pathogens

As well as *Escovopsis*, there are additional reports of other exploiters of the attine fungus-farming symbiosis, despite the production of antibiotics by mutualistic bacteria associated with the ants and defensive MG secretions. Black yeast in the genus *Phialophora* were shown to live on the body of attine ants exploiting the resource base meant to nourish mutualistic actinobacteria (Little & Currie 2007). Their presence matches a wider observation of black yeasts in the order Chaetothyriales that have been isolated from across the family of ants (Formicidae), including in species that maintain carton nests or domatia (Voglmayr & al. 2011, Vasse & al. 2017). *In vitro* experiments suggested that the black yeast associated with *Apterostigma* can outcompete *Pseudonocardia* for nutrients as well as directly parasitise the cuticular actinobacteria (Little & Currie 2008), although the interaction seems complex. The black yeast was unable to directly affect the ants or the *Leucoagaricus* fungus garden, but their presence made the colony as a whole more susceptible to *Escovopsis* infection, presumably because *Pseudonocardia* defence was compromised. Black yeasts have predominantly been found in extreme environments (De Hoog 2014) and their ability to withstand those environments may allow them to survive on attine ants where they are likely to encounter metapleural gland secretions and symbiont derived secondary metabolites (Little & Currie 2008).

Other yeasts have also been documented to be associated with attine ants (Craven & al. 1970, Carreiro & al. 1997, Pagnocca & al. 2008, 2010, Rodrigues & al. 2009, Mendes & al. 2012). These yeasts likely play a role in detoxification of the plant material, which would benefit both the *Leucoagaricus* cultivar and the farming ants (Mendes & al. 2012). They may also play a protective role in preventing other fungal pathogens from getting a foothold in the nest (Rodrigues & al. 2009). Yeasts and filamentous fungi were also found on the body of gynes (dispersing virgin queens), whereas *Escovopsis* was not present, suggesting that they are transported in a passive fashion to new nests (Pagnocca & al. 2008).

Filamentous fungi other than *Escovopsis* have also been observed in the nests of attine ants (reviewed by Pagnocca & al. 2012). When the farming ants are removed from nests the fungus gardens are quickly overgrown by other fungi. This is believed to involve mainly opportunistic species, whereas *Escovopsis* endures as a specialised infectious agent for a greater length of time in the presence of ants tending the nest. The presence of additional fungal species is most likely connected to the live plant material used to grow *Leucoagaricus* cultivars, suggesting that the colony-wide microbiome of fungus gardens is not fully static and determined to some extent by the forage material available to the colony (Fisher & al. 1996). Another potential pathogen of attine ants are filamentous *Syncephalastrum* fungi, which have been shown able to infect laboratory colonies

of *Atta sexdens rubropilosa* (Barcoto & al. 2016), though the importance of these fungi in infecting wild attine ant colonies is unknown.

Finally, *Ophiocordyceps* fungi have been well documented to infect ants of the Camponotini tribe causing the well-known "zombie ant" phenomenon (Hughes & al. 2011, de Bekker & al. 2014), but they have also occasionally been found to infect leafcutter ants both in natural settings and laboratory experiments (Hughes & al. 2009). Infections were documented from several Panamanian isolates, some of which induced the characteristic *Ophiocordyceps* stroma growing out of the back of the head. Although rare, it does suggest that *Ophiocordyceps* may be able to overcome taxonomic barriers to infect a wider range of ant genera. As we mentioned above, both *Ophiocordyceps* and *Escovopsis* share the ability to produce alkaloids, which can act as ion channel blockers during infection (de Bekker & al. 2015, Heine & al. 2018).

Summary of recent insights in symbiotic partnerships of leafcutter ants

The attine ant fungus-farming symbiosis is one of the best-known examples of obligate multipartite mutualism and has been intensively studied for more than 25 years. During the last ten years, advanced molecular techniques have allowed a series of novel insights in the chemical characteristics of the evolutionary arms race between the mutualistic partners and their fungal parasites in the genus *Escovopsis*. It is becoming increasingly clear that these arms race dynamics are likely to drive complex co-adaptation processes even though most studies continue to be based on two-partner interactions. The intricacy of novel discoveries leave little doubt that the attine fungus-growing ants will continue to provide a cutting edge and experimentally tractable model, not only for studying mutualisms and microbiome formation, but also for analysing multi-partite co-evolutionary interactions. The recent work that this review is primarily focused on shows that the specialised cultivars and *Pseudonocardia* mutualists make potent antifungals that can be effective in controlling parasitic *Escovopsis* mycoparasites. However, the *E. weberi* strains that specialise on infecting the gardens of the most derived genera, *Atta* and *Acromyrmex*, have streamlined genomes able to maintain virulence factors and toxins such as shearinines. These compounds can kill the cuticular *Pseudonocardia* bacteria that assist *Acromyrmex* species in controlling *Escovopsis* infections while also adversely affecting foraging, grooming and weeding behaviour of *Atta* and *Acromyrmex* workers. Despite these recent advances there is still much to be clarified. Just a handful of the secondary metabolites encoded by *Escovopsis* species have been characterised and we still know nothing about the putative secreted or volatile compounds produced by the *Leucoagaricus gongylophorus* cultivar that inadvertantly induce accelerated growth of *Escovopsis* hyphae towards host fungus gardens. Neither do we know what compounds the tiny subcuticular glands that nourish cuticular actinobacteria produce. In addition, much of the knowledge gained about the chemical ecology of this complex mutualism has been obtained via *in vitro* experiments and needs to be validated under field conditions. Recent technological advances, including stable isotope probing combined with next generation sequencing and imaging mass spectrometry, should now allow the study of these molecules *in vivo* to exactly determine the chemical

interactions that occur between these multiple symbiotic partners. We expect that future studies will reveal an even greater breadth of chemical compounds produced by the various mutualists and parasites involved in the attine ant fungus-farming symbiosis, and will help to unravel their adaptive functions in much greater detail than possible at present.

Acknowledgements

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