

# Hotspots for symbiosis: function, evolution, and specificity of ant-microbe associations from trunk to tips of the ant phylogeny (Hymenoptera: Formicidae)

Jacob A. RUSSELL, Jon G. SANDERS & Corrie S. MOREAU



## Abstract

Ants are among the world's most abundant and dominant non-human animals. Yet in spite of our growing knowledge of microbes as important associates of many animals, we have only begun to develop a broad understanding of the ants' microbial symbionts and their impacts across this diverse family (Hymenoptera: Formicidae). With an impressive degree of niche diversification across their ~ 140 million year history, evolution has performed a range of natural experiments among the ants, allowing studies of symbiosis through a lens of comparative biology. Through this lens it is gradually becoming clear that specialized symbioses can be gained or lost in conjunction with important shifts in ant biology, ranging from dietary ecology to investment in chemical defense. Viewing symbiosis across the ant phylogeny has also lent an additional insight – that the presence of specialized and ancient microbial symbionts is a patchily distributed attribute of ant biology. In fact, recent evidence suggests that several groups of ants harbor very few microbial symbionts – at least those of a eubacterial nature. These combined findings raise the possibility that the importance of symbiosis has fluctuated throughout the evolutionary history of the ants, making "hotspot" lineages stand out amongst potential symbiotic coldspots. In this review, we discuss these phenomena, highlighting the evidence for symbiont turnover and symbiotic hotspots that has accumulated largely over the past decade. We also emphasize the types of bacteria and fungi that can be found more sporadically across a range of ants, even beyond apparent hotspot taxa. An emerging theme from the literature suggests that several ant-enriched or ant-specific microbial lineages are common associates of far-flung hosts from across this insect family, suggesting that a recurring symbiotic menagerie has sojourned across the ants for quite some time. In summary, the weight of the evidence suggests the importance of symbiosis across several ant taxa, supporting a growing appreciation for the major role of symbiosis in animal biology. Yet apparently low-density bacterial communities in some ants raise questions about symbiont ubiquity and the forces governing the microbial populations that colonize these hosts and the world's many eukaryotes beyond.

**Key words:** Symbiont, microbiome, specialization, cospeciation, mutualism, *Wolbachia*, nitrogen-recycling, review.

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*Jacob A. Russell (contact author), Department of Biology, Drexel University, Philadelphia, Pennsylvania, 19104, USA.  
E-mail: jar337@drexel.edu*

*Jon G. Sanders, Department of Pediatrics, University of California San Diego, La Jolla, California, 92093, USA.*

*Corrie S. Moreau, Department of Science and Education, Field Museum of Natural History, Chicago, Illinois, 60605, USA.*

## Introduction

While recombination and gene duplication are distinguishing tools in the adaptive arsenals of eukaryotes, symbioses with microorganisms have also contributed major innovations toward eukaryotic evolution. This is evidenced today by the large numbers of plants, animals, fungi, and protists relying upon intimately associated microbes, called symbionts, for various biological functions. The successes of these associations can be seen in the vast diversity and biomass of eukaryotes exploiting niches made accessible by their microbiota, whether these be deep sea hydrothermal vents, nitrogen-limited diets, or oxygen-rich environments. With technological advances and improving phylogenetic infrastructure, we are beginning to appreciate the variety and nature of these eukaryote-microbe symbioses across model and non-model organisms alike (RUS-

SELL & al. 2014). And as such, we are gaining insight into the fascinating stories of fidelity, function, and integration that characterize at least a subset of nature's host-microbiome interactions (DOUGLAS 2010).

With expanding notions of symbiont ubiquity and influence (MCFALL-NGAI & al. 2013), it is important to consider just where we do understand: 1) the compositions of symbiotic communities; 2) whether animal-associated microbes are highly specialized, long-standing symbionts or whether they colonize other hosts or free-living habitats; and 3) the contributions made by symbionts toward eukaryotic host function. In the functional realm, 'omics-scale technologies have begun to transform our knowledge base (ENGEL & al. 2013, SANDERS & al. 2013); but even as this revolution unfolds, our understanding of symbiont

## Box 1: Symbiosis, mutualisms, and holobionts.

Symbioses are intimate and prolonged interactions between different species. It is now virtually a truism to state that symbioses are highly important to eukaryotes. But the apparent ubiquity of symbiotic interactions encompasses a vast range of interaction types and strengths. Simple interactions can sometimes be empirically characterized, as when binary partners are separated and assayed independently (FELDHAAR & al. 2007). More complex systems, such as extracellular gut microbial communities, will necessarily be more difficult to fully deconstruct. Difficulty in characterizing more complex interactions does not mean they cannot have important or even profound fitness consequences. However, this complexity can obscure the particular and even contradictory roles being played by different symbiotic constituents. Consequently, the nature of the functional and fitness relationships between symbiotic partners in these systems needs to be considered with great caution – not assumed.

Simple pairwise interactions between partners have frequently been considered in a theoretical framework of costs and benefits that borrows from economics and game theory (ARCHETTI & al. 2011). By modeling interactions as exchanges of costs and benefits, a formal taxonomy of interaction types can be proposed, ranging from purely negative (amensalism or spite) to exploitative (parasitism) to mutually beneficial (mutualism or cooperation).

Of these, mutualisms in particular have received a great deal of study, both because of their apparent ubiquity and importance, and because they present an apparent evolutionary conundrum: If benefits provided to another organism are costly, what prevents selection from favoring "cheaters" that skip out on paying that cost (SACHS & al. 2004)? A number of highly integrated symbioses have been shown to match theoretical resolutions to this problem, with mechanisms for partners to correctly identify and bond with symbionts across generations. Some interactions maintain faithful symbiont passage from parent to offspring (i.e., vertical transmission), establishing partner fidelity and aligning host and symbiont evolutionary interests (SACHS & WILCOX 2006). In other, environmentally acquired symbioses, hosts screen symbionts through partner choice mechanisms (NYHOLM & MCFALL-NGAI 2004, KALTENPOTH & al. 2014); symbiont-relevant functions may then be policed through host-delivered rewards and sanctions (KIERS & al. 2003, SALEM & al. 2015).

With the growing recognition that important interactions with symbiotic microbes could be virtually ubiquitous (MCFALL-NGAI & al. 2013), it is attractive to translate these concepts for 1:1 species interactions to the more common, multi-species symbiotic systems. Given that an organism's phenotype (and consequently its fitness) depends not just on its own genotype but on its interactions with symbionts, we can consider the phenotypic expression of the whole as a "holobiont". To the extent that the genomes of these interacting organisms are somehow coordinated or integrated, the genetic antecedent to this collective phenotype can be described as the "hologenome".

The degree to which these concepts are accurate models of biological reality, or even useful ones, is a matter of vigorous recent debate (MORAN & SLOAN 2015, DOUGLAS & WERREN 2016, MCFALL-NGAI 2016, ROSENBERG & ZILBER-ROSENBERG 2016, THEIS & al. 2016). Of particular concern is whether the potential for mismatches in interests among symbiont community members render a singular descriptor meaningless, and, further, whether loose or nonexistent cross-generational linkages among symbiont genomes mean that the collective hologenome cannot be considered an evolutionary unit, as evolution requires both selection and heritability. Indeed, a strong case can be made that labels of mutualism and parasitism cease to be useful for complex symbiotic communities, where the strength and valence of many interactions may be heavily contingent on the composition of the community (MUSHEGIAN & EBERT 2015).

Symbioses between ants and microbes appear to span much of this range of complexity and integration. While some (especially on the simpler end) may be amenable to classification into categories of mutualism and parasitism, many may not – and all such interactions may have fitness effects that vary across environments or among related species.

function remains fairly limited. Our comprehension is firm for eukaryotes with strong medical, agricultural, or economic relevance (CHU & al. 2013, WANG & al. 2013, BENNETT & MORAN 2015, BRUNE & DIETRICH 2015, MORAN 2015); for those with conspicuous, long-recognized symbioses (WEST & al. 2002, NYHOLM & MCFALL-NGAI 2004, GOODRICH-BLAIR 2007, FELLBAUM & al. 2012, HENTSCHER & al. 2012); and for those with lifestyles amenable to laboratory research (DOBSON & al. 2015). Yet on the whole, we lack awareness of what host-associated microbes are doing in a daunting range of eukaryotic taxa, including those in hosts as important and ubiquitous as mycorrhizal fungi (DESIRO & al. 2014).

Mysteries enshrouding function have not stopped organismal biologists from cataloguing the composition of symbiotic communities (KALTENPOTH & STEIGER 2014) or from studying symbiont distributions over space, time, and host phylogeny (BALDO & al. 2008, HIMLER & al. 2011). Simple and affordable techniques like PCR screening and Sanger sequencing have long facilitated these goals, while more recent innovations such as next generation amplicon sequencing of conserved ribosomal RNA genes have made major contributions. Such approaches have told us much about the ecology and evolution of symbioses across a growing range of eukaryotes, establishing a basic comprehension in groups with under-studied function. Phylo-

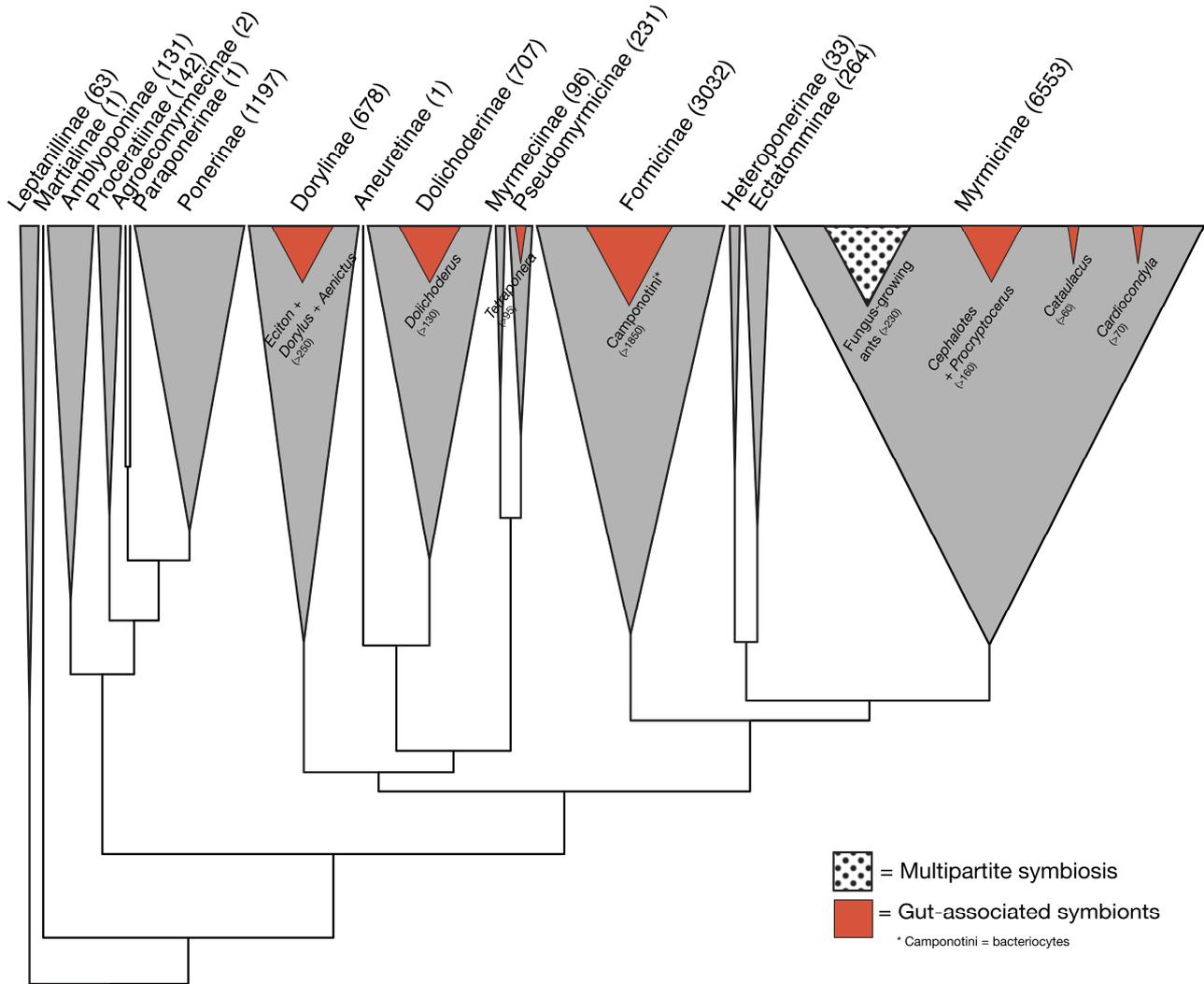


Fig. 1: Symbioses with specialized bacteria or fungi are highly prevalent or ubiquitous within several diverse clades of ants. Lineages with stable and ancient symbioses are highlighted on this ant phylogeny (modified from MOREAU & BELL 2013), and information on ant clade diversity is provided (BOLTON 2014). Note that the fungus-growing (i.e., attine) ants' symbioses are different than those of the remaining focal ant groups, which harbor bacteria within the gut cavity or in specialized cells called bacteriocytes (i.e., *Blochmannia* in the Camponotini, and *Westeberhardia* in *Cardiocondyla*). While attines, camponotines, and *Cephalotes* and *Procrystocerus* appear to engage in ubiquitous symbioses, specialized army ant-associated gut symbionts vary in prevalence across the AenEcDo sub-group of the Dorylinae, while the phylogenetic limits of core gut symbionts across *Cardiocondyla*, *Cataulacus*, *Dolichoderus*, and *Tetraponera* remain somewhat undefined. Beyond these groups *Wolbachia* appear concentrated within several ant taxa, suggesting the potential for the "hotspot" list to grow. While we recognize the likelihood for future documentation of integrated, persistent, specialized symbioses in other ant groups (e.g., *Formica*; *Plagiolepis*), the above focal lineages stand out in comparison to many ants with low bacterial densities and a lack of evidence for specialized, ancient, and integrated symbioses.

genetics has also been a powerful tool. Not only has it helped to identify correlations between host ecology and symbiosis (DELSUC & al. 2014, MIKAELIAN & al. 2015). It has also aided in understanding the dynamics of symbiosis, with revelations as to whether hosts and microbes engage in loose, fairly specialized, or near-exclusive pairings for substantial time periods (SCHILTHUIZEN & STOUTHAMER 1997, CLAY & SCHARDL 2002, TAKIYA & al. 2006). In addition to these molecular and analytical approaches microscopy has long been a powerful tool for the study of symbiosis, elucidating mechanisms of symbiont acquisition and integration (NYHOLM & MCFALL-NGAI 2004, OHBAYASHI & al. 2015), and hence illuminating how mu-

tualisms with environmentally acquired microbes can represent evolutionarily stable strategies (Box 1).

With growing applications of these diverse tools to study symbioses, it comes with some surprise that there remains a good deal of symbiotic dark matter, and not just in a functional sense. Indeed, we lack an understanding of the identities of symbiotic partners and how they vary over space and time across numerous inconspicuous eukaryote hosts, especially fungi and protists. But even well-studied macroscopic animal taxa can fit this billing, including ants, for which we have developed just a modest degree of symbiotic knowledge across a diverse range of potential hosts. A group with ~ 140 million years of evo-

lutionary history (BRADY & al. 2006, MOREAU & BELL 2013, MOREAU & al. 2006), ants (Hymenoptera: Formicidae) have evolved an impressive variety of diets and lifestyles, undergoing drastic transformations in colony size, caste specialization, and species interactions. As they have diversified and radiated across the world's terrestrial niches several ants have evolved symbiotic habits. But beyond historically well-studied groups like the fungus-growers or carpenter ants, knowledge on the nature and functions of symbioses between ants and microbes has been slow to accumulate. A recent wave of publications has begun to change this, painting ants as a fascinating system for the broader understanding of symbiosis. Indeed, microscopy-, molecular-, and systematics-based studies have uncovered a good deal on the ecology and evolution of ant-microbe symbioses, establishing function as the next great frontier.

In this review, we summarize recent research on ant-microbe symbioses, picking up where Zientz and colleagues left off in their 2005 review on this same topic (ZIENZT & al. 2005). While we aim to report on the breadth of known ant-microbe associations, we also discuss an emerging theme from our observations – that ant-microbe symbioses may often not reach the levels of ubiquity, integration, or importance that are assumed to characterize animals and their microbiomes (ZILBER-ROSENBERG & ROSENBERG 2008). Indeed, several diverse, deep-branching ant taxa with highly prevalent and conserved symbioses are scattered across the Formicidae phylogeny amidst lineages with little evidence for such associations. We highlight where such symbioses appear concentrated and how they have changed over time in conjunction with ant biology. Our more suggestive conclusion, however, is that microbial symbionts of a eubacterial nature may be "influential passengers" (O'NEILL & al. 1998) in just a subset of these successful social insects.

### **Ancient, specialized, integrated**

Research across the ants long ago identified the existence of conspicuous, microbially based symbioses (BLOCHMANN 1888). Hosts of symbiotic microbes have often stood out due to their behavior, derived anatomy, and large resident microbial masses (HÖLLDOBLER & WILSON 1990, COOK & DAVIDSON 2006, CURRIE & al. 2006). But with the application of molecular and phylogenetic tools we now see that they stand out in another way: Their symbioses involve a limited range of host-restricted microbes which have engaged with ants for eons, with up to ~ 40 - 55 million years of specialized partnerships. One species-rich taxon that aptly fits this description includes the fungus-growing ants, formerly of the tribe Attini (a.k.a. attines). The hyper-diverse carpenter ants and their relatives (tribe Camponotini, a.k.a. camponotines) make up a second such group, while the sister genera *Cephalotes* and *Procryptocerus* of the former tribe Cephalotini (a.k.a. cephalotines) show a roughly similar history of ubiquitous and specialized symbioses. A symbiosis with resemblance to that seen in camponotines has recently been described for ants from the genus *Cardiocondyla*, and while the age of this association remains unclear, the intracellularity and transovarial transmission for the participating microbes (KLEIN & al. 2015) suggest levels of integration and specialization typical of other ant hotspots.

Beyond these three groups, we also see evidence for specialized symbiotic associations in army ants from the subfamily Dorylinae. While their possibly specialized microbes show lower ubiquity, their phylogenetic depth and breadth of distributions suggest relationships that could date in excess of ~ 80 million years (FUNARO & al. 2011). Finally, based on microscopy and molecular findings to date, diverse ants from three disparate genera – *Catantolus*, *Tetraponera*, and *Dolichoderus* – are proposed to engage in enriched, specialized symbioses with bacteria. While all groups seem united by persistent symbioses, these proposed symbiotic "hotspots" (highlighted in Fig. 1) show key differences in the functions (when known), localizations, and, in most cases, the identities of their microbial symbionts. To emphasize just how symbioses have changed and functioned over time, we devote the next sections of our review to a discussion of these symbiotic hotspots. We then zoom out to consider both the possibility of symbiotic coldspots and the broader landscape for symbiosis across the remaining Formicidae.

### **Fungus-farming ants and their multi-partite symbioses**

**An agricultural symbiosis:** With their long history of study, fungus-growing ants (former tribe Attini) represent one of the more charismatic examples of symbiosis in the animal kingdom. A Neotropical group with over 200 species, these ants have cultivated basidiomycete fungi for food for roughly 55 million years (SCHULTZ & BRADY 2008, WARD & al. 2015). Nearly all fungal cultivars come from the Agaricaceae (formerly Lepiotaceae; a.k.a. gilled mushrooms), while attines from one species group in the genus *Apterostigma* have domesticated unrelated basidiomycetes from the Pterulaceae (coral mushrooms) (MUNKACSI & al. 2004). These intimate interactions involve ant cultivation of fungal monocultures (MUELLER & al. 2010) that are seeded by founding queens who acquired the fungus in their natal colonies (HÖLLDOBLER & WILSON 1990). Such vertical transmission helps to align ant and cultivar interests, and to promote stable, mutualistic associations over time (Box 1). While some horizontal transfer and repeated domestication of free-living fungi have occurred (VO & al. 2009, MEHDIABADI & SCHULTZ 2010), attines and their cultivars do exhibit a modest degree of lineage tracking, such that related cultivars tend to partner with related hosts (SCHULTZ & al. 2015). The causes of such tracking are unclear. Possible explanations include partner choice, driven by tendencies of major workers to eliminate alien fungal cultivars from their nests (IVENS & al. 2009). Costs of cultivating novel cultivars – reflecting, perhaps, ant adaptations toward specific cultivar lineages – may also slow the rates of partner swapping (SEAL & MUELLER 2014). Yet switching to novel cultivars could occasionally be beneficial (KELLNER & al. 2013), raising questions about the net effects of colony-level natural selection in shaping long-term trends of ant-cultivar lineage tracking.

Fungal cultivars are "fed" food substrates by their ant hosts, which can vary from fresh leaves and flowers in the case of the leaf-cutters, to a mix of arthropod frass, arthropod carcasses, fruits, flowers, and seeds in attine taxa beyond (DE FINE LICHT & BOOMSMA 2010). In turn, the cultivar fungi help to break down complex carbohydrates contained within such substrates, making carbon more readily available while further enhancing the availability of

nitrogen and lipids for at least some fungus farmers (PINTO-TOMAS & al. 2009, SUEN & al. 2010, DE FINE LICHT & al. 2014). The associations between higher attine lineages (e.g., *Sericomyrmex*, *Trachymyrmex*, *Atta*, and *Acromyrmex*) and their cultivars have been argued to exhibit features consistent with greater specialization and coevolution. Central to these points are the gongylidia produced by their cultivar fungi. These swollen hyphal structures are argued to provide heightened nutrition to ant hosts (DE FINE LICHT & al. 2014) and higher attines, including leaf-cutters, use fecal droplets to deposit enzymes derived from gongylidia within their gardens (RONHEDE & al. 2004, KOOIJ & al. 2014). Some of these enzymes further aid in the breakdown of garden substrate; others detoxify plant-borne compounds that would otherwise be harmful to the ants (DE FINE LICHT & al. 2013). While the exclusivity and mode of shared evolutionary histories are not fully resolved for the leaf-cutters and their cultivars (MIKHEYEV & al. 2010), it is intriguing to see such highly modified fungi and ant behaviors are associated with these ants' use of fresh leaf material and with major transitions in colony size, caste specialization, and life span. Indeed it is arguably this symbiosis that has allowed leaf-cutters to become major defoliators within tropical systems (HERZ & al. 2007).

**Antagonists of the mutualism:** Evolving in association with the attine-fungal mutualism are specialized weed-like pathogens from the genus *Escovopsis* (Fungi; Ascomycota; Sodiariomycetes). Found, thus far, exclusively within attines' fungus gardens, these pests can overgrow cultivar fungi within gardens, inhibiting the ants' production of food (CURRIE & al. 1999a). While they are nearly ubiquitous associates of the attine-fungus mutualisms, *Escovopsis* have not been found in the gardens of yeast agriculturalists from the genus *Cyphomyrmex* (MEHDIABADI & SCHULTZ 2010), and they may be rare in at least some taxa beyond (ISHAK & al. 2011a). Fine-scale specialization was argued to characterize these antagonisms (e.g., GERARDO & al. 2006a), with early reports of *Escovopsis* lineages tracking those of the ant host and fungal cultivar (CURRIE & al. 2003, GERARDO & al. 2004). But lineage tracking is certainly not absolute, and *Escovopsis* can switch hosts in the lab and field (CURRIE & al. 2003, GERARDO & al. 2006b, TAE-RUM & al. 2007, MEHDIABADI & SCHULTZ 2010). Furthermore, recent studies with broader sampling regimes suggest even less specialization than originally reported (MEIRELLES & al. 2015).

It is clear, then, that more work is needed to understand *Escovopsis* diversity and the potential for pathogen specialization, whether governed by ant or fungal cultivar attributes. What is also clear is that the natural impacts of *Escovopsis* are not well understood. While certainly capable of parasitic effects in the lab (CURRIE 2001), comparable field studies are needed to appreciate whether this prevalent fungus places strong pressures on attines and under which particular environmental conditions. For example, *Escovopsis* may be rare or absent from some attine species or populations (RODRIGUES & al. 2011, MEHDIABADI & SCHULTZ 2010, ISHAK & al. 2011a). Furthermore, surveys across host species and geographic locales have uncovered diverse microfungi in a substantial fraction of attine gardens (RODRIGUES & al. 2008, RODRIGUES & al. 2011), revealing that *Escovopsis* are not the only non-cultivar fungi within these communities. Finally, findings

that common non-*Escovopsis* microfungi are associated with garden decline under lab conditions (SEAL & MUELLER 2014) raise the possibility that pressures by alternative pathogens may be an important part of attine-cultivar ecology and evolution.

**A specialized defensive, biological control mutualism?** While the challenges of demonstrating symbiotic function in a natural context are well-appreciated (OLIVER & al. 2014, but see JAENIKE & al. 2010), it is at least unequivocal that the attine-fungus symbiosis is crucial to the life and death of attine ants and, for leaf-cutters, perhaps their highly specialized fungi as well. The unclear life-or-death relevance of *Escovopsis* pathogens in the field is, contrastingly, a partial hindrance to understanding a third major player, or group of players, in the attine-fungal cultivar symbioses: antibiotic-producing cuticular actinomycetes (CALDERA & al. 2009). Proposed as a major arm of attine fungal garden defense (CURRIE & al. 1999b), these biocontrol agents would seem to complement other attine mechanisms for weed control, including garden grooming and weeding during growth stages (CURRIE & STUART 2001), along with behavioral processing of garden substrates prior to placement in gardens (DE FINE LICHT & BOOMSMA 2010). While the specifics of these cuticular symbioses are not fully clear, there are several emerging trends that at least help to define important areas for future research.

One unequivocal attribute of attine biology is that workers typically possess a white coating of bacteria on their cuticles, which are often most visible on the laterocervical plates of the ventral thorax in some attine lineages. It is here where the bacteria colonize crypts connected to ducts from nearby glands (CURRIE & al. 2006). These glands are proposed to nourish the bacteria and could even go some ways toward promoting the preferential retention of antibiotic producing symbionts. In so doing, they may serve as a mechanism for both partner choice and sanctioning of cheaters (SCHEURING & YU 2012).

While conspicuous in appearance in most attines except in *Atta* and *Sericomyrmex* (CURRIE & al. 1999b, MUELLER & al. 2008, FERNANDEZ-MARIN & al. 2009), the identities and roles of these cuticular bacteria have been debated in the literature. Initially described as members of the genus *Streptomyces* (CURRIE & al. 1999b), it was later discovered that many of the dominant, cultivable cuticular microbes belong to the genus *Pseudonocardia* (CAFARO & CURRIE 2005). These bacteria produce antibiotics that can suppress the growth of *Escovopsis* in vitro (CURRIE & al. 1999b), an observation that has been used to suggest a major role for *Pseudonocardia* in garden defense. But while single *Pseudonocardia* strains may dominate some cuticles and colonies (ANDERSEN & al. 2013; Tab. 1), other antibiotic-producing bacteria are certainly found in and on various attine workers and their gardens. Furthermore, *Pseudonocardia* can suppress a range of fungi in addition to *Escovopsis* in in-vitro-assays, including their own fungal cultivars (SEN & al. 2009, BARKE & al. 2010). On top of this, *Pseudonocardia* have been retained in *Cyphomyrmex* ants with yeast agriculture, in spite of the likely absence of *Escovopsis* from yeast fungus gardens (MEHDIABADI & SCHULTZ 2010). Some *Pseudonocardia* also show relatedness to free-living bacteria from the environment (CAFARO & CURRIE 2005, MUELLER & al. 2008, MUELLER & al. 2010), indicating that there is no single

Attine species	Colony	#/age/ origin	Abundant 16S rRNA sequence groupings								
			Pseudonocardia Ps1 (Otu001)	Pseudonocardia Ps2 (Otu002)	uncl. Actinomycetales (Otu003)	Pseudomonas (Otu004)	Staphylococcus (Otu005)	uncl. Entomoplasmatales (Otu006)	Pelomonas (Otu007)	uncl. Rhizobiales (Otu008)	all other OTUs
<i>Acromyrmex echinator</i>	150	1/C/L	0.0015	0.9635	0.0015	0	0.0039	0.0078	0	0.0029	0.0190
		1/M/F	0.0006	0.8264	0.0202	0.0092	0	0	0.0024	0.0006	0.1406
		1/M/L	0.0015	0.9458	0.0021	0	0.0036	0.0072	0.0015	0	0.0382
	160	1/C/L	0.9704	0.0005	0	0	0	0.0072	0	0	0.0220
		1/M/F	0.6903	0	0.0540	0.0079	0	0.0002	0	0.0002	0.2474
		1/M/L	0.8871	0.0012	0.0460	0	0.0039	0	0.0019	0	0.0600
	280	1/C/L	0.0095	0.9583	0.0011	0	0	0.0032	0.0005	0.0079	0.0195
		1/M/F	0.0081	0.2960	0.0118	0.0978	0	0	0	0.0004	0.5859
		1/M/L	0.0036	0.4409	0.0755	0	0	0.2851	0	0	0.1949
	282	1/C/L	0.9848	0	0.0012	0	0.0015	0	0	0	0.0125
		1/M/F	0.9502	0.0005	0.0005	0.0059	0.0024	0.0003	0	0	0.0402
		1/M/L	0.9023	0	0.0049	0	0.0007	0.0533	0.0007	0.0078	0.0304
	322	1/C/L	0.0268	0.9338	0.0020	0	0	0.0020	0.0007	0	0.0348
		1/M/F	0.0031	0.6496	0.0038	0	0	0.0244	0	0	0.3191
		1/M/L	0.0072	0.9398	0.0262	0	0	0.0018	0	0	0.0250
	342	1/C/L	0.0015	0.8158	0	0	0	0	0	0.1254	0.0573
		1/M/F	0	0.0619	0.0004	0.0023	0	0	0.0002	0	0.9351
		1/M/L	0	0.9110	0	0	0	0	0.0021	0.0186	0.0683
	356	1/C/L	0.0004	0.9388	0.0017	0	0.0017	0	0.0037	0	0.0537
		1/M/F	0.0013	0.3611	0.0432	0.1539	0.0200	0	0	0	0.4205
		1/M/L	0.0077	0.8667	0.0384	0.0141	0	0	0.0008	0	0.0723
480	1/C/L	0.0010	0.9453	0	0	0.0020	0.0108	0	0.0020	0.0391	
	1/M/F	0.0002	0.0850	0.0444	0.0081	0.0018	0.0564	0.0002	0	0.8039	
	1/M/L	0.0126	0.5289	0.0108	0	0.0027	0.1540	0.0010	0.0609	0.2291	
528	1/C/L	0.9824	0	0.0004	0	0	0.0018	0	0	0.0154	
	1/M/F	0.7747	0	0.0362	0	0	0	0.0008	0	0.1882	
	1/M/L	0.9589	0	0.0057	0	0	0	0.0057	0	0.0298	
<i>Acromyrmex volcanus</i>		1/M/L	0.6152	0.1470	0.0526	0.0345	0.0381	0.0181	0.0109	0.0091	0.0744
<i>Cyphomyrmex costatus</i>		5/M/L	0.0002	0.0998	0.0119	0.2380	0.0012	0	0.0007	0.0002	0.6480
		5/M/L	0.0591	0.5742	0	0.0853	0.0028	0	0.0011	0.0006	0.2769
<i>Cyphomyrmex longiscapus</i>		3/M/L	0	0.0001	0	0.0006	0	0	0	0	0.9993
<i>Trachymyrmex zeteki</i>		3/M/L	0.0012	0.0003	0	0.0025	0.0018	0	0	0	0.9942
		3/M/L	0.0028	0.0001	0	0.0040	0.0001	0.0041	0	0.6085	0.3804

Tab. 1: Laterocervical plates of leaf-cutter ants are dominated by colony-specific *Pseudonocardia* strains. Data are from ANDERSEN & al. (2013). For *Acromyrmex echinator*, the focal species, we include only those data from colonies with three sampled workers, organizing these to show variation between colonies. Each row presents the 16S rRNA amplicon sequence data from the laterocervical plates of a single worker from the lab or field and at either the immature (callow) or mature adult stage. Information in column 3 (# / age / origin) shows the number of workers sampled for each sequence library, whether workers were immature ("C") or mature ("M"), and whether the colony came from the lab ("L") or was sampled directly from the field ("F"). Numbers in cells are the proportions of 454 16S rRNA amplicon sequence reads classifying to the various 97% OTUs represented by each column. Data are shown for only the eight most abundant OTUs. The first two columns represent the two dominant *Pseudonocardia* 97% OTUs. In more fine-scaled analyses it appeared that each is made up by a single strain, or sequence type, after accounting for sequencing error. Colonies were dominated by only one *Pseudonocardia* type, and differences persisted for ants reared in the lab for substantial durations. While other bacteria were found in these locations, they were less abundant and more sporadic. These same *Pseudonocardia* OTUs were also found on two other sympatric species in this study, suggesting that recurring symbionts are stable players in attine biology.

attine-associated symbiont lineage. It is also known that other microbes in attine gardens can suppress *Escovopsis* under lab conditions (e.g., RODRIGUES & al. 2009).

When coupled with a recent study on *Escovopsis* evolution (MEIRELLES & al. 2015), the above findings argue against a rigid view of one-to-one arms-race style coevolution between attines, *Pseudonocardia*, and *Escovopsis*, as proposed in earlier studies (CURRIE & al. 2003). They also raise the potential for alternative *Pseudonocardia* functions, like ant defense (MUELLER 2012, SAMUELS & al. 2013), and the involvement of other players in the war over garden control. When coupled with the in vitro nature of *Pseudonocardia* antibiotic production and *Escovopsis* inhibition studies, it has become clear that the centrality of this microbe in natural garden defense awaits further study. In Box 2, we expand upon these discussions, highlighting knowns and unknowns in this intriguing example of ant-bacteria ectosymbiosis.

#### Horizons in the study of fungus-farming symbioses:

Recent insights into the microbial world associated with ant fungus gardens (PINTO-TOMAS & al. 2009, AYLWARD & al. 2014) suggest interesting avenues for research in this system. Also promising are discoveries of anomalous evolutionary events providing opportunities to apply the comparative method. While fungus growing arose just once in the history of attines, their symbioses have shown occasional convergence and evolutionary reversals – it will be fascinating to understand how other traits have evolved in concert with these events. Indeed, findings of increased reliance on metapleural gland secretion for gardening in *Atta* and *Sericomyrmex*, provide one example, as both have lost most of their cuticular *Pseudonocardia* (FERNÁNDEZ-MARIN & al. 2009). It is also the case that a group of *Apterostigma* ants have acquired the *Leucoagaricus gongylophorus* cultivar species previously thought to be exclusive to leaf-cutters (SCHULTZ & al. 2015). How have the ants, their ectosymbionts, and their fungi been modified in association with this domestication? Has the range of garden pathogens shifted to resemble those more common in leaf-cutter gardens? If not, how have holdover pathogens, including *Escovopsis*, evolved to exploit a novel fungus cultivar? Another finding of interest involves the independent origins of gongylidia-like hyphal structures by lower-attine fungal cultivars reared by *Mycoceturus smithii* (see MASIULIONIS & al. 2014). How has this event impacted the nutritional symbiosis and have these ants evolved any features that parallel those seen for higher attine agriculture? Strong foundational knowledge and re-

search tools have us well-poised to address these questions and many more within these fascinating symbiotic systems. And although the prospects for widespread, one-to-one coevolution have dimmed in this system, it is still clear that attine-fungus-ectosymbiont associations have had major impacts on attine evolution and the range of ecosystems inhabited by these charismatic ants.

#### A simple, highly derived symbiosis within the Camponotini

Aside from attine symbioses, the most deeply understood symbiotic system across the Formicidae involves carpenter ants and their relatives from the Camponotini. For over 40 million years (WERNEGREEN & al. 2009), members of this highly diverse (> 1850 species) and cosmopolitan group have evolved with intracellular bacteria from the genus *Blochmannia*. These symbionts inhabit midgut-associated bacteriocytes, specialized cells used to house intracellular microbial partners in a variety of invertebrate-bacteria symbioses (MORAN & TELANG 1998).

Genomes from two representative *Blochmannia* strains were published over ten years ago, laying the groundwork for our understanding of symbiotic function (GIL & al. 2003, DEGNAN & al. 2005). While contemporary studies had told the tale of long-term codiversification between camponotines and *Blochmannia* (SAUER & al. 2000, DEGNAN & al. 2004), genomics revealed reduced genomes with minimal gene sets. In the face of this genome reduction, the retention of pathways for the biosynthesis of essential amino acids, sulfur metabolism, nitrogen recycling, and co-factor biosynthesis were conspicuous, pointing toward nutritional roles for these bacteria. A more recent study on symbiont genomes from additional lineages suggests that not all nutritional pathways have remained intact throughout *Blochmannia*'s history (WILLIAMS & WERNEGREEN 2015). Parallel gene loss in distinct *Blochmannia* lineages presents opportunities for future studies on the correlations between such losses and the dietary ecology of the host ants. Such a comparative approach could be informative, helping to understand the causes and consequences of genomic variability in an otherwise static symbiosis.

The mostly static nature of the camponotine-*Blochmannia* symbiosis is promoted by limited acquisition of new genes within *Blochmannia* genomes (DEGNAN & al. 2005, WILLIAMS & WERNEGREEN 2015) along with strict partner fidelity, promoted by transovarial transmission (SAUER & al. 2002). Indeed there is little if any evidence for hori-

**The attine-cultivar-*Pseudonocardia* holobiont and its implications.** While still some ways from convincing demonstration, recent evidence is at least consistent with roles for *Pseudonocardia* in garden defense. Furthermore, recent questions on the composition of cuticular communities (SEN & al. 2009) have been partially clarified, affecting our understanding of whether stable, *Pseudonocardia*-driven phenotypic variation should exist among colonies and whether it might respond to natural selection, fitting a model where the holobiont serves as a unit of selection (Box 1).

To begin, 16S rRNA amplicon sequencing data reveal stable domination of laterocervical plates of *Acromyrmex echinator* workers by a single, dominant *Pseudonocardia* strain (ANDERSEN & al. 2013), fitting with earlier culture-based findings (POULSEN & BOOMSMA 2005). While they do co-inhabit the cuticle with other bacteria, perhaps even more so as the ants age, the dominant *Pseudonocardia* strain of a particular colony persists for years under laboratory rearing (Tab. 1; derived from ANDERSEN & al. 2013). Interestingly, lab-transferred strains can dominate the cuticles of workers from non-native colonies that originally hosted different *Pseudonocardia*; however, the growth of transferred strains is impacted by colony identity, suggesting that ant-encoded genetic variation may partially govern patterns of *Pseudonocardia* strain distribution within and across attine species (ANDERSEN & al. 2015). Regardless of the drivers, *Pseudonocardia* and fungal cultivar transmission from founding queens to offspring (CURRIE & al. 1999b) would lend the cohesion necessary for the attine-cultivar-*Pseudonocardia* holobiont to serve as a unit for natural selection (e.g., SLOAN & MORAN 2015). And while this certainly does not demonstrate coevolution, let alone coevolution involving *Escovopsis*, it does suggest the interesting potential for symbiont-driven evolution in response to important pathogens that target either the fungal cultivars or the ants themselves.

While short-term fidelity may characterize these symbioses, whether attines and *Pseudonocardia* show long-term cohesion is another question. Indeed there was no strong signal of codiversification among these ants and their ectosymbionts in a recent comprehensive analysis; patterns instead suggested restricted partnerships between some attine groups and particular *Pseudonocardia* clades (CAFARO & al. 2011). Furthermore, when a large number of free-living bacteria are included in phylogenetic treatments, ant-specific *Pseudonocardia* groupings appear as small, shallow-branching entities (MUELLER & al. 2010).

The apparent absence of cospeciation in this system should, perhaps, come as little surprise. Defensive microbial symbionts of other insects undergo horizontal transfer, and populations may harbor a variety of microbes effective against the same class of natural enemy (ŁUKASIK & al. 2013, OLIVER & al. 2014, FLÓREZ & al. 2015). Pathogens are moving targets, and the most nimble defensive symbioses may be those with a capacity to acquire novel defensive capabilities. Attines may fall in the middle of this spectrum, given the clear persistence of dominant strains within colonies of some attine species (ANDERSEN & al. 2013) – presumably those transmitted by queens during colony founding – combined with occasional horizontal transfer and domestication from free-living populations. This could create some capacity for adaptive evolution acting at the colony level while at the same time allowing the ants to engage in associations with tried and true symbionts showing effectiveness against their major enemies and aligned interests with their ant hosts. Indeed a recent model (SCHEURING & YU 2012) has even suggested that some amount of vertical transmission for cuticular bacteria could improve the abilities of attines to "select" free-living antibiotic producing partners, protecting themselves against take-over and exploitation by cheaters. Under this model the most important role of the vertically inherited microbes would not be direct defense against pathogens, but instead recruitment of the best defenders. This suggests a new and interesting framework for the *Pseudonocardia*-attine association that warrants empirical study.

**What do *Pseudonocardia* do?** In returning to the idea of *Pseudonocardia* as a garden defender, indirect evidence has accrued to suggest effectiveness against *Escovopsis*. For example, in a study across seven attine genera, ant-associated *Pseudonocardia* showed slightly greater in vitro suppression of *Escovopsis* growth than did either free-living *Pseudonocardia* or *Streptomyces* (CAFARO & al. 2011). An additional study (POULSEN & al. 2010) using similar in vitro assays of *Escovopsis* inhibition showed an ectosymbiont by pathogen strain interaction – there was no single *Pseudonocardia* strain ranking most (or least) effective against the battery of examined *Escovopsis* pathogens, nor was there a single *Escovopsis* isolate with universal susceptibility (or counter-resistance) to inhibition by all *Pseudonocardia* isolates. When the authors examined a representative subset of these ectosymbiont-pathogen combinations in vivo, they found parallels with their in vitro discoveries, suggesting that the identity of the harbored *Pseudonocardia* strain on attine cuticles may somehow govern the range of *Escovopsis* strains successfully thwarted from garden take-over within that colony. Whether this is indeed the case, and if so whether it occurs through specific *Pseudonocardia*-produced antibiotics vs. specific recruitment of defenders effective against different *Pseudonocardia* (SCHEURING & YU 2012), are possibilities in need of further testing. Indeed, direct evidence for antibiotic production by *Pseudonocardia* in an in vivo context would be an important breakthrough in this system, in its own rite. What is also uncertain is the question of whether attines, their cultivars, and *Escovopsis* truly co-evolve and what roles, if any, *Pseudonocardia* play in this process.

In considering defensive potentials for *Pseudonocardia* or other cuticular bacteria, we can also look to the recent discovery that both behavioral and chemical traits used for garden defense show trade-offs with attines' investment

in ectosymbionts. For instance, across the genus *Trachymyrmex*, species with low levels of ectosymbiont cover exhibit higher rates of metapleural gland grooming, in which potent ant-produced antimicrobials are added by workers to infected garden regions (FERNANDEZ-MARIN & al. 2013). Similar findings have been obtained for *Atta* and *Sericomyrmex* ants, as those with greater reliance on metapleural gland secretions for garden pest control show reduced investment in symbioses with cuticular bacteria (CURRIE & al. 1999b, MUELLER & al. 2008, FERNANDEZ-MARIN & al. 2009, FERNANDEZ-MARIN & al. 2015). But while these findings implicate cuticular bacteria in protection of attines' fungus gardens, they of course fall short of painting *Pseudonocardia* as the central player in effecting garden defense.

Other findings have come similarly close in implicating bacteria – but not directly *Pseudonocardia* – as important garden defenders. For instance, spores of *Escovopsis* and *Trichoderma* garden pests are removed by *Trachymyrmex* cf. *zeteki* workers, passing to their infrabuccal pockets. They are clearly killed in these locations, and there appears to be a rise in actinomycete presence in these pockets for *Escovopsis* treated vs. untreated colonies. Furthermore, the most common actinomycete morphotype cultivated from infrabuccal pockets was shown to inhibit *Escovopsis* growth, in vitro, suggesting that these pocket-associated microbes play a role in garden defense (LITTLE & al. 2006). Unfortunately, the identities of the responsible microbes have not been established, and no direct follow-ups to this work have been published, to our knowledge. A similar mystery surrounds other prior results, for instance one suggesting worker-deposition of unidentified actinomycetes onto fungus gardens (MANGONE & CURRIE 2007). Here, too, the lack of DNA sequencing prevents us from implicating any particular bacterium in cultivar defense. To summarize, while bacteria are clearly important in garden defense, and while *Pseudonocardia* could be at the center of such defense in many attine-fungus garden systems, (1) the sites of active battlefronts for antibiotic-producing *Pseudonocardia*, (2) the enemies they target, and (3) their propensities to fight alone or in alliance with other microbes, are matters awaiting further in vivo assessment. Also in question is just how much and where these attributes vary across the attine phylogeny, as the clearly demonstrated dynamics for attine-*Pseudonocardia* symbioses shown to date are not consistent with a monolithic model for these widespread interactions.

zontal transfer or loss of *Blochmannia* in their prolonged history with camponotines, suggesting complete interdependence for both parties. Somewhat surprisingly, *Blochmannia* removal does not have a large impact on the performance of adult workers (SAUER & al. 2002). Instead the importance of these symbionts appears to play out during earlier stages of development. Interestingly, workers without *Blochmannia* rear fewer brood to adulthood, suggesting their importance in colony growth (ZIENZ & al. 2006, FELDHAAR & al. 2007). Studies of symbiont abundance and both bacteriocyte number and occupation show a build-up of *Blochmannia* leading up to and through pupation, followed by a decline in early adulthood (SAUER & al. 2002, WOLSCHIN & al. 2004, STOLL & al. 2007). Measures of gene expression by *Blochmannia* reveal heavy devotion to nitrogen recycling during ant pupation, with a shorter bout of gene expression for pathways involved in amino acid synthesis around this same time (ZIENZ & al. 2006). High expression of nitrogen metabolism genes appears to be part of a regulatory switch toward transcription of potentially beneficial symbiosis genes at the expense of those with more standard housekeeping functions (STOLL & al. 2009).

Experimentation has shown that these symbionts do indeed provide essential amino acids to adult workers when they consume urea (FELDHAAR & al. 2007). When considering this alongside genomic evidence for aromatic amino acid synthesis by *Blochmannia* (GIL & al. 2003), and the need for costly aromatic amino acids at the time of cuticular sclerotization in young adults, one would conclude that *Blochmannia* play a major role in helping camponotines to meet the demands imposed by ant development. Whether this is a ubiquitous function, and whether *Blochmannia* roles vary across host phylogeny and ecology are questions awaiting further study. With the extra-

ordinary taxonomic and ecological diversity contained within the Camponotini, there should hence be no shortage of fodder for future symbiosis-themed research.

#### **A second cuticle-building symbiosis?**

*Blochmannia*-camponotine symbioses show resemblance to at least one other ant-bacterium association, documented recently in the genus *Cardiocondyla*. The symbiont, "*Candidatus Westeberhardia cardiocondylae*" (henceforth *Westerberhardia*), was discovered in genome sequencing efforts for the invasive ant *Cardiocondyla obscurior* (see KLEIN & al. 2015). Colonizing mid-gut associated bacteriomes, the symbiont's tiny genome was surprisingly low in gene density, suggesting it to be in a transitional stage of genome degradation. Retained within the 533 kb *Westerberhardia* genome are all of the genes in the shikimate pathway, enabling synthesis of aromatic amino acid pre-cursors including 4-hydroxyphenylpyruvate. Host metabolism could take the hand-off from symbionts at this stage, using this latter compound to directly manufacture tyrosine. Like other aromatic amino acids, tyrosine is crucial in the maturation of the adult worker cuticle. So the conspicuous retention of this pathway in spite of rampant genome shrinkage, combined with the degradation of bacteriocytes and symbiont decline in young adult workers, led the authors to propose that *Westerberhardia* functions similarly to *Blochmannia*, providing hosts with the resources required for successful development. While some lab-reared *C. obscurior* colonies lacked *Westerberhardia*, the symbiont was found in another species, *C. wroughtoni* (see KLEIN & al. 2015), and a prior study had detected a highly similar 16S rRNA sequence in *C. emeryi* (see RUSSELL & al. 2009b). One might thus postulate that this enteric bacterium has been a symbiont of *Cardiocondyla* ants for some time.

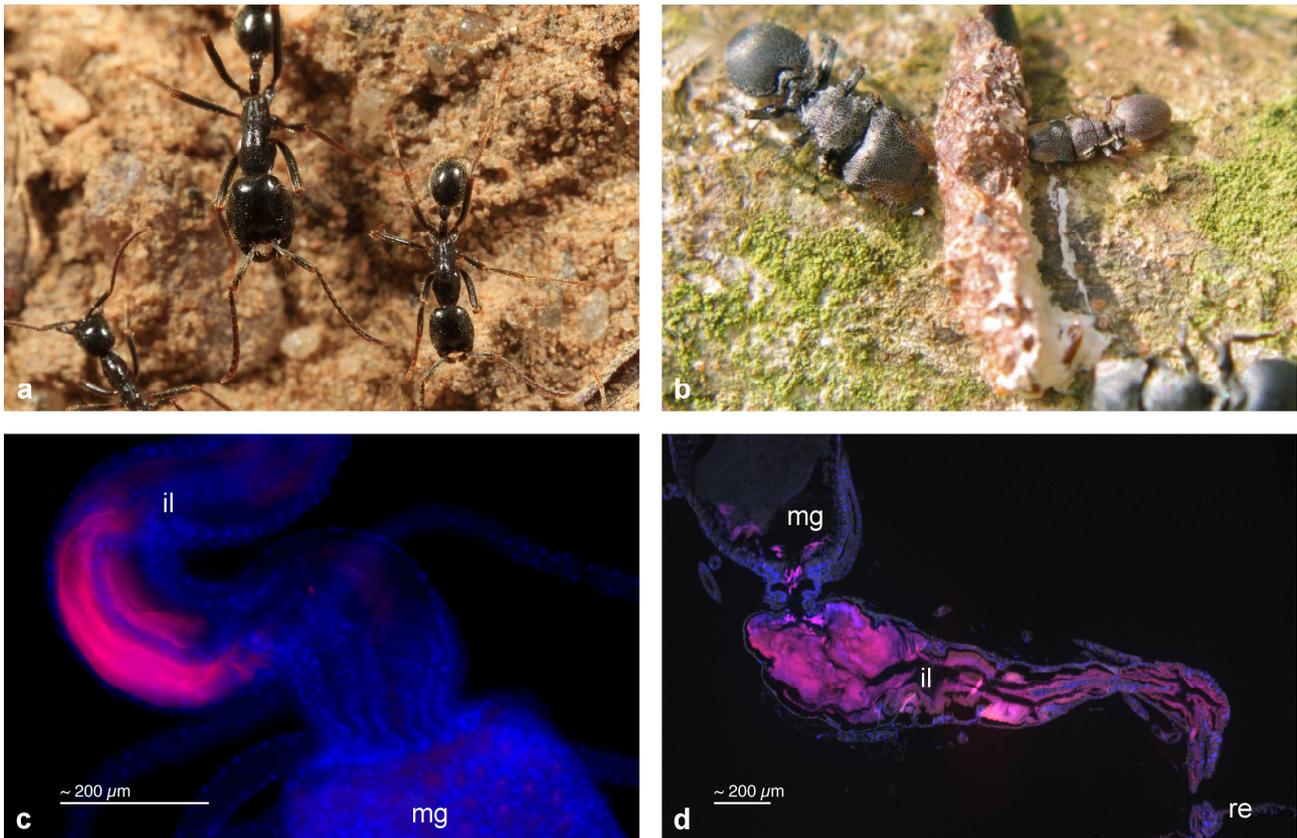


Fig. 2: Ants harboring specialized bacterial gut symbionts, and their localization within digestive tracts. (a) *Labidus* sp. workers represent a range of army ants (genera *Aenictus*, *Dolichoderus*, *Cheliomyrmex*, *Labidus*, *Eciton*, *Neivamyrmex*, and *Nomamyrmex*) harboring apparently specialized Firmicutes and Entomoplasmatales gut bacteria. (b) Workers of two *Cephalotes* species feeding on bird droppings represent dozens of congeneric species and the sister genus *Proccryptocerus*, which play host to specialized communities of gut bacteria. (c) FISH micrograph of gut tissues from a *Labidus praedator* worker showing localization of bacteria (magenta) in the ileum. (d) FISH micrograph of gut tissues from a *Cephalotes* sp. worker showing bacteria (magenta) in the midgut and at especially high concentrations in the ileum. For both FISH images: mt = Malpighian tubules; mg = midgut; il = ileum. Magenta fluorescence shows localization of bacteria based on a eubacterial 16S rRNA probe; blue fluorescence represents the signal from host nuclei. Image credits: (a) Daniel Kronauer; (b) Scott Powell; (c) and (d) Piotr Łukasik.

### Gut symbioses at opposite ends of the food chain

#### The low end: symbionts in ants with nitrogen poor diets:

Attine and camponotine symbioses have been recognized for decades (BUCHNER 1965, HÖLLDOBLER & WILSON 1990), but more recent studies have illustrated that several unrelated ants harbor large numbers of extracellular bacteria in their guts (e.g., Fig. 2b; BILLEN & BUSCHINGER 2000, BUTION & CAETANO 2010). Several of these ants have been noted to come from herbivorous or indirectly herbivorous groups, feeding on plant sap or insect honeydew – both nitrogen-poor and carbon-rich food sources (reviewed in COOK & DAVIDSON 2006). Two separate studies also found several of these ants to exhibit heavy: light nitrogen isotope ratios that overlapped with those of known insect herbivores (BLÜTHGEN & al. 2003, DAVIDSON & al. 2003). It was argued that nutrient-provisioning symbionts of such ants could play a major role in their success within tropical tree canopy habitats, enabling them to capitalize upon abundant, renewable foods with limited nitrogen (DAVIDSON & al. 2003). Accordingly, COOK & DAVIDSON (2006) noted the tendencies of these ants to

harbor microbes in portions of the gut innervated with Malpighian tubules, proposing nitrogen recycling as one mechanism for gut microbe function in these systems.

Parallel research on these ant groups has suggested a means by which hosts could promote the spread of potentially beneficial bacteria within a colony. Specifically, oral-anal trophallaxis behaviors used in the transmission of termite gut symbionts (MCMAHAN 1969) have been observed in *Cephalotes*, *Proccryptocerus*, and beyond (WILSON 1976, WHEELER 1984, COOK & DAVIDSON 2006, LANAN & al. 2016). In these ants, callow workers appear to emerge from pupation with few detectable gut bacteria (ROCHE & WHEELER 1997). They then consume anal secretions from mature adults, receiving a likely inoculum of symbionts. Such behavioral transfer to the female reproductive caste (gynes) would allow these systems to mimic those with transovarial symbiont transmission, making gut bacteria heritable components of ant biology that traverse generations. This would promote partner fidelity and could favor long-term co-divergence or lineage tracking, explaining the persistence of core symbiont taxa across many tens of millions of years in some of these ants (see be-

low). Additional mechanisms, such as the highly selective proventricular filter in *Cephalotes* (LANAN & al. 2016), may help to reinforce this fidelity, keeping symbiont communities intact. Such robustness and an effectively vertical route for microbe transfer could enable symbiont-driven, colony level phenotypes to respond to natural selection. So what kinds of bacteria colonize the guts of these ants? How do they vary within and across colonies and species? And what do they do? In the last decade, researchers have made considerable progress in addressing the first two questions.

One of the first sequence-based studies of gut bacteria from non-camponotine "herbivores" revealed an intriguing pattern whereby unrelated ants from two ant genera – *Dolichoderus* and the *nigra* species group of *Tetraponera* – harbored related bacteria from a single lineage in the order Rhizobiales. This lineage, in turn, showed close affinity to bacteria from the genus *Bartonella* (STOLL & al. 2007). Shortly after this a diagnostic PCR survey for these bacteria was performed across hundreds of ant species, showing a broad distribution for *Bartonella*-like, ant-specific Rhizobiales across the Formicidae phylogeny. *Dolichoderus* and *Tetraponera* were again found to be common hosts, while both *Cephalotes* and *Cataulacus* were newly added to this list (RUSSELL & al. 2009b). Data were consistent with host-specificity, with some clustering of these Rhizobiales microbes into ant genus- or tribe-specific clades.

When Rhizobiales frequencies within each surveyed ant genus were analyzed against nitrogen isotope ratios from ant tissues, a significant negative correlation was discovered, suggesting enrichment of these bacteria in ants at the bottom of the food chain (RUSSELL & al. 2009b). This was a phylogenetically independent trend, as the focal genera came from separate clades on the ant tree – thus, multiple origins of nitrogen-poor diets amongst several ant genera are associated with the origins of Rhizobiales symbioses. The timing and relative order of dietary and symbiotic shifts have yet to be worked out in detail. But, regardless, the patterns discovered are at least consistent with a role for such microbes in the origins or maintenance of this trophic niche (RUSSELL & al. 2009b) – one that could be key to the abundance of several diverse ant groups in tropical forest canopies (DAVIDSON & al. 2003).

While some time has passed since the publication of these findings, there has to date been no clear demonstration of function for Rhizobiales or any other gut bacteria of ants (see JAFFE & al. 2001, however, for a study on the potential fitness impacts of *Cephalotes* gut bacteria). And while *nifH* nitrogenase genes were detected in ants with Rhizobiales (STOLL & al. 2007), there was no evidence for in vivo nitrogen fixation in Rhizobiales-bearing *Cephalotes* or for the identified *nifH* genes actually belonging to the Rhizobiales symbionts (RUSSELL & al. 2009b). Regardless, the localization of ant-specific Rhizobiales to mid- and hind-gut tissues (STOLL & al. 2007, RUSSELL & al. 2009b), and their persistence in lab-reared *Cephalotes varians* workers eating artificial sugar diets (RUSSELL & al. 2009b) have revealed that these bacteria are persistent gut symbionts of *Cephalotes* guts. Furthermore, the presence of known nitrogen provisioning *Blochmannia* in the two most "herbivorous" ant genera (based on nitrogen isotopes) lacking Rhizobiales suggested a broader correlation between extensive use of nitrogen-poor diets

and associations with known or suspected nutritional symbionts (RUSSELL & al. 2009b).

As for many other animals, extracellular gut symbionts of ants exist as members of multi-species communities (ENGEL & MORAN 2013), and adult workers from *Cephalotes* and the sister genus *Procryptocerus* were among the first to exemplify this. With shallow community sampling through Sanger sequencing of 16S rRNA clone libraries, a diversity of Proteobacteria were discovered, as were representatives from the phylum Verrucomicrobia (order Opitutales) (RUSSELL & al. 2009b), belonging to the recently named genus, *Cephaloticoccus* (LIN & al. 2016). Like Rhizobiales, these bacteria were localized to the gut, persisting in lab-reared workers. A subsequent study expanded the range of sampled *Cephalotes* hosts, helping to reveal some regularity in the communities across this group (ANDERSON & al. 2012). Core bacteria of *Cephalotes* were found to hail from divergent, host-specific clades on bacterial 16S rRNA phylogenies, suggesting long-term persistence and specialization. In this same study at least two additional ant-specific lineages, both from the proteobacterial order Burkholderiales, were found in genera beyond *Cephalotes* (i.e., *Dolichoderus* and *Tetraponera*). As both novel hosts had been similarly inferred to be nitrogen-limited canopy ants (DAVIDSON & al. 2003), the study suggested further convergence in gut microbiota across ecologically similar hosts.

Since this time, four next generation sequencing studies of bacterial 16S rRNA genes have confirmed the existence of several core bacteria from the aforementioned taxa in cephalotine guts; they have also continued to find that the majority of core symbionts come from *Cephalotes*-specific lineages on 16S rRNA phylogenies (KAUTZ & al. 2013b, HU & al. 2014, SANDERS & al. 2014, LANAN & al. 2016). While not yet fully established, early returns are consistent with co-diversification between some gut microbiota and *Cephalotes* species (SANDERS & al. 2014), suggesting ancient partner fidelity for some portion of the gut community. Combined with the near ubiquity of some core symbiont lineages across the cephalotines, it is clear that these stable and ancient symbioses warrant functional study.

In spite of the possibly static nature for this symbiosis across the cephalotines, the colonies of some *Cephalotes* species do vary in the relative abundance or presence / absence of core symbiont species (HU & al. 2014, SANDERS & al. 2014). Patterns of between-colony and geographic variation have also been uncovered through examining symbiont strain distributions, as inferred from the various 16S rRNA genotypes found across workers (see Fig. 3; from data in HU & al. 2014). While the implications are not yet clear, the biology of the system thus far suggests that variation in symbiont strain composition among colonies could be stable and heritable. This would make gut bacteria sources of maternally inherited, potentially adaptive genetic variation, perhaps allowing the *Cephalotes* holobiont to serve as a unit of selection (Box 1).

**The high end: symbionts in ants with nitrogen rich diets:** In a random sampling of phylogenetically disparate ants, three bacterial taxa were most often recovered in universal bacterial PCR screens and subsequent Sanger sequencing – Rhizobiales from the aforementioned *Bartonella*-like lineage, common insect-associated intracellular symbionts from the genus *Wolbachia*, and representatives

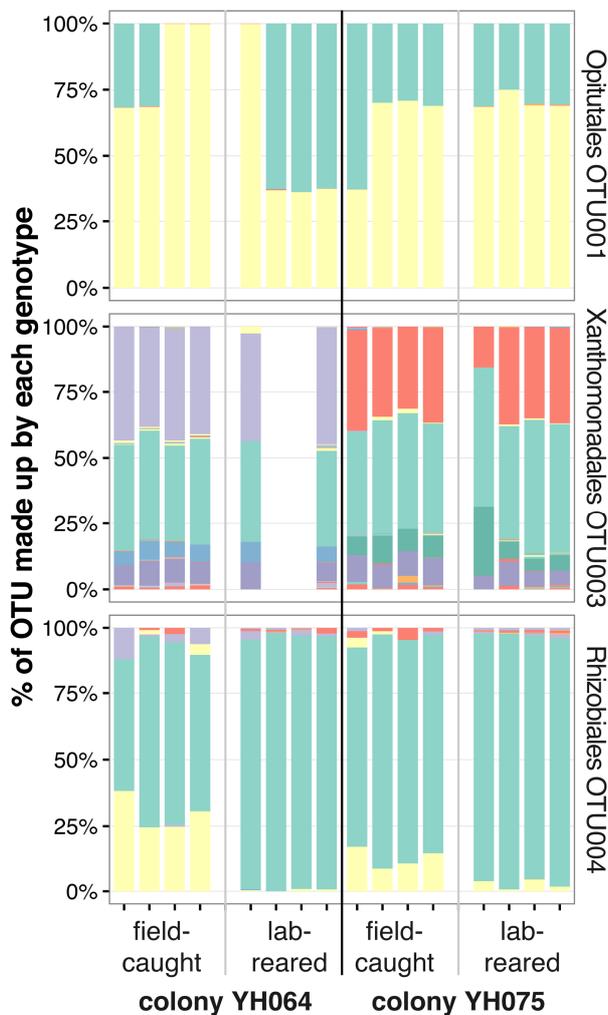


Fig. 3: Variation in strain composition across colonies and rearing environments for three core species of specialized gut bacteria in the workers of *Cephalotes varians*. Data are from HU & al. (2014) and were derived from three 97% OTUs of 16S rRNA sequences. Each column represents the bacterial strain composition for a single OTU from a single adult worker gut, based on analysis of 454 amplicon sequencing data. Workers came from one of two colonies collected in Key West, Florida. Specimens were either immediately preserved after field collection or reared in the lab for several months on artificial diets of 30% sucrose water. (a) Strain composition for OTU1, the dominant core symbiont, *Cephalotococcus capnophilus* (order Otitales) (avg. # sequences from this OTU per worker = 1124.5). As for panels (b) and (c), each color represents a unique and vetted 16S rRNA sequence genotype. Among the two focal colonies, sequences derived from the *C. capnophilus* OTU showed no clear between-colony differences or impacts of diet. (b) Strain composition for OTU3, a core symbiont from the order Xanthomonadales (avg. # sequences from this OTU per worker = 572.4). For this species, workers from the two focal colonies harbored stable differences in strain composition, revealing between-colony differences in microbiota (note that two lab-reared workers from YH064 did not have any representatives from this species). (c) Strain composition for OTU4, a core symbiont from the order Rhizobiales (avg. # sequences from this OTU per worker = 334.1). Relative abundance values for strains from this species were impacted by rearing environment.

from various clades within a third group known as the Entomoplasmatales (RUSSELL & al. 2009b). This bacterial order includes known arthropod-associated symbionts and pathogens from the genus *Spiroplasma*. Accordingly, subsequent diagnostic PCR screening for Entomoplasmatales recovered a number of *Spiroplasma* associates from amongst the hundreds of surveyed ant species (FUNARO & al. 2011). Several other non-*Spiroplasma* lineages were identified as well, including a fairly large ant-specific clade with closer relatedness to *Entomoplasma* and *Mesoplasma*.

When combining results from broad, ant-wide sampling with those from in-depth screening in a few focal host taxa, it was discovered that army ants from the former subfamilies Aenictinae, Dorylinae, and Ecitoninae (now collectively lumped within the Dorylinae: BRADY & al. 2014) were enriched for associations with an army ant-specific clade of Entomoplasmatales bacteria (FUNARO & al. 2011). Screening of dissected tissues suggested mid- and hind-guts to be a common site of localization for army ant associates (see Fig. 2c for microscopy evidence of bacterial masses in army ant guts). But unlike Rhizobiales, which were found in all surveyed mature adults from infected *Cephalotes*, *Dolichoderus*, and *Tetraponera* species (RUSSELL & al. 2009b), Entomoplasmatales bacteria showed intermediate prevalence both within species and even some colonies (FUNARO & al. 2011). So despite: 1) deep 16S rRNA divergence amongst members of this Entomoplasmatales lineage, and 2) broad distributions for these bacteria across an > 80 million year old ant group with disparate geographies (BRADY & al. 2006), the symbiosis appears neither ubiquitous nor obligate (FUNARO & al. 2011). Nevertheless, these bacteria are a recurring staple of army ant history, raising questions on their means of acquisition and impacts upon the colony.

Since this time it has been shown that army ants are not the only predators associating with these bacteria. Inclusion of GenBank sequences from an unpublished study allowed Kautz and colleagues to discover that ants from the subfamily Ponerinae harbor members of the army ant Entomoplasmatales clade (KAUTZ & al. 2013a). Interestingly, this instance of army ant-ponerine symbiont sharing is reinforced by a second finding – at least one Ponerinae species (genus *Leptogenys*) harbors a bacterium from a deeply divergent Firmicutes lineage, consisting otherwise of only army ant associates (FUNARO & al. 2011).

There is clearly a good deal of work needed to characterize these potential symbioses, including research on microbial persistence, localization, evolution, and function. But together, these findings do raise an interesting possibility – specialized, prevalent, and ancient symbionts appear enriched in unrelated ant groups at extreme ends of the food chain. Might this suggest a general importance for symbiotic microbes in the use of imbalanced diets? Or might other lifestyle attributes of these ants provide a better explanation for these trends? The answer should be intriguing either way.

#### Where else do we find microbial symbionts?

In considering the above patterns in relation to the ant phylogeny, we begin to see the emergence of hotspots for microbial symbioses across these insects. We define hotspots as deep-branching host lineages (i.e., whole tribes or genera, or at least large portions thereof) with a high prevalence of specialized bacteria or fungi, and with ap-

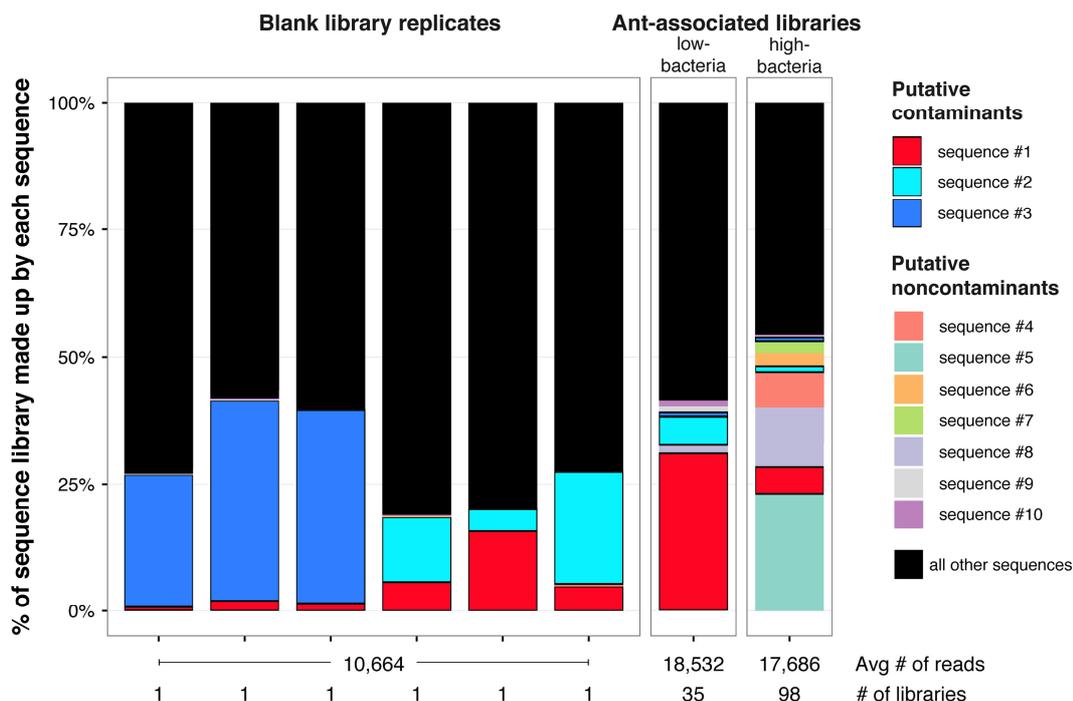


Fig. 4: Contaminant sequences and their distributions across 16S rRNA amplicon sequence libraries from invasive Argentine ants (data from HU & al., in press). Six replicate "blank" DNA extractions (i.e., no biological material added) were used for amplicon sequencing, as were 133 extractions from *Linepithema humile* (adult workers in nearly all cases). Distributions of the 10 most abundant unique 16S rRNA sequences (generated through Illumina amplicon sequencing) were examined. Individual libraries are shown for each blank, while pooled results are shown for ant-associated libraries from specimens with strong vs. weak (or absent) 16S rRNA amplification. Bacteria represented by red, blue, and cyan portions of bar graphs were abundant in three of six blank libraries and also present in libraries generated from whole ant DNA extractions. Contaminants appear more problematic for ant extractions yielding weak-to-no amplification of 16S rRNA genes with universal eubacterial primers.

parent investment in the morphological, physiological, and / or behavioral features required to house or perpetuate these symbioses. While attines, camponotines, and cephalotines clearly fit this bill, the other groups highlighted in Figure 1 are proposed as hotspots, each requiring some degree of further study to understand the ages and prevalence of symbioses, and the degrees of ant investment in the sheltering and transfer of their specialized inhabitants.

Collectively, the ~ 2845 described species within these lineages (BOLTON 2014) make up ~ 22% of the ~ 13,000 described species within the Formicidae. It would thus appear that a sizeable fraction of ant diversity is associated with ancient, ant-specific fungi or bacteria. These may often be integral to host functioning, but of course much work awaits to assess function outside of the attines and camponotines. So what do we know about symbioses beyond these proposed hotspot groups? With the exception of widespread *Wolbachia* symbionts, the answer to this question was, until recently, "very little". This is rapidly changing, and we devote much of the remaining manuscript to a discussion of new findings of relevance to the field.

**Do all ants invest heavily in symbioses with microbes?** Before moving on to discuss instances of symbiosis across the ants, it is important to note a realistic possibility: that not all ants are highly invested in symbioses with bacteria. The breadth of the concept of symbiosis presents a recurring problem to discussions on this subject. Certainly, all animals likely engage in intimate inter-

actions with microbes, even interactions with profound relevance to fitness. But even when these fitness effects can be empirically demonstrated, as in *Drosophila* (e.g., RIDLEY & al. 2013), the interactions can be diffuse: non-specific (CHANDLER & al. 2011), labile (WONG & al. 2013), and of limited abundance (BRODERICK & al. 2014). For this reason, the concept of investment – evidenced by specificity of partnerships, prevalence or persistence of symbionts across hosts, and morphological or behavioral specialization – is one that we find useful. Most of the ant systems described above show notable investment in their microbial symbioses. Several are also characterized by conspicuously high symbiont biomass, which placed groups like carpenter ants squarely on the radar of myrmecologists over 100 years ago (BLOCHMANN 1888).

With the increased focus on symbiosis across the ants, it is beginning to appear that these invested, high biomass symbioses are exceptional. Indeed, one published study has documented low bacterial density in the ant *Crematogaster rochai*, with an estimated number of bacterial 16S rRNA gene copies that was hardly distinguishable from that of "blank" extractions (RUBIN & al. 2014). In that study, only one out of 32 extractions from that species successfully generated sequences in Illumina amplicon sequencing efforts, revealing challenges to the study of ant-associated microbiota. A similar finding was obtained in one of our laboratories when attempting to amplify bacterial 16S rRNA from the weaver ant, *Oecophylla smaragdina* (J.A. Russell, unpubl.).

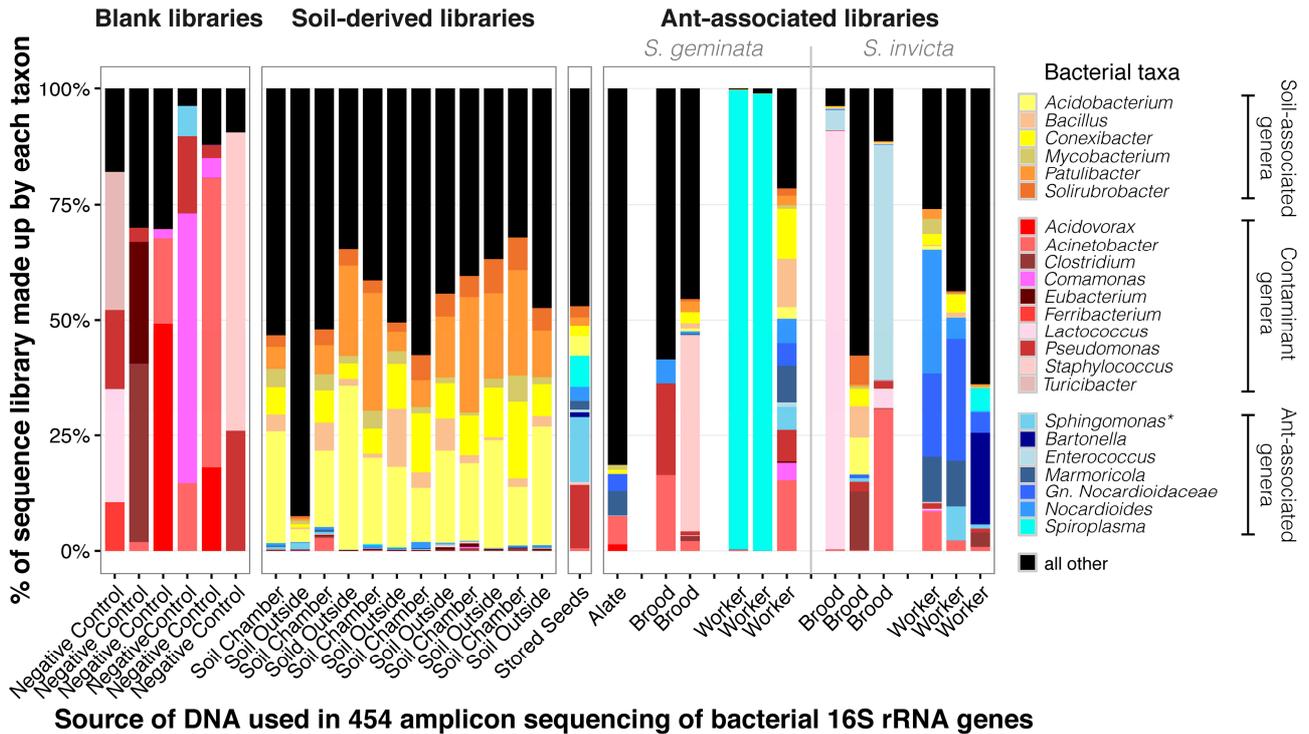


Fig. 5: Disentangling contaminants from stable ant-associates. Bacteria detected from *Solenopsis* ants, their associated habitats, and negative controls. Here each bar represents a single sequence library. Classifications were derived from the BLAST search results of ISHAK & al. (2011b: supplementary tab. 3). The proportions of all classifiable sequences made up by various genera and higher order taxa are shown here. Taxa making up 10% or more of at least one "blank" library are shown in various shades of red and pink. Those with lower prevalence or complete absence from blank libraries with at least 4% representation in two or more ant libraries are shown in shades of blue. Soil-associated genera meeting this same criterion (i.e., absent or rare in ant and blank samples,  $\geq 4\%$  in two or more soil libraries) were highlighted with yellow and orange colors. Rarer taxa were binned into the "other" category, represented by black. In short, one finds a modest fraction of ant-derived 16S rRNA libraries (sampled with 454 amplicon sequencing technology) to be made up by recurring ant-associated taxa that are unlikely to be soil- or lab / field-borne contaminants. Note, "\*" designates a genus (*Sphingomonas*) making up over 6% of reads from one blank library, calling into question its likelihood as a non-contaminant ant-associate.

Two additional ant groups exhibit potential hallmarks of low bacterial densities. In the first instance, Argentine ant workers have been found to yield weak-to-no-amplification with universal bacterial 16S rRNA primers, in spite of amplification with primers targeting ant genes (HU & al., in press). Those workers yielding weak signal gave rise to 16S rRNA amplicon libraries that were dominated by contaminants – i.e., sequences abundant within libraries from blank DNA extractions (Fig. 4). Recent ground-truth experiments on serially diluted *Salmonella* cultures suggest this pattern is a hallmark of low starting bacterial quantities, again indicating that amplicon sequencing studies of bacterial 16S rRNA will be challenging for hosts with low-symbiont densities (SALTER & al. 2014). In light of this, sequencing from blank extraction controls alongside DNA from targeted specimens may be essential in distinguishing between likely symbiotic associates versus contaminants. Such a study design has identified substantial overlap between bacterial genera sampled from *Solenopsis* species with those from blank DNA extractions and soil specimens (Fig. 5; from data in ISHAK & al. 2011b). While this may reflect bio-

logical reality – i.e., that *Solenopsis* associate with symbionts related to those in soil and laboratory reagents – it is also consistent with the above expectations of increased contaminant representation in samples with low bacterial densities.

These findings dovetail with two additional datasets. The first (J.G. Sanders, P. Łukasik, M.E. Frederickson, J.A. Russell, R. Koga, N.E. Pierce, unpubl.), utilized a combined approach of qPCR, SYBR green DNA staining and fluorescence microscopy, and fluorescent in situ hybridization targeting bacterial 16S rRNA. Focusing on ants from terrestrial and arboreal habitats in Peru, it was shown that the majority of surveyed ant genera harbored very few detectable bacteria. Exceptions included *Cephalotes*, *Camponotus*, and *Dolichoderus*, three groups already known for associations with dense symbiotic communities whose symbiont densities were far above those from nearly all other groups. The second stems from our past experience in ant-bacteria research – universal PCRs with eubacterial primers (described in RUSSELL & al. 2009b) fail to amplify bacterial 16S rRNA genes in a large percentage of ants from a wide range of taxa collected throughout the

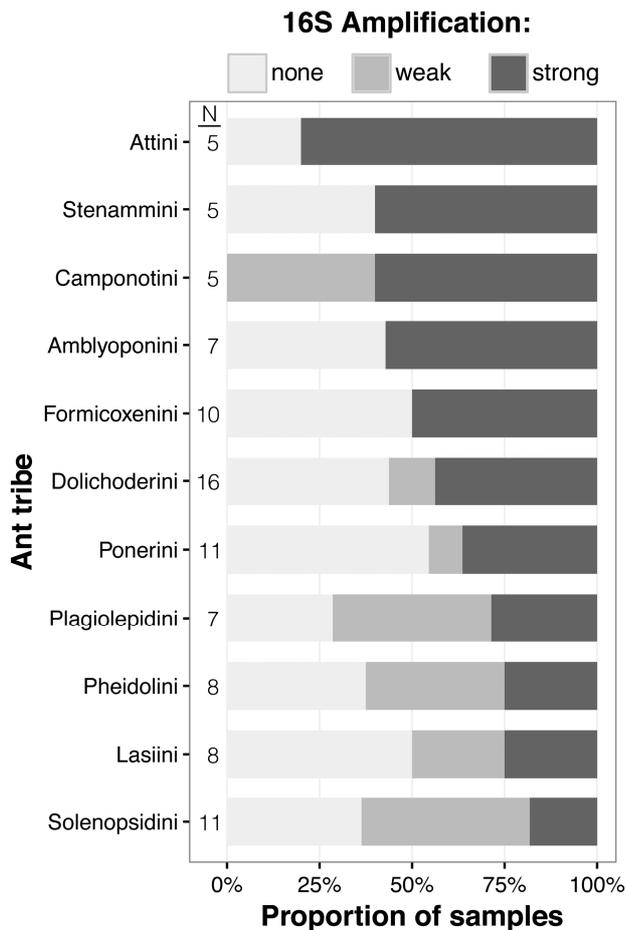


Fig. 6: PCR with universal bacterial 16S rRNA primers routinely fails to yield strong amplification. DNA from whole workers or gasters was surveyed using the universal 16S rRNA primers 9Fa and 1513R. Results are shown for individual extractions and separated into well-sampled taxa. The total % of ant extractions with no universal PCR signal was 35.4% (n = 144 sampled specimens). Further details on screening can be found in Table S1.

world (Fig. 6; Tab. S1, as digital supplementary material to this article, at the journal's web pages). Specimens targeted in these efforts were largely the same as those included in a phylogenetic study of the Formicidae (MOREAU & al. 2006); given the successful use of these samples to generate sequence data for the ant phylogeny study, we would argue that the issue with failed bacterial 16S rRNA amplification was not sample template quality or the presence of PCR inhibitors. The amplification of positive controls in nearly all of the reactions used to generate the data in Figure 6 further argues that the results are not due to an inefficient PCR assay with high rates of stochastic failure. More recent surveys of ants collected from the Florida Keys and southeastern Texas provide similar results, suggesting also that it is not only the adult stage that exhibits such rare amplification. When considering these results, we argue that specialized and enriched symbioses are not the norm for many ants. Instead, a substantial percentage of workers from across many ant species harbor low bacterial densities.

**More hotspots? – *Wolbachia* symbioses:** Suggestions that at least some ants harbor few bacterial symbionts

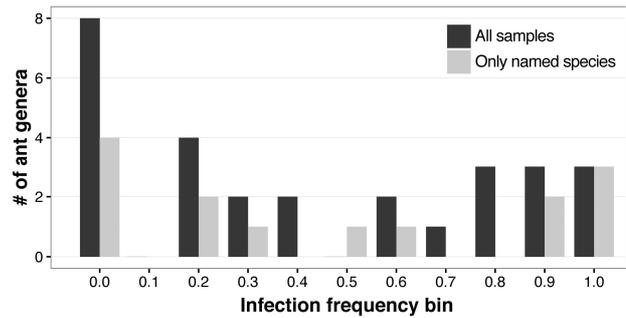


Fig. 7: Are there hotspots and coldspots for *Wolbachia* symbioses? *Wolbachia* are often very rare or very common in sampled ant genera when compared to the ant-wide prevalence of 34 - 36% infected species. Frequency histograms illustrate data from a systematic review of *Wolbachia* frequencies across ants and other insects (RUSSELL & al. 2012). X-axis frequency bins represent the % species infected with *Wolbachia* for the genera of interest. The Y-axis shows the number of genera with the given infection rates. Data are shown only for those genera with at least five surveyed species. In black are all data (*Wolbachia* presence / absence across identified and unidentified ant species), while data in gray include only *Wolbachia* presence / absence from identified ant species, minimizing potential impacts of pseudoreplication.

make symbiotic hotspots all the more conspicuous. But there are indeed other symbioses across a wider diversity of ants than those described above. The one example with the greatest resemblance to the hotspot phenomenon involves intracellular *Wolbachia* symbionts (class: Alphaproteobacteria) and their patchy distributions across ant genera (Fig. 7). Members of the *Wolbachia* genus are broadly distributed across the arthropods, making them among the most successful symbionts of animals on the planet. These bacteria are found in ~ 34 - 36% of ant species, a frequency comparable to those in several other insect groups (RUSSELL 2012, RUSSELL & al. 2012). Some *Wolbachia* make their living in arthropods through the manipulation of host reproduction (WERREN & al. 2008). However, several strains have recently been shown to impact insect defense and disease vector competency (HEDGES & al. 2008, TEIXEIRA & al. 2008, MOREIRA & al. 2009, MARTINEZ & al. 2014). Other functions have been uncovered as well, including nutritional roles through B-vitamin synthesis (HOSOKAWA & al. 2010). Due to experimental challenges we know little about the impacts of *Wolbachia* on ants (see Box 3 for one speculation). But it is now becoming clear that the range of possibilities is quite broad, suggesting a need to look beyond initial suspicions of reproductive manipulation (VAN BORM & al. 2001, RUSSELL 2012).

Throughout its range of invertebrate hosts, *Wolbachia* transfer appears to almost always be maternal and internal, with the symbionts colonizing eggs or developing embryos inside of females (FRYDMAN & al. 2006). On occasion, however, *Wolbachia* do move horizontally between species as evidenced through experiments and host symbiont phylogenetic incongruence (VAN MEER & al. 1999, HUIGENS & al. 2000). Among the ants we see no clear evidence for co-diversification between these symbiotic parties. But New World ants are enriched for *Wolbachia* from

Box 3: Does symbiotic variation across development hint at *Wolbachia* function in leaf-cutters and wood ants?

While we know little of *Wolbachia* function, an interesting pattern has been reported in which these symbionts are lost from *Atta*, *Acromyrmex*, and *Formica* workers at some point during adulthood (KELLER & al. 2001, VAN BORM & al. 2001, WENSELEERS & al. 2002, VILJAKAINEN & al. 2008, FROST & al. 2010). Since sterile workers are dead end hosts for transovarially transmitted symbionts, the finding is still fully consistent with maternal transfer maintaining these bacteria over time – indeed gynes, the transmitting hosts, appear to harbor higher frequencies than workers and males (VAN BORM & al. 2001, WENSELEERS & al. 2002). While loss of transovarially transmitted *Wolbachia* from non-transmitting castes may in some ways seem logical, none of the other ant-*Wolbachia* associations described beyond attines and wood ants have been shown to exhibit this same pattern (RUSSELL & al. 2012). So might this phenomenon signify a unique function for the enigmatic *Wolbachia* of these hosts?

To illustrate one possibility, we re-iterate that *Blochmannia* and *Westeberhardia* symbionts show similar trends of loss, where workers de-invest in these symbioses somewhat early on in adulthood. In these systems bacteria seem crucial to their hosts' maturation, providing building-blocks (e.g., aromatic acids or their pre-cursors) used in the construction of adults' cuticles. Might *Wolbachia* play a similar role in attines and wood ants? A game plan for exploring such a function has been established through prior research on camponotines and *Cardiocondyla* (GIL & al. 2003, WOLSCHIN & al. 2004, DEGNAN & al. 2005, ZIENTZ & al. 2006, STOLL & al. 2009, STOLL & al. 2010, KLEIN & al. 2015) and through work on a parallel cuticle-building symbiosis between weevils and their *Sodalis* symbionts (VIGNERON & al. 2014).

just a few lineages made up, mostly, of New World ant-associates. Ants from Europe, Asia, Africa, and Australasia associate with a different range of *Wolbachia* altogether, including one well-represented clade that appears slightly less host-specific (TSUTSUI & al. 2003, RUSSELL & al. 2009a). This geographic split seems unusual amongst insect-*Wolbachia* associations of insects, raising questions about the drivers of divergent symbioses that take place on the opposite sides of our largest oceans.

As suggested above, patterns in the distributions of *Wolbachia* across the ants suggest that there may be hotspots – and coldspots – for infection, as there are few modestly sampled ant genera with infection frequencies resembling the ant-wide average (~ 34 - 36%) (Fig. 7; data from RUSSELL & al. 2012). For example, we see rarity in groups like *Dolichoderus* (0 / 15 surveyed species) and *Dorylus* (0/21 surveyed species), but high prevalence in *Aenictus* (14 / 16 surveyed species), *Tetraoponera* (8 / 10 surveyed species), *Formica* (9 / 10 surveyed species), and potentially *Acromyrmex* (5 / 5 surveyed species). Given the absence of ant genus-specific *Wolbachia* clades, these patterns appear to involve congeners independently acquiring, and keeping, a range of horizontally transferred bacteria that are often from more generalized ant-enriched lineages (RUSSELL 2012). This suggests that physiological, behavioral, or ecological traits distinguishing ant groups may also make them variably suited to serve as hosts for *Wolbachia* symbionts. Comparative studies could hold the key to understanding this phenomenon, but of clear importance will be studies attempting to unlock the impacts of enigmatic *Wolbachia* on these diverse insects. It will also be important to understand whether *Wolbachia* hotspot hosts truly invest in their symbioses or whether they may simply be those most susceptible to manipulation by a crafty group of bacterial endosymbionts.

**Other transovarially transferred symbionts:** Throughout history, dozens of bacterial lineages have evolved lifestyles as transovarially transmitted bacteria of insects (MORAN & al. 2008). Some colonize bacteriocytes, while others localize to hemolymph and other insect tissues. Like camponotines and some *Cardiocondyla*, a few other ant

groups harbor symbiotic bacteria in midgut-associated bacteriocytes that they possibly passage through transovarial transfer. In *Plagiolepis* ants (BUCHNER 1965, DASCH & al. 1984) for example, the apparent bacteriocyte colonists appear related to heritable *Sodalis* symbionts of other insects (SAMESHIMA & al. 1999, WERNEGREN & al. 2003, RUSSELL & al. 2012). While this phylogenetic placement will require further study, a lineage with modest bootstrap support was found to consist of bacteria from two *Plagiolepis* species and another genus, *Euprenolepis*, from the same tribe (RUSSELL & al. 2012), raising the question of whether this could be an ancient symbiosis with clear host investment (i.e., a hotspot). Outside of these examples from the Plagiolepidini, bacteriocytes have been reported in ants of the genus *Formica* (BUCHNER 1965, DASCH & al. 1984), although the identities of their putative bacteriocyte colonists are different from those in *Plagiolepis* (SAMESHIMA & al. 1999). Very little is known about the biology of these symbioses, suggesting fertile ground for future study.

Across the ants, *Wolbachia* are by far the most prevalent and broadly distributed transovarially transferred symbionts (RUSSELL & al. 2012). But recent studies, have detected additional candidates like *Arsenophonus* or *Spiroplasma*, across multiple species (NOVAKOVA & al. 2009, FUNARO & al. 2011, ISHAK & al. 2011a, b, RUSSELL & al. 2012, SEBASTIEN & al. 2012). Complicating interpretation is the fact that bacteria from these clades can exhibit several lifestyles, ranging from gut colonists, arthropod pathogens, or hemipteran-vectored pathogens of plants (CLARK 1982, BRESSAN & al. 2012). Their status as heritable symbionts, thus, remains to be demonstrated. *Serratia symbiotica*, a protective symbiont found mostly in aphids (RUSSELL & al. 2003), and *Cardinium hertigii*, a widely distributed reproductive manipulator (DURON & al. 2008), are two recently identified symbionts from one ant species, *Formica cinerea* (see SIRVIO & PAMILO 2010). While a recent study suggested *S. symbiotica* association with *Camponotus* gut tissues (HE & al. 2011), we know of no instances in which *Cardinium* makes its living as a gut inhabitant, a plant pathogen, etc. This makes it at least

plausible that this species is a low-frequency, heritable symbiont of some ants (RUSSELL & al. 2012); but again, one with unknown function.

Additional symbionts with a possible transovarial transfer route have been found in ants (TUFTS & BEXTINE 2009), including some with relatedness to the genus *Asaia* (KAUTZ & al. 2013a). Such bacteria are interesting in several ways – in *Anopheles* mosquitoes, they appear to suppress *Wolbachia* populations within co-infected hosts (HUGHES & al. 2014). This may explain why *Wolbachia* are rare or possibly absent from the genus *Anopheles* (RUSSELL & al. 2012). The appearance of *Wolbachia* coldspots across the ants makes this finding all the more interesting, so do the findings by KAUTZ & al. (2013a) suggesting low *Wolbachia* frequencies in the genus *Pseudomyrmex* where *Asaia* frequencies were highest. A second point of interest is that *Asaia* belong to a group of acetic acid-generating bacteria, which thrive under the high osmolarity and low pH conditions in the guts of insects with sugar-rich diets. Prior studies have suggested nutritional function for one such microbe cultured from *Tetraponera* ants (SAMADDAR & al. 2011). So the detection of the bacterium in *Pseudomyrmex* workers with extensive feeding on extrafloral nectar (KAUTZ & al. 2013a) suggests an interesting candidate function for these associations.

To summarize, while we have little definitive evidence for transovarial transfer of most bacteria in ants, there is no shortage of candidates. In spite of this, ants may lack or rarely harbor heritable symbionts found commonly in other insects, including those hosted by the insects with which ants frequently interact. For instance, *Hamiltonella defensa* – a common protective symbiont of aphids – was not found in over 200 surveyed ant species (RUSSELL & al. 2012). Frequent ant consumption of aphid honeydew, where *H. defensa* can be found (DARBY & DOUGLAS 2003), makes this finding somewhat surprising, suggesting strong barriers to this symbiont's acquisition or persistence. As heritable symbiotic menageries show some divergence between arthropod groups, this may suggest more general limits to the distributions of even some of the world's best-travelled symbionts (RUSSELL & al. 2012).

**An expanding catalog of multi-partite gut symbioses:** Few of the world's symbionts exist as microbial monocultures – instead, the vast majority are members of multi-species microbial communities, sharing their hosts and interacting with one another through both direct and indirect means. While the above sections occasionally discussed such multi-partite symbioses, several of the focal studies examined just one or a few microbial players at a time. With the advent of next generation amplicon sequencing methods, it is now much easier to explore entire communities from almost any host – or, by our experience, at least those with modest symbiont densities. Two recent investigations have applied such tools to help expand our understanding of ant-associated microbes in the attine system.

In the first of these studies SAPOUNTZIS and colleagues (2015) explored bacterial gut communities from three Panamanian *Acromyrmex* species. From amplicon sequencing of bacterial 16S rRNA across pools of worker guts they found communities to be fairly simple, with domination by only one of four common species, including the usual suspect *Wolbachia*. Traditionally, intracellular *Wolbachia* have not been thought to live inside the gut lumen

(but see ANDERSEN & al. 2012), and it is possible they were associated with gut tissues from the body cavity; this possibility was at least partially supported given minimal evidence for *Wolbachia* in the gut via Fluorescence In Situ Hybridization (FISH) microscopy and the very rare detection of *Wolbachia* in fecal fluid. Two other dominant species came from the order Entomoplasmatales, localized by FISH and electron microscopy to the gut cavity (ileum and rectum), Malpighian tubules, and fat body. Also discovered were *Bartonella*-like Rhizobiales, which appeared localized to biofilms within the ileum and rectum. Rhizobiales and one of the two Entomoplasmatales were stable in lab reared *Acromyrmex* workers on artificial diets, even surviving treatment with some antibiotics (SAPOUNTZIS & al. 2015). Given their findings of NifH proteins in the same gut compartments colonized by Rhizobiales, the authors propose a role for these microbes in nitrogen-provisioning to the ant hosts, envisioning internally housed nutritional mutualists that benefit attines through the same means as fungus garden-dwelling proteobacteria (PINTO-TOMAS & al. 2009).

Applying similar sequencing tools, LIBERTI & al. (2015) explored the overlap of bacterial communities among attines and their social parasites. Their findings suggest that social parasites of the ant genus *Megalomyrmex* share Entomoplasmatales and Rhizobiales symbionts with their various attine hosts. The identified Entomoplasmatales hailed from various sub-lineages within the *Spiroplasma platyhelix* clade, which is enriched with representatives found across the Formicidae (FUNARO & al. 2011). PCR screening across the attine *Sericomyrmex amabilis* and its *Megalomyrmex* social parasite (*M. symmetochus*) indicated that the presence of these bacteria in host ants and gardens was a strong predictor of presence in *M. symmetochus* workers, as there were no instances of this social parasite possessing the bacterium when the host or fungus garden did not. Host attines and their *Megalomyrmex* associates tended to share Entomoplasmatales strains, as evidenced by identical 16S rRNA genotypes, suggesting recent symbiont exchange amongst members of the interacting colonies. In a similar vein, it was shown that Rhizobiales strains were also shared among hosts and social parasites, although presence in host colonies was not a strong predictor of presence in their associated *Megalomyrmex*. The fungus garden could serve as a conduit for symbiont exchange, given consumption of the fungal cultivar by *Megalomyrmex*, and molecular detection of both Rhizobiales and Entomoplasmatales in the studied attine gardens (LIBERTI & al. 2015). These symbionts have also been detected in attine fecal droplets (SAPOUNTZIS & al. 2015), suggesting a route for deposition onto gardens.

One of the other noteworthy trends from these recent attine-based studies has been the high variability amongst microbial communities from different attines (LIBERTI & al. 2015, SAPOUNTZIS & al. 2015). While symbiont communities may sometimes be rather stable and quite similar among members of a single attine population (i.e., as for *Trachymyrmex septentrionalis*; see ISHAK & al. 2011a), different attine colonies and species can engage in symbioses with very different complements of bacterial symbionts. The high variability of these symbioses sets them apart from those of herbivorous ants, like *Cephalotes*, and their symbiotic gut microbiota. Other ants may trend more

Box 4: Mutualists, commensals, or pathogens? For sporadically distributed symbionts we currently just cannot say.

While symbiont ubiquity may suggest obligate associations in groups like *Cephalotes* and *Camponotus*, the more sporadic occurrence of certain bacteria (Rhizobiales, *Spiroplasma*, etc.) in several ant species highlights our limited knowledge of the nature and function of symbionts across this group. *Bartonella*, the bacterial genus most closely related to the ant-associated Rhizobiales clade, is considered a pathogen in mammalian hosts (BREITSCHWERDT & KORDICK 2000); pathogenic relationships are also common in the Entomoplasmatales, the order to which *Spiroplasma* and *Entomoplasma* belong (RAZIN & al. 1998). Without evidence we can no more assert antagonism than we can mutualism. But given the ability of amplicon-based approaches to generate signal from even minute quantities of initial gene copies, the potential functional implications of non-ubiquitous microbial ant symbionts should be considered cautiously – perhaps especially so in the symbiotically "cold" branches of the ant phylogeny.

Indeed, sporadically distributed commensal, or even facultatively pathogenic, microbes could very well be the evolutionary antecedents of the candidate mutualists that are enriched within symbiotic hotspots. Transitions from both free-living and parasitic lifestyles to mutualistic ones appear to be relatively common among bacteria (SACHS & al. 2011). Even *Wolbachia*, long considered principally a reproductive manipulator, can produce outcomes ranging from parasitism to mutualism in insect hosts (e.g., HOSOKAWA & al. 2010).

By the same token, while the extensive morphological specialization and compositional stability of symbiotic organs in ants like *Camponotus* strongly suggest a conserved mutualistic role, assays of the functional importance of its *Blochmannia* endosymbionts have been performed for only a few members of this extremely species-rich and ecologically diverse genus (e.g., FELDHAAR & al. 2007, DE SOUZA & al. 2009). As with the conditional mutualism observed for *Laboubenia* cuticular fungus in *Lasius*, the realized fitness effects of even these highly-integrated symbioses could vary across situation and phylogeny, with contingency upon complex interactions with other microbes and the environment. Hence in the era of cheap and ubiquitous microbial sequence profiling, special care must be taken in the functional interpretations of these relationships.

toward the attine model of high variability within (SANDERS & al. 2014) or among related species, again making symbiont stability in hotspot groups like *Cephalotes* all the more conspicuous.

**Rhizobiales: not just for herbivores:** The high degree of symbiont variability among some closely related ants is further exemplified by emerging trends for *Bartonella*-like Rhizobiales. While clearly enriched in groups with nitrogen-poor diets, these bacteria have been found in a growing number of ants from distinct trophic niches. Fungus-feeding attines and their *Megalomyrmex* social parasites provide some examples, and bacteria from this same ant-specific Rhizobiales clade were also recently found in the yellow crazy ant, *Anoplolepis gracilipes* (see SEBASTIEN & al. 2012). Higher trophic level groups like ponerines and para ponerines appear to possess them too, including *Harpeganthos saltator* (see BONASIO & al. 2010) and the bullet ant, *Paraponera clavata* (see LARSON & al. 2014). Yet variable Rhizobiales presence within some attine species (LIBERTI & al. 2015), within *P. clavata* (see LARSON & al. 2014), and amongst *A. gracilipes* populations (SEBASTIEN & al. 2012) presents a unique pattern in relation to well-sampled herbivorous host groups. *Cephalotes* are certainly the best studied from this latter category, and here Rhizobiales are found across most if not all lineages, with potential ubiquity in some species (RUSSELL & al. 2009b, KAUTZ & al. 2013b, HU & al. 2014, SANDERS & al. 2014). The evidence is, thus, beginning to frame these *Bartonella*-like Rhizobiales as widespread, facultative gut symbionts that have been co-opted into more specialized and, perhaps, obligate symbioses by ants with nitrogen-poor diets.

The impacts of facultative Rhizobiales symbionts remain mysterious. Given their relatedness to *Bartonella* pathogens, we cannot rule out the possibility they are pathogenic to some ant hosts (Box 4). Yet the more com-

mon working hypothesis is that these bacteria are beneficial, nitrogen-provisioning symbionts. Such a perspective was adopted in a recent study of the omnivorous ant *Paraponera clavata* (see LARSON & al. 2014). Capitalizing on the variable presence / absence of the *Bartonella*-like Rhizobiales in this species, the authors examined variation in this symbiont's prevalence across habitat types and in response to diet. Diet had a clear effect as the percentage of Rhizobiales-bearing workers increased in colonies presented with sucrose supplements for two weeks, but not those provisioned with only prey or water. In unmanipulated ant populations, one out of two cross-habitat comparisons suggested higher Rhizobiales prevalence in workers from habitats with potentially greater access to sugar-rich foods (i.e., extrafloral nectar). The apparent increase in Rhizobiales prevalence after two weeks of altered diets was an interesting finding, and it is consistent with these bacteria somehow becoming more abundant in the guts of workers in response to what they eat. At question is whether these changes reflect proliferation of low-density microbes in direct response to dietary sugar or whether ant-mediated control favors Rhizobiales population growth under certain conditions. It is unlikely that this question will be resolved soon given the recalcitrant nature of ants, including bullet ants, as models for manipulative lab research. The lower-hanging fruit here, instead, involves the question of whether these bacteria provision *P. clavata* with nitrogen or other nutrients and, if so, just how they do so. Experiments with isotope-labeling and metatranscriptomics could go some way towards addressing this important question.

**Emerging cases of fungiculture across the ants:** With several ants showing convergence in symbiotic lifestyles, it is important to establish that such patterns extend beyond ant-bacterial symbioses. Indeed, ants have repeatedly evolved to cultivate fungi – for food or for architectural

support. To illustrate these associations we turn to the diverse suite of domatia-colonizing plant ants. Long thought to rely on plant-derived food resources and, in some cases, occasional prey, some plant ants house fungi inside their domatia. Recent studies have shown larvae of several such ant species consume these fungi (BLATRIX & al. 2012), while at least one species is known to "feed" its fungi, most likely through waste deposition (DEFOSSEZ & al. 2011). More than one fungal symbiont may be harbored within a single domatium's fungus patch, yet the ants studied to date tend to have one dominant domatia symbiont, and symbionts at least for now seem somewhat specific to their ant hosts based on phylogenetic analyses (BLATRIX & al. 2013). Pointing further toward the specificity and active management of these interactions are findings that domatia of several non-mutualist plant ant species lack the fungal patches that are nearly ubiquitous within those of mutualistic plant ants like *Petalomyrmex phylax* or *Axinidris afer*; this becomes all the more conspicuous when considering that non-mutualists colonize domatia on the same plants that play host to mutualist ants (DEFOSSEZ & al. 2009). Nutritional benefits received by the plants suggest that this interaction could be a direct, three-way mutualism (DEFOSSEZ & al. 2011). Findings of these fungi in sealed off nests occupied by only queens and her first-born brood further elucidate the life history of these symbioses, hinting that queens either bring fungi with them (via vertical transmission) or that they selectively acquire them from the environment early on in the colony's life (BLATRIX & al. 2013).

Phylogenetic analyses, based on fungal ITS sequences, have placed several domatia fungi from unrelated plant ants into the order Chaetothyriales, a group referred to commonly as the black yeasts (BLATRIX & al. 2013, NEPEL & al. 2014). Found also in the gardens of attine ants, where their roles may be antagonistic (LITTLE & CURRIE 2008), black yeasts appear central to an additional set of mutualisms in which their fungal mycelia provide architectural support for ant constructed carton galleries. Used as nests, runways, or ambush hunting grounds (DEJEAN & al. 2005), these cartons contain a mix of substances like soil or masticated plant material, which are held together by the fungi (SCHLICK-STEINER & al. 2008). Reports indicate that the carton gallery fungi are groomed (MAYER & VOGLMAYR 2009) and that queens from some species may bring them on their nuptial flights (SCHLICK-STEINER & al. 2008), using these inherited microbes to establish structures in or near their new nests. Phylogenetic analyses suggest that several of the "carton gallery" fungi are scattered amongst the Chaetothyriales, not unlike those serving as domatia symbionts. Ant cultivators may harbor one or a few dominant carton gallery fungi (SCHLICK-STEINER & al. 2008, RUIZ-GONZALEZ & al. 2011). But in some cases, a large number of distantly related Chaetothyriales species may co-exist within a single carton structure (MAYER & VOGLMAYR 2009). At present, many of the ant-associates appear to group into ant-specific lineages, suggesting that the selected fungi are at least partly specialized on the ants or vice versa (NEPEL & al. 2014). More intense sampling and comparisons to free-living fungi will be important in assessing the true degree of specificity.

Unlike some symbioses between ants with low nitrogen diets and their gut bacteria, domatia and carton gal-

lery fungal symbionts appear to have been acquired by ant lineages near the tips of the Formicidae phylogeny. In addition to the hosts named above, these fungi are known in species within groups like *Allomerus*, *Azteca*, *Crematogaster*, *Pseudomyrmex*, and *Tetraponera*. But by no means have they been found to be broadly characteristic of these genera. The fact that these symbioses have evolved on numerous occasions presents a useful opportunity to understand the aspects of ant biology associated with the investment in fungiculture. One such correlate could involve active means for maintaining healthy fungal cultivars. Indeed some authors have reported on the presence of cuticular actinomycetes or proteobacteria from these alternative fungus growers (SEIPKE & al. 2013, HANSHEW & al. 2015). While some produce antibiotics of potential use in the maintenance of healthy fungus crops (SEIPKE & al. 2012, GAO & al. 2014), there is no evidence that these substances are actively used in defense of domatia or carton gallery fungi, nor is it clear that the presence of such bacteria on cuticles is a phenomenon restricted to fungus growing ants. Nevertheless, the idea of convergence in "weed control" by multiple fungus growing lineages beyond the attines is an intriguing prospect that warrants further study.

**A new cuticular ectosymbiont engaged in a conditional mutualism:** The cuticle is the first line of defense for many host-pathogen interactions. Thus it is perhaps of little surprise that this location can support communities of microbes that may be important in protection (HARRIS & al. 2009, KUENEMAN & al. 2014). While cuticular *Pseudonocardia* have been proposed to directly defend ants (SAMUELS & al. 2013), another example of a likely cuticular defensive symbiont was recently identified in *Lasius* ants. The defender, a fungus known as *Laboulbenia formicarum*, had been found on a range of ants in prior studies where it was argued to be mildly parasitic. But recent discoveries suggested high prevalence within invasive populations of *Lasius neglectus* (see KONRAD & al. 2015). While costly under starvation, this fungus appears to protect *L. neglectus* against infection by *Metarhizium* fungal pathogens through up-regulating the host ants' innate immunity and promoting increased grooming behaviors (KONRAD & al. 2015). Whether other ants frequently employ microbes for their own defense or whether the *L. neglectus* story is fairly unique is a question waiting to be explored. Either way, the recency of the association and the likelihood of novel pressures on the pathogens of *L. neglectus* suggest potential for this system as a model for natural studies of defensive symbiosis.

## Conclusions and future directions

The past 15 - 20 years have netted fascinating insights into previously hidden or obscure symbioses between ants and their microbes. While the understanding of function is still in its infancy for many, we have learned that these parties show a continuum of fidelities that may range all the way from regular environmental acquisition (MAYER & VOGLMAYR 2009) to exclusive vertical transfer (SAUER & al. 2000). Across this spectrum, phylogenetic signatures of ants' symbionts often suggest that microbes show a history of interactions with members of the family Formicidae and, sometimes, with specific sub-lineages nested within. These recurrent associations suggest opportunities

for reciprocal influence, with ants and specific symbiont clades shaping each other's evolution. Whether such influence is uni-directional, bi-directional, diffuse, or specific will vary across systems and is a question requiring direct investigation. But as argued earlier, not all ants seem to be equally engaged in symbioses with microbes. Based on this observation, hotspots for microbial symbioses can be defined as groups that have engaged with specialized symbiotic bacteria or fungi for tens of millions of years, investing in mechanisms for their housing and transfer. Significantly younger hotspots may also exist, and one might easily imagine that the current absence of such nascent symbioses arises due to the current lack of in depth taxon sampling required for their demonstration. To date, hotspots appear to harbor high-density microbial communities. So do the emerging patterns of low-density bacterial communities across ants suggest that many are coldspots for symbiosis? Or may it be the case that at least some of these ants truly "invest" in symbioses with small numbers of associated bacteria? Also, can we conclude that symbiotic bacteria are unimportant in these groups? While the answers to these questions are not yet clear, trends do reveal that symbiotic associations in some ant lineages vary strongly from colony to colony or species to species, at the very least suggesting a lack of constancy and, perhaps, a lack of strong symbiotic integration. So although we cannot rule out some investment by such ants or the importance of their interactions with bacteria, their symbioses clearly differ from those of several hotspot lineages.

Systematic microbial surveys and symbiont quantification will be important in testing the hypothesis that some ant lineages invest little in microbial symbioses, as will studies on the behavioral, anatomical, and physiological mechanisms used by ants to support or combat bacteria. It will be furthermore important to establish whether potential coldspots for bacterial endo- and ecto-symbionts are truly devoid of other types of microbes and of externally housed symbionts from the nest environment. Indeed, much of the work performed to date has been bacterially biased, meaning we know next to nothing of potentially beneficial protists, archaea, or viruses. Even fungi have largely gone unexplored in molecular surveys, with identified cases being likely those that are most conspicuous; this knowledge gap is not unique to ants, plaguing many insects and animals beyond (GIBSON & HUNTER 2010). It is clear that some insects can harbor specialized or mutualistic symbionts from these understudied microbial groups (NODA & al. 2007, BEZIER & al. 2009, PAUL & al. 2012, KALTENPOTH & STEIGER 2014). As such, our abilities to argue forcefully for symbiotic hotspots and coldspots will remain limited until broader surveys are applied. In our search for undiscovered symbionts, the field will benefit from moving beyond the eubacterial 16S rRNA surveys that so recently became accessible. With dropping costs and increasing infrastructure for data analysis, shotgun metagenomic approaches should become highly useful, in this sense, as they are not limited to the detection of specific microorganisms.

There are many other knowledge gaps when it comes to microbial symbioses among the ants. Perhaps most glaring is the lack of study on the microbiota of ant larvae (see EILMUS & HEIL 2009 for an exception). Serving as the colony stomach in many species, larvae digest the

solid food particles that cannot pass through the narrow proventricular restriction at the crop-midgut junction of adult workers from many ant species (COOK & DAVIDSON 2006). How do larvae process these foods, what roles do microbes play in such processing, where do these microbes come from, and how do their functions impact the colony? Low larval symbiont densities in other social insects (MARTINSON & al. 2012) hint at one possible reason why few larval stage symbionts have moved onto the radar. But larvae, like queens, are often not included in molecular screens of ants due to collection difficulties. Concerted efforts are, thus, needed to understand whether these juveniles may be relatively devoid of symbionts or whether we know so little because we have simply spent such little time looking. In a similar vein, it will be important to consider whether potential coldspot ant species remain viewed in this light when larvae and queens are examined more thoroughly for their symbioses.

Broader questions about the impacts of caste differentiation on the symbiont distributions in ant colonies are intriguing and surprisingly underexplored; so are questions on the capacities for ants to engineer the microbiomes of their "built environments", and on the precise nature of correlated evolution between ant size, anatomy, stoichiometry, and physiology in relation to ant diet and microbial symbioses. In this age of advanced sequencing, phylogenetic, and statistical capabilities, it is our hope that we are primed for a breakout in these realms. Indeed, the time may be ripe for a coordinated ant microbiome initiative. With so much to learn about these ecosystem engineers and the many invasive pests among their ranks, there is clearly much to be gained through such investment. The successful human microbiome project (TURNBAUGH & al. 2007) provides an ambitious template, with the potential to guide research on the hidden microbial dimension of these fascinating and dominant social insects.

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