The ants (Hymenoptera: Formicidae) are unique and enigmatic hosts of prevalent *Wolbachia* (Alphaproteobacteria) symbionts

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Abstract

Intracellular bacteria from the genus *Wolbachia* are the most prevalent microbes found among the arthropods, shaping the ecology and evolution of their hosts through selfish and mutualistic means. The ants comprise an interesting group of *Wolbachia* hosts given their status as eusocial haplodiploids with commonly biased sex ratios. Although experimental hurdles have obscured *Wolbachia*'s impacts on the ants, published findings have still managed to document unique aspects of ant-*Wolbachia* interactions, including exceptional levels of multiple infection, symbiont curing, and potential effects of host mobility on the spread and evolution of these heritable symbionts. To further develop hypotheses on the significance of *Wolbachia* and the forces that shape their distributions across the ants and beyond, I synthesize the results of a growing body of literature that has largely focused on symbiont prevalence. Results from surveys across ~ 455 species indicate that ants are common hosts, although infection levels differ among ant genera and likely across taxa with different modes of colony founding. A meta-analysis of *Wolbachia* distributions across ~ 2,600 other arthropod species revealed a similar pattern of variation among lower-level taxa, suggesting that the ecological and evolutionary shifts governing infection dynamics have occurred on recent timescales. Phylogenetic analyses suggest that ant-associated *Wolbachia* symbionts show some degree of host specificity. However, related strains are generally distributed across ants from multiple tribes and genera, while ants from different locations harbor strains from distinct lineages on the *Wolbachia* phylogeny. Combined, the trends and attributes of these symbioses reveal potential causes of *Wolbachia* proliferation, while hinting at the impacts of enigmatic microbial symbionts across this diverse group of hymenopterans.

Key words: Symbiosis, colony founding, reproductive manipulation, coevolution, review.

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Introduction

Recent discoveries have established ants as common hosts of microbial symbionts, revealing that bacteria and fungi play roles in their nutritional ecology (FELDHAAR & al. 2007, STOLL & al. 2007, DE SOUZA & al. 2009a, PINTO-TOMAS & al. 2009, RUSSELL & al. 2009b). As several of these microbes are specialized and ancient ant associates (SAUER & al. 2000, CURRIE & al. 2003, ZIENTZ & al. 2005, RUSSELL & al. 2009b), it is clear that coevolved, mutualistic symbionts have been significant players in the ants' evolution. In addition to these beneficial and host-specific microbes, ants also harbor more enigmatic and generalist bacteria, with unknown effects on host biology (PELOQUIN & GREENBERG 2003, LI & al. 2005, LEE & al. 2008, TUFTS & BEXTINE 2009, FUNARO & al. 2011). Among this cohort, heritable intracellular bacterial symbionts from the genus *Wolbachia* (Alphaproteobacteria) have, by far, been the most commonly documented microbes to date.

Wolbachia: consequences, spread, and evolution. Although *Wolbachia* spread depends largely on maternal transmission, their abilities to move horizontally have helped *Wolbachia* to colonize numerous crustaceans, arachnids, and nematodes, along with a majority of the world's insect species (HILGENBOECKER & al. 2008). And throughout their 100 plus million year journey across these invertebrates (BANDI & al. 1998), *Wolbachia* have left a distinctive footprint, shaping their hosts' development, behavior, speciation, genome evolution, and genetic diversity (CHARLAT & al. 2003). While recent discoveries have revealed *Wolbachia* to be more versatile than originally thought (WEEKS & al. 2007, HEDGES & al. 2008, TEIXEIRA & al. 2008, KREMER & al. 2009, HOSOKAWA & al. 2010), much of their impact has likely extended from their abilities to manipulate host reproduction. In total, *Wolbachia* can execute one of four types of reproductive manipulation, each favoring their spread through the increased production, survival, or reproduction of infected female hosts (reviewed in WERREN & al. 2008).

Among these selfish, manipulative strategies, the most common is cytoplasmic incompatibility (CI), whereby "incompatible" matings produce few or no offspring. In their simplest form (unidirectional CI), such matings arise when infected males fertilize *Wolbachia*-free females, resulting in improper condensation and fragmentation of the paternal set of chromosomes during the first mitotic division (BREEUWER & WERREN 1990). But incompatibility can also arise when male and female mates harbor *Wolbachia* strains with mismatching modification and rescue capabilities (bidirectional CI). The distinction between these

CI varieties is an important one, as they should have opposite effects on divergence and diversification: While unidirectional CI can promote introgression across species or population boundaries (e.g., ROUSSET & SOLIGNAC 1995), CI in its bidirectional form is more likely to strengthen, or even drive, reproductive isolation (e.g., BORDENSTEIN & al. 2001).

Beyond the well-documented CI phenotype, *Wolbachia* are also capable of inducing thelytokous parthenogenesis within some haplodiploid arthropods (i n d u c t i o n o f p a r t h e n o g e n e s i s), enabling asexual reproduction by infected female hosts. Furthermore, genetic males from a limited range of arthropods can be feminized by their *Wolbachia* symbionts (f e m i n i z a t i o n). And finally, *Wolbachia* are known to kill infected males from several coleopteran, dipteran, and lepidopteran species (m a l e - k i l l i n g). While all four of these phenotypes can influence host sex ratios, they may also affect the genetic diversity and evolution of mitochondrial genomes (e.g., JIGGINS 2003, SHOEMAKER & al. 2004, CHARLAT & al. 2009), revealing long-term consequences of reproductive manipulation.

Justification for ant-*Wolbachia* explorations. As much as we know about the consequences of *Wolbachia* across several groups of arthropods, our knowledge on *Wolbachia*-ant interactions is largely confined to distributional trends. Indeed, the ants are the most heavily sampled of all invertebrate orders, which provides a great opportunity to explore the biological attributes that correlate with infection. In contrast, knowledge on the phenotypic, ecological, and evolutionary consequences of *Wolbachia* infection within the ants remains limited, which is unfortunate in several respects. First, we have little understanding of how *Wolbachia* shape the development, reproduction, and, possibly, the defensive biology of these insects, which include several invasive pests and over 12,000 species. Second, the ants possess several unique attributes that make them an interesting group of potential *Wolbachia* hosts. For instance, frequent sex ratio bias across the ants could either result from *Wolbachia* or render their tricks ineffective. Additionally, the presence of sterile workers, and thus dead-end hosts, raises questions of whether *Wolbachia* are selected to persist, and maintain function, within the adult worker caste. Finally, since ants vary in their modes of colony founding and the associated propensity for queens to disperse short vs. long distances (KELLER 1998), species with different founding modes are likely to also differ in their susceptibility to genetic drift. As drift may promote the establishment of manipulative and slightly deleterious *Wolbachia* symbionts (e.g., STOUTHAMER & al. 1999), it is conceivable that behavioral differences could drive differences in *Wolbachia* prevalence across ant taxa.

In the following passages I revisit these and several other facets of ant biology, examining their significance as mediators of ant-*Wolbachia* symbioses. In doing so, I review the trends of *Wolbachia* infection within individuals, colonies, species, and higher-level ant taxa, placing these latter findings into a broader context through a meta-analysis on *Wolbachia* prevalence across the arthropods. I also dissect the evolutionary histories of these symbioses, discussing the evidence for host specificity and geographic isolation. From there I shift my focus to the consequences of these interactions, citing known effects of *Wolbachia* on their ant hosts, while considering the plausibility of other potential phenotypes. Through synthesizing these results – the culmination of nearly 15 years of research – I not only attempt to explain the forces that drive symbiont prevalence, but to also suggest important avenues for future research on these unusual, yet elusive symbioses.

Distributions of *Wolbachia* across the ants and beyond

Despite the current inability to perform manipulative experiments, we can still learn a good deal about the interactions between ants and *Wolbachia* through studying the distributions and evolution of these heritable symbionts. The utility of the former approach can be illustrated by noting that *Wolbachia* are not found in all species and that their prevalence appears to vary across invertebrate taxa. Such variation enables us to identify host attributes that may govern the spread of these influential microbes. In the following section, I attempt to achieve just that, exploring the prevalence of *Wolbachia* across the ants and beyond.

Prevalent *Wolbachia* infections across the ants. Although first detected in ants in 1995 (WERREN & al. 1995a), it was not until WENSELEERS & al.'s (1998) publication that we gained in-depth knowledge on the prevalence and distributions of *Wolbachia* across the ant family Formicidae. Through use of diagnostic PCR screening (see Box 1 for the limitations of this approach), the authors detected *Wolbachia* infections in 25 / 50 ant species from West Java and West Sumatra (n = 6 workers screened per colony; N = 1 colony screened per species). At the time, this was the highest reported infection level for any arthropod taxon, suggesting a unique status of the ants as hosts of these microbes.

Eleven years after this publication, an even broader study used diagnostic PCR to screen 329 ant colonies (typically, n = 1 worker screened per colony), spanning well over 200 species from six continents, 137 genera, 43 tribes, and 17 of 21 ant subfamilies (RUSSELL & al. 2009a). In total, the authors found *Wolbachia* in 29.1% of the surveyed ant colonies – a significantly lower infection rate than the 50% estimate reported by WENSELEERS & al. (1998). It is possible the discrepancy is partially due to differences in methodology, sampling effort (within species), or sampling locale. However, when comparing frequencies within the 29 genera targeted by both investigations, neither study exhibited a bias toward higher infection rates (i.e., six genera had higher *Wolbachia* frequencies in WENSELEERS & al. 1998, six had higher frequencies in RUSSELL & al. 2009a, and 17 were equivalent). As such, it is more likely that differential sampling across taxa with varying infection levels accounts for the frequency differences between these two surveys.

Aside from these publications, the majority of explorations on ant-associated *Wolbachia* have adopted a more taxon-specific approach, focusing on infection dynamics within individuals, colonies, populations, species, and close relatives from the same genera or tribes. Highlights from this work (see Tab. 1 and Appendix 1, as digital supplementary material to this article, at the journal's web pages) have revealed prevalent infection within several genera, variation in within-population infection frequencies, intracolony polymorphism, and frequent instances of multiple infection. Due to their significance, the details and implications of these findings are discussed throughout subsequent sections of this contribution.

Box 1: Methods of *Wolbachia* detection.

Although occasionally inferred from phenotypic assays, the most reliable means of *Wolbachia* detection can be achieved through molecular screening. Diagnostic PCR with *Wolbachia*-specific primers has been the molecular method of choice, thanks in part to its speed and affordability. While the presence of a properly sized PCR product can often be taken to indicate that these symbionts are present, especially when coupled with sequence confirmation, it should be noted that negatives can arise due to improper primer binding (i.e., in the event of a divergent annealing site) or, alternatively, low titer *Wolbachia* infections (e.g., ARTHOFER & al. 2009).

Another complication of *Wolbachia* screening is the inability to truly assess a species as being uninfected. Since symbionts can be found in host populations at very low frequencies (e.g., SHOEMAKER & al. 2003), diagnosing symbiont absence would require an inordinate amount of intraspecific sampling. One implication is that most diagnostic PCR estimates of cross-species infection frequencies are lower than actual infection rates. For instance, diagnostic PCR surveys across the arthropods, targeting 1 - 10 individuals per species, have detected *Wolbachia* in 15 - 20% of the surveyed species (WERREN & al. 1995a, WERREN & WINDSOR 2000). But a meta-analysis accounting for sampling intensity and documented levels of (intraspecific) polymorphism estimated that 66% of the world's arthropod species are infected (HILGENBOECKER & al. 2008).

In light of these facts, it is important that the limitations of diagnostic PCR surveys should be kept in mind when interpreting estimates of infection levels across arthropod taxa. It should also be noted that the infection frequencies reported from the meta-analysis in this study (e.g., Fig. 1) are strict assessments of presence / absence based on surveys of just one-to-a-few individuals per species. As such, they undoubtedly underestimate the prevalence of *Wolbachia* within the targeted host taxa.

Wolbachia beyond the ants: a meta-analysis across the arthropods. While the findings of RUSSELL & al. (2009a) suggested a less remarkable infection rate across the ants, earlier studies had similarly diminished the ants' status as record-holders. For instance, fig wasps were shown to be enriched for *Wolbachia,* with 59% infection in Panama (26 / 44 infected species, SHOEMAKER & al. 2002) and 67% in Australia (41 / 61 infected species, HAINE & COOK 2005). Gall-forming wasps from roses also appeared enriched for *Wolbachia*, as 62% of 21 surveyed species were found to be infected (PLANTARD & al. 1999). Looking beyond the Hymenoptera, KYEI-POKU & al. (2005) reported that 19 / 19 surveyed louse species (order Phthiraptera) were infected, while BALDO & al. (2007) found *Wolbachia* in 12 / 12 surveyed scorpion species from the genus *Opistophthalmus*, suggesting the potential for near-ubiquity within these lineages.

While *Wolbachia* prevalence from these groups clearly exceeds even the highest estimates seen for the ants (WENSELEERS & al. 1998), we currently lack a comprehensive picture of where the ants rank among the other arthropod hosts. And similarly, we also lack knowledge on *Wolbachia* frequencies across the many well-sampled arthropod taxa. Fortunately the publication of dozens of *Wolbachia* surveys over the past two decades has provided plenty of data to address this shortcoming. By summarizing these findings, we can not only place our knowledge on ant infection rates into a broader and more quantitative context, but we can also begin to generate hypotheses on the host phenotypes or environmental variables that promote *Wolbachia* spread.

To accomplish this, I compiled screening results from 91 publications on *Wolbachia* distributions across various arthropods. Due to an expected bias against publication of negative results, data included in this meta-analysis came from studies that used diagnostic PCR to screen for *Wolbachia* across multiple species (i.e., studies on single species are expected to be especially biased toward positive

findings and were, thus, not included). Results of *Wolbachia* infection status across ~ 3054 species were compiled into a large table that has been published as a supplement to this paper (Appendix 2, as digital supplementary material to this article, at the journal's web pages). Proportions of infected species, based on current sampling efforts (Box 1), were then computed for well-sampled orders, families, and genera (i.e., $n \geq 10$), as illustrated in Figure 1.

Several trends are immediately apparent from this analysis. First, some arthropod groups appear to seldomly host *Wolbachia* symbionts. Most notably, less than 20% of species from the Amphipoda, Coleoptera, Ixodida, and Odonata are infected. In contrast, groups such as Isoptera (termites), Phthiraptera (lice), Scorpiones (scorpions), and Thysanoptera (thrips) are comparatively enriched for *Wolbachia* infection, with rates ranging from 47.7 - 90.9%. As a family, the Formicidae exhibited an infection rate of 34.1% (~ 455 species with, typically, one colony and one worker examined per species), ranking 22nd out of 64 families with at least 10 surveyed species. The infection frequency was nearly equivalent across the remaining hymenopteran species (34.8%; n = 531 species), suggesting that, as a whole, the ants fit the typical profile for this order.

With this large dataset in hand, we can begin to consider the attributes of *Wolbachia* hosts that correlate with symbiont frequency (% infected species, based on current screening efforts). Previous authors have speculated that traits such as eusociality or haplodiploidy might affect *Wolbachia* frequencies (WENSELEERS & al. 1998, VAVRE & al. 2000); but although ants, termites, and thrips are all infected at modest-to-high rates, so are non-social arthropods such as lice and scorpions. In addition, the Vespidae (0% of 16 species) and Apidae (22.2% of 18 species) harbor *Wolbachia* at low rates, further suggesting that eusociality is not a major driver of *Wolbachia* prevalence. Haplodiploidy (broadly defined, e.g., NORMARK 2003) is also not a faithful predictor of *Wolbachia* prevalence. For instance, while 56.3% of thrips species (order Thysanoptera) are infected,

Tab. 1: Patterns of *Wolbachia* infection (in ants) across several hierarchical levels.

Hierarchical level	Findings	Implications
Across orders and families	1) *Wolbachia* are prevalent across lice, scorpions, thrips, fig wasps, crickets, etc. (Fig. 1; Appendix 2). 2) Dragonflies, beetles, and some social Hymenoptera (e.g., Vespidae and Apidae) are rarely infected (Fig. 1; Appendix 2). 3) The Formicidae rank 22nd out of 64 well-sampled families (Fig. 1; Appendix 2).	1-3) Frequency variation at these levels should be interpreted with caution due to variation at lower taxonomic levels (e.g., genera).
Across genera	1) Extensive variation in *Wolbachia* prevalence across genera, even within families (Fig. 1; Appendix 2). 2) High infection rates across *Acromyrmex*, *Formica*, *Solenopsis*, and *Tetraponera* species (Fig. 1; Appendix 2).	1-2) Recent changes in hosts, symbionts, and / or environmental factors may be responsible for differences in frequencies among lower-level taxa.
Across species	1) Ant species relying on independent colony founding rarely harbor *Wolbachia* (WENSELEERS & al. 1998; Appendix 3; Fig. 2).	1a) Increased population viscosity and genetic drift (or unidentified correlates of dependent founding) may favor *Wolbachia* spread. 1b) Common infection in dispersal-limited ant species provides a possible explanation for heightened geographic isolation among ant-associated *Wolbachia*.
Within species	1) Infection frequencies range from 2 - 93% within *Solenopsis invicta* (SHOEMAKER & al. 2003, AHRENS & SHOEMAKER 2005). Low infection rates (< 50%) within some attine species (FROST & al. 2010). 2) High infection levels (> 90%) within some *Acromyrmex*, *Formica*, and *Solenopsis* species (VAN BORM & al. 2001, WENSELEERS & al. 2002, VILJAKAINEN & al. 2008, DE SOUZA & al. 2009b, FROST & al. 2010). 3) Possible loss from invasive populations of *S. invicta* (SHOEMAKER & al. 2000, BOUWMA & al. 2006, YANG & al. 2010) and *L. humile* (TSUTSUI & al. 2003, REUTER & al. 2005).	1) Low-to-intermediate infection levels, coupled with between-population variation, could be due to weak CI or conditional fitness benefits that vary over time and space. 2) High infection levels are consistent with expectations for CI with high penetrance (TURELLI & HOFFMANN 1995). 3) Reintroduction of *Wolbachia* could be considered as a means for biological control of invasive ants, if they are indeed deleterious.
Within colonies	1) Polymorphic infection within colonies for some *Acromyrmex*, *Atta*, and *Formica* species (VAN BORM & al. 2001, KELLER & al. 2001, WENSELEERS & al. 2002, VILJAKAINEN & al. 2008, FROST & al. 2010). 2) No evidence for polymorphism within *Solenopsis invicta* colonies (SHOEMAKER & al. 2003).	1) Likely symbiont curing from adult workers. This may be an adaptation (on the part of manipulative *Wolbachia* or their hosts) that ensures efficient production of sexual females. 2) Loss is either not adaptive in all contexts or does not occur due to evolutionary lag.
Within individuals	1) Frequent multiple infection (REUTER & KELLER 2003, VAN BORM & al. 2003, AHRENS & SHOEMAKER 2005, DEDEINE & al. 2005, VILJAKAINEN & al. 2008, DE SOUZA & al. 2009b) in *Acromyrmex*, *Atta*, *Formica*, and *Solenopsis*. 2) Up to eight strains in *Solenopsis daguerri*, setting the record for number of strains in a single individual (DEDEINE & al. 2005). 3) Supergroup B may be most commonly found in individuals with a Supergroup A strain.	1-2) Ants could be melting pots for genetic exchange. 3) Do Supergroup B strains "piggy-back" upon Supergroup A infections?

haplodiploid scale insects (families Coccidae, Dactylopiidae, Diaspididae, and Margarodidae) exhibit a 15.4% infection rate, while 28.6% of whitefly species (Aleyrodidae) are infected. Furthermore, haplodiploid mites from the Mesostigmata and Prostigmata are respectively infected at unremarkable rates of 35.7% and 25.6%. We are, therefore, left to invoke other factors that govern the dynamics of *Wolbachia* spread. As suggested below, these traits have likely evolved on more recent timescales than either eusociality or haplodiploidy.

***Wolbachia* beyond the ants: variation across lower taxonomic levels.** Although arthropod orders show clear differences in their infection levels (Fig. 1A), a close examination shows that this obscures substantial variation across related families and genera (Figs. 1B & 1C). At the family level, this trend is exemplified within the Orthoptera (grasshoppers, crickets, and katydids) – the cross-species infection frequency within this order is not extraordinary (26.3%), yet this value is a composite of apparently prevalent infection within the Gryllidae (72.7%) and
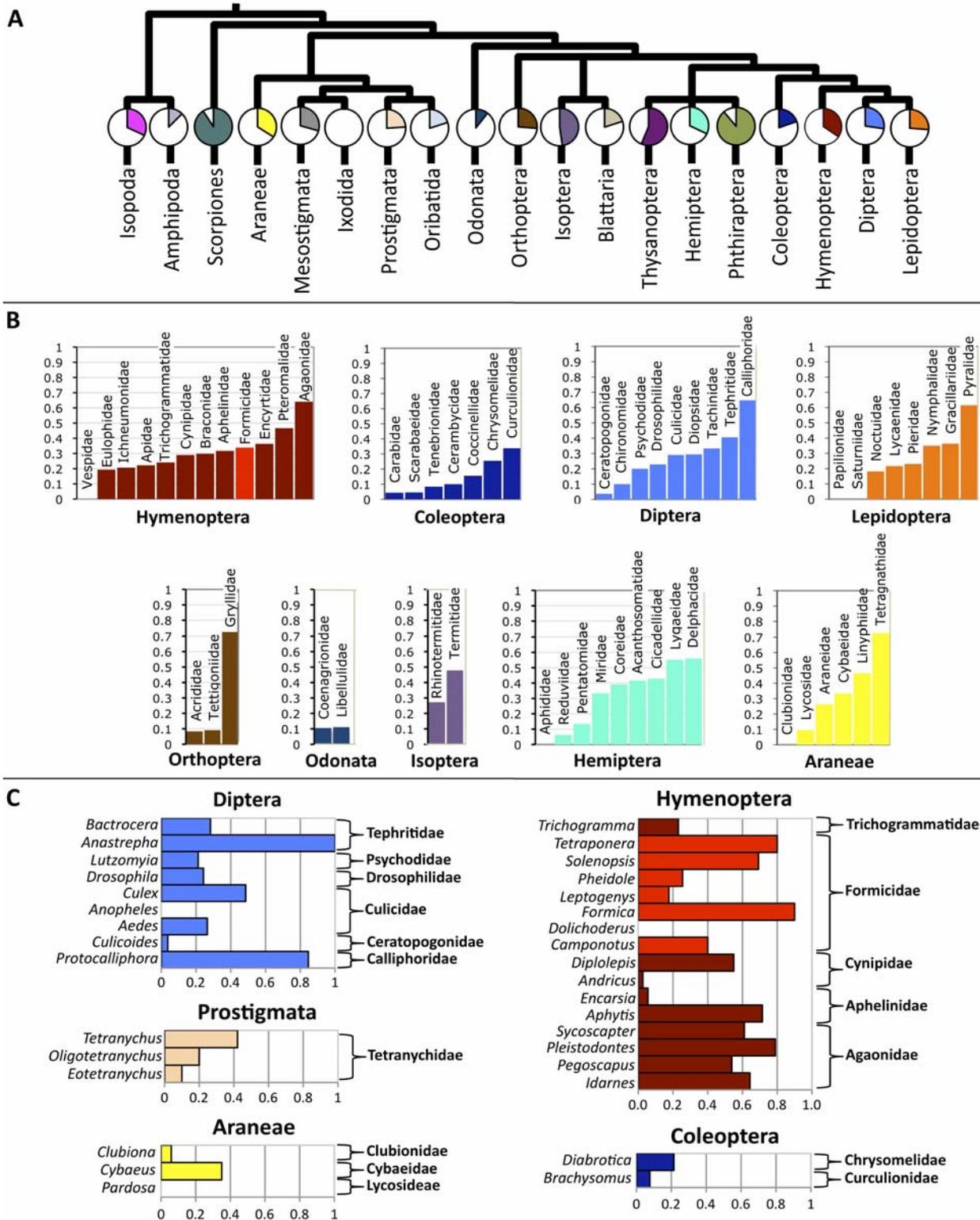
Fig. 1: *Wolbachia* frequency across arthropod taxa. The results of diagnostic PCR screening across 91 publications were compiled (Appendix 2) and graphed to illustrate the prevalence of *Wolbachia* symbionts across host orders (A), families (B), and genera (C). Note that graphs illustrate the deleterious inducers of infected species detected to date. For the sake of brevity and accuracy, only taxa with at least 10 surveyed species have been illustrated. To highlight how frequencies vary among related taxa, infection rates (proportions of infected species) were depicted within families (B) and genera (C) belonging to orders with at least two taxa with $n \geq 10$ surveyed species. Note that color coding is consistent between sections A - C, and that ant data are highlighted in red to distinguish them from the remaining hymenopteran taxa. The schematic tree depicted in section A was assembled based on information from the Tree of Life website (http://tolweb.org/tree/).

rare infection levels in both the Acrididae (8.3%) and Tettigoniidae (9.1%). Similarly, the estimate of *Wolbachia* prevalence for the Hymenoptera (34.4%) conceals variable infection rates among ant, bee, and wasp families, which ranged from 0% (Vespidae) to 64.1% (Agaonidae) infection across species.

At the genus level, variation is also extreme within the mosquito family Culicidae (order Diptera; 29.1% infection): 0% of species from the genus *Anopheles* (n = 35) were infected compared to 48.6% infection within the genus *Culex* (n = 35). Gall wasp species from the Cynipidae (order Hymenoptera; 29.0% infection) showed similar variation, with frequencies ranging from 2.6% among *Andricus* species (n = 38) to 55.0% within the genus *Diplolepis* (n = 20).

This general trend is also exemplified within the ants. For instance, the Formicinae had an infection frequency of 46.2%. Within this subfamily, infection levels of well-sampled tribes (n > 10) varied from 30.4% (Camponotini; n = 23) to 71.4% (Formicini; n =14), with the high infection prevalence among the Formicini being driven by the genus *Formica* (9 / 10 infected). Infection rates among several other well-sampled ant genera also varied substantially (Fig. 1C), matching findings from a recent study on *Wolbachia* in the attines (FROST & al. 2010).

Combined, these observations reveal that generalizations of infection levels within higher level taxa (e.g., subfamilies and tribes) must be qualified until the genera contained within can be sampled with adequate effort. This variation also indicates that more accurate estimates of infection prevalence across the ants and other arthropods will require screening across lower level taxa in proportion to their total diversity.

A possible correlate of *Wolbachia* prevalence across the ants. As mentioned above, frequency variation among lower-level taxa suggests that the attributes promoting *Wolbachia* prevalence have likely evolved on recent timescales. One promising determinant that appears rather labile across the ants was first suggested by WENSELEERS and colleagues (1998), who drew comparisons between *Wolbachia* presence and several behavioral and ecological attributes of the studied ants, including queen number per colony, nesting habits (i.e., arboreal vs. soil), reproductive mode (i.e., queens vs. workers; winged vs. wingless queens), and mode of colony founding. Interestingly, the authors noted that *Wolbachia* were rarely found among species engaging in obligately independent colony founding (1 / 8 infected species), in which inseminated, alate queens disperse by flight to establish colonies individually (see KELLER 1998 for a review on colony-founding modes). Ant species with other (or mixed) modes of colony founding – including budding, fission, and pleometrosis (founding by multiple queens) – were more commonly infected (11 / 22 species harbored *Wolbachia*), although the difference between these groups showed only borderline significance (WENSELEERS & al. 1998).

Using a more up-to-date dataset on infection frequencies and colony founding (Appendix 3, as digital supplementary material to this article, at the journal's web pages), I revisit this relationship (for more detail, see Appendix 1). In essence, although the trend still remains, it is non-significant, being clouded by prevalent infection in one group of independent founders (i.e., leaf-cutter ants) and small num-
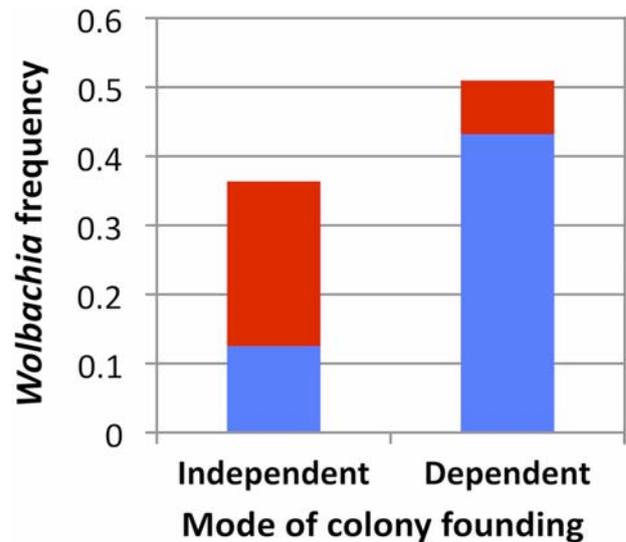

Fig. 2: *Wolbachia* infection across dependently vs. obligately independently founding ant species. Based on literature reviews of the *Wolbachia* and ant literature, ant species previously screened for *Wolbachia* symbionts were classified as either obligately independent founders or dependent founders that tend to utilize budding or fission (with rare pleometrosis) to found colonies (Appendices 1 and 3). The proportions of infected species within these two classes were then graphed. To account for the effects of extensive sampling within the genus *Formica* (dependent founders) and two groups of Attini genera (*Atta* and *Acromyrmex*; independent founders; for a review on the Attini, see MEHDI-ABADI & SCHULTZ 2010), the relative contributions of each toward total infection frequencies were illustrated with red shading. Thus, blue bars reveal infection levels without these data.

bers of independently founding species (Fig. 2). Yet, if one removes the heavily sampled ant genera from this analysis, infection is significantly lower across the obligately independently founding species (p = 0.0342). Additionally, a concentrated changes test (MADDISON 1990, MADDISON & MADDISON 2003) examining the frequency of *Wolbachia* acquisition across the ant phylogeny detected a borderline significant trend of *Wolbachia* rarity within independently founding lineages (p = 0.065; see Appendix 1). While these results indicate that the validity of this correlation requires further examination, it appears that the original findings of WENSELEERS & al. (1998) have held up in subsequent studies, when we look beyond the attines. And since independently founding queens typically disperse through flight, while those from most of the analyzed non-independent founders disperse via foot (i.e., budding or fission), it appears that host queen mobility is correlated with *Wolbachia* infection.

Evolutionary histories of *Wolbachia*-ant associations

With the distributional patterns of *Wolbachia* symbionts in mind, it becomes important to address the evolution of ant-*Wolbachia* interactions. Just how specific and ancient are these symbioses? Do the ants harbor an exclusive group of related *Wolbachia* strains that have radiated onto species from this family? And have these bacteria coevolved with their ant hosts? If so, are *Wolbachia* specific to particular genera or tribes, or do they appear to be more broadly speci-

alized on the ants? Several molecular studies have addressed these topics, yielding one of the most detailed depictions of arthropod-Wolbachia evolution assembled to date.

The degrees of specificity and timescales of infection across the arthropods. *Wolbachia* symbionts undergo faithful maternal transmission with few directly observed instances of natural horizontal transfer (but see HUIGENS & al. 2000, HUIGENS & al. 2004a). Given this, one could envision two extremes in terms of their specificity. First, they could codiversify with their hosts, being maintained by strict maternal transmission for millions of years. At the other extreme, *Wolbachia* could undergo rampant host switching between unrelated arthropods, showing no evidence for fidelity to particular taxa. Generally speaking, the first scenario can be ruled out for several reasons, most notably because *Wolbachia* and arthropod host phylogenies are not congruent (e.g., O'NEILL & al. 1992, WERREN & al. 1995b, SCHILTHUIZEN & STOUTHAMER 1997, but see RAY-CHOUDHURY & al. 2009). The rarity of cospeciation implies that these symbionts are gained and lost on timescales that are shorter than the lifespan of most host species (SHOE-MAKER & al. 2000).

A handful of studies have used molecular clocks and coalescent modeling in attempts to quantify these timescales, finding that the times since *Wolbachia* invasion and spread within host species typically range from 3,000 - 50,000 years (SHOEMAKER & al. 1999, JIGGINS 2003, RASGON & al. 2006, JAENIKE & DYER 2008, DUPLOUY & al. 2010). To date, only one study has attempted to date the time since *Wolbachia* invasion within an ant species, estimating that several mtDNA lineages of *Solenopsis invicta* were invaded in excess of 50,000 years ago (AHRENS & SHOEMAKER 2005).

Although *Wolbachia* persist for short durations, occasionally moving between distant relatives, several studies have noted that related arthropod hosts tend to harbor related *Wolbachia* strains, or at least strains with related alleles (SCHILTHUIZEN & STOUTHAMER 1997, BOUCHON & al. 1998, VAN MEER & al. 1999, JIGGINS & al. 2002, DITTMAR & WHITING 2004, KYEI-POKU & al. 2005, BALDO & al. 2007, 2008). These suggest some degree of fidelity, hinting at a potential for diffuse coevolution between *Wolbachia* symbionts and particular arthropod taxa.

Specificity across the ants: *Wolbachia* **supergroups.** With the above trends in mind, we can now assess the evidence for specificity among ant-*Wolbachia* interactions, reminding ourselves that distributional patterns for these symbionts will generally not extend from cospeciation. Instead, findings of related strains in related ant hosts would indicate that horizontal transfer most readily occurs among close relatives, possibly due to the sharing of transmission vectors (e.g., parasites) or to physiological specialization on similar host backgrounds (see RUSSELL & al. 2009a for a review, but see LACHAISE & al. 2000, SHOEMAKER & al. 2000, and JIGGINS 2003, for an alternative explanation – symbiont introgression – that applies in some cases).

Early attempts to genotype the *Wolbachia* strains of ants were made through the use of diagnostic PCR primers that distinguished between Supergroups A (Sg-A) and B (Sg-B) – the two lineages that are most common across the insects (WERREN & WINDSOR 2000). These efforts detected Sg-A *Wolbachia* in a majority of infected ants, observing that Sg-B strains were rare and most often found

as co-infecting strains within hosts harboring Sg-A (WEN-SELEERS & al. 1998, VAN BORM & al. 2001). Although subsequent investigations suggested a possibly higher frequency for Sg-B (SHOEMAKER & al. 2000, TSUTSUI & al. 2003, VAN BORM & al. 2003), their reliance on a single gene (i.e., *wsp*) with a propensity to undergo extensive recombination (JIGGINS & al. 2001, WERREN & BARTOS 2001, BALDO & al. 2010) called these results into question (e.g., AHRENS & SHOEMAKER 2005).

Fortunately, *Wolbachia* strains from singly infected ants have been typed using a Multi Locus Sequence Typing method (BALDO & al. 2006), helping to better address the abundance of Sg-B (RUSSELL & al. 2009a, FROST & al. 2010). This MLST approach involves sequencing across five housekeeping genes that are scattered across the *Wolbachia* genome (BALDO & al. 2006). With data from multiple genes in-hand, subsequent phylogenetic and strain typing analyses can consider the weight of evidence across loci when deciphering whether sequence relatedness (or divergence) has arisen due to clonal inheritance (and mutation) or recombination.

In total, strain typing with MLST produced results similar to those from earlier diagnostic screening studies (WEN-SELEERS & al. 1998, VAN BORM & al. 2001): Out of 54 MLST typed *Wolbachia* strains from ants with single infections, 52 belong to Sg-A, while the remaining two fall within Sg-B and -F (BALDO & al. 2006, RUSSELL & al. 2009a, FROST & al. 2010). Looking more broadly, Sg-A strains are generally more prevalent across the Hymenoptera when compared to Sg-B (WEST & al. 1998, WERREN & WINDSOR 2000). This suggests that these insects may be predisposed to infection by a limited range of symbionts, or that one of these symbiont clades either gained or lost the ability to easily colonize and invade populations of hymenopterans.

Specificity across the ants: MLST typing and phylogenetics of ant-associated *Wolbachia***.** Although informative, the classification of *Wolbachia* strains to supergroups cannot reveal finer-scale evolutionary dynamics. Indeed, sequence divergence at ftsZ and 16S rRNA genes suggests that Supergroups A and B diverged between 58 and 67 million years ago (WERREN & al. 1995b). Since this split, members of these supergroups have proliferated across a wide range of insects, mites, spiders, and isopods, providing a testament to their abilities to undergo host switching. Given these facts, it is clear that phylogenetic analyses depicting the histories within supergroups are necessary if we truly wish to understand the evolution of *Wolbachia*-host interactions.

Phylogenies based on the *wsp* gene were the first to reveal relatedness among *Wolbachia* strains within the Formicidae, suggesting that distantly related ants harbor related strains from ant-specific clades (SHOEMAKER & al. 2000, VAN BORM & al. 2001, TSUTSUI & al. 2003, REUTER & al. 2005, DE SOUZA & al. 2009b). But due to the aforementioned problems with single-gene phylogenies (especially those using *wsp*), two published MLST studies on ant-associated *Wolbachia* have given us more accurate insights (RUSSELL & al. 2009a, FROST & al. 2010).

In brief, phylogenies constructed with sequence data from five housekeeping loci supported the general trends garnered from *wsp* sequences – that is, *Wolbachia* from the ants tend to form monophyletic clades (RUSSELL & al.

2009a, FROST & al. 2010). Statistical tests indicated that this trend was non-random, suggesting that the trait of ant association is constrained on the *Wolbachia* phylogeny (RUSSELL & al. 2009a). Furthermore, both Analysis of Molecular Variance statistics and extensive allele sharing among strains from ants supported the phylogenetic results, providing strong evidence for fidelity in the associations between ants and their *Wolbachia* guests (RUSSELL & al. 2009a).

Although the two aforementioned studies have made progress in characterizing *Wolbachia* evolution, 46 Sg-A strains were recently typed using the MLST system (STAHL-HUT & al. 2010). These have not been analyzed along-side strains from the ants, necessitating a re-assessment of the observed trends. To accomplish this, I have performed a phylogenetic analysis on 132 published Sg-A strains, using the program ClonalFrame (DIDELOT & FALUSH 2007), as described previously (RUSSELL & al. 2009a). In spite of the additional data (from mushroom-associated insects, mostly dipterans), the published trends remain in tact (Fig. 3) – ants commonly harbor *Wolbachia* from ant-specific, or predominantly ant-specific, clades. In fact, 26 of the 52 ant associates from Sg-A fell into one of six ant-specific clades. It is interesting to note that those from the same genera do not appear to be infected with taxon-specific strains of these symbionts. For instance, *Pheidole* and *Acromyrmex* species harbor strains from several clades consisting of strains from other ant genera.

The topology of this updated MLST phylogeny also supports a previously documented trend that has, thus far, only been seen for ants. Specifically, while several closely related *Wolbachia* strains from *Drosophila* or Lepidoptera showed cosmopolitan distributions, groups of related symbionts from ants are confined to either the New World and Old World / Oceania regions (RUSSELL & al. 2009a). Among these geographically isolated strains, trends of host association were greatest for those from New World ants (20 / 30 New World *Wolbachia* strains clustered into one of four ant-specific clades, while 6 / 22 strains infecting ants from Old World and Oceania locations grouped into one of two ant-specific lineages). These findings echo a previous *wsp*-based discovery (TSUTSUI & al. 2003), illustrating a greater degree of specificity for symbioses between *Wolbachia* and New World ants.

Combined, these patterns suggest some interesting and unique aspects of ant-*Wolbachia* interactions. First, New World (and, possibly, Old World) ants have diffusely co-evolved with separate lineages of *Wolbachia* symbionts that do not, at present, appear specialized on particular ant lineages. This has not involved codiversification, nor does it appear to be driven by introgression of *Wolbachia* strains among relatives. Instead, the trends of host specificity are likely due to horizontal transfer being most successful when symbionts move between relatives. A literature review supports this notion, suggesting that *Wolbachia* from other arthropods are indeed most capable of persisting in close relatives of their natural hosts after experimental horizontal transfer (RUSSELL & al. 2009a).

Second, the tendency for *Wolbachia* to more commonly infect budding and fissioning ant species suggests that ant hosts of *Wolbachia* are relatively immobile (i.e., queens of these species establish new colonies by foot). Such limitations, combined with some degree of host specificity,
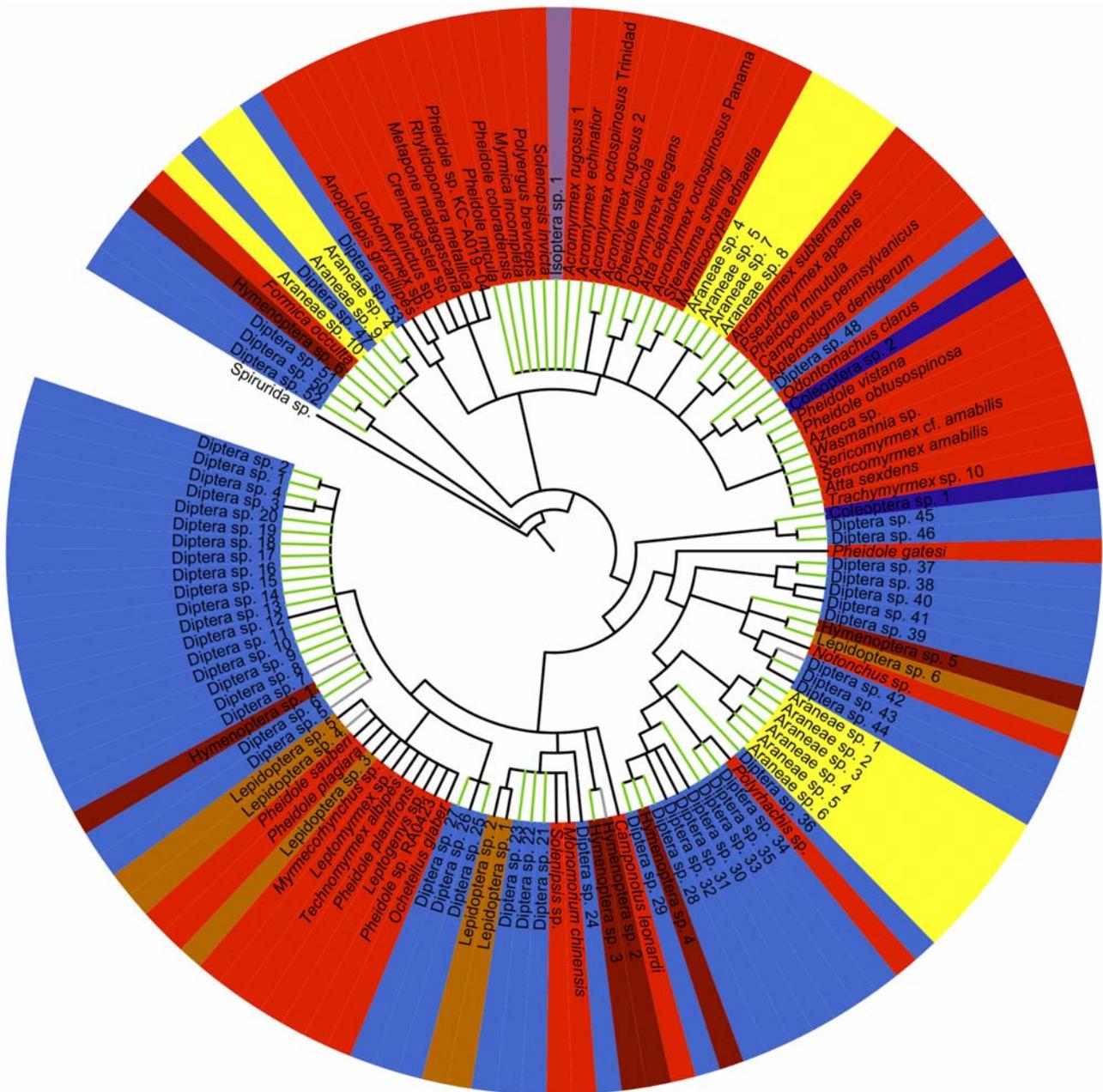
may have promoted separate coevolutionary trajectories for ant-associated strains from opposite sides of the Atlantic and Pacific Oceans. Since the extent of transoceanic isolation does not appear to be as great for *Wolbachia* of other, more vagile hosts (i.e., fruit flies, moths, and butterflies; see RUSSELL & al. 2009a), host mobility appears as an early candidate mechanism shaping both the evolution and proliferation of *Wolbachia*. Future studies will help to reveal whether these trends remain a unique feature of ant-*Wolbachia* interactions, or whether they more generally extend to arthropod taxa with limited dispersal.

Consequences of *Wolbachia* infection in ants

Having suggested that the ants are commonly infected with *Wolbachia* from ant-specific lineages, we can now address the most important question from the perspective of a myrmecologist: What are the consequences of *Wolbachia* infection? A satisfactory answer to this question will suggest ways by which *Wolbachia* shape both the ecology and evolution of over one third of the species within the Formicidae. In raising this question, several related questions immediately come to mind: For example, have *Wolbachia* earned their living through reproductive manipulation? Or might they be mutualistic partners that benefit host fitness? Furthermore, have aspects of the ants' eusociality promoted any novel interactions that are not found in non-social insects? Below, I review several studies that have attempted to address these issues, highlighting the most likely consequences and the largest shortcomings of our current knowledge on *Wolbachia*'s impacts within the Formicidae. In Appendix 4 (as digital supplementary material to this article, at the journal's web pages), I also provide a synopsis of this research in the form of a table depicting the weight of the evidence supporting these candidate phenotypes.

Sex ratio and *Wolbachia* infection in ants. Early work on *Wolbachia* in ants was often pitched in the context of potential host-symbiont conflicts involving optimal sex ratio. It was argued that these microbes could play a previously unforeseen role in the production of female-biased colonies due to their known abilities to alter the relative numbers of males and females (WENSELEERS & al. 1998, KELLER & al. 2001).

Formica exsecta represented an intriguing early candidate host for *Wolbachia*-driven sex ratio manipulation, due to its production of split sex ratios, whereby colonies typically produce a majority of either male or female sexuals. While relatedness asymmetry explained some variation in this trait (i.e., higher male production in colonies where workers have lower relatedness), this mechanism failed to explain sex ratio variation within some populations (CHAPUISAT & KELLER 1999), suggesting that *Wolbachia* or some other factor could play a role. PCR screening confirmed that *Wolbachia* were, indeed, present within *F. exsecta*. However, the authors found that there was no evidence for higher symbiont prevalence in female-producing colonies (KELLER & al. 2001). This finding resembles those from other species exhibiting split sex ratios, including ants with (i.e., *Formica truncorum*: no correlation between within-colony *Wolbachia* prevalence and sex ratio; WENSELEERS & al. 2002) and without *Wolbachia* (DEBOUT & al. 2010). And while the presence of different *Wolbachia* strains in colonies with different sex ratios could still enable a symbiont-driven mechanism (e.g., RIEGLER & STAUFFER 2002),

Fig. 3: MLST phylogeny depicting the relatedness among Supergroup A *Wolbachia* strains. The tree was constructed based on an alignment of five housekeeping genes using the program ClonalFrame (DIDELOT & FALUSH 2007), which uses multi-locus sequence datasets to reconstruct microbial genealogies. More details on the approach used here can be found in RUSSELL & al. (2009a). To illustrate host taxonomy, external nodes are color-coded according to the scheme in the legend (the same as that used for Fig. 1). Geographic origins from either the New World or the Old World / Oceania are illustrated with green vs. black branch shading, respectively. Gray branches reveal unknown origins. *Wolbachia* from ants are named after their hosts, while those from other arthropods are named after their hosts' orders.

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there is currently little evidence that implicates *Wolbachia* in the manipulation of ant sex ratios.

Do *Wolbachia* manipulate ant reproduction? Although *Wolbachia* infection can have several effects on host fitness and phenotypes (DEDEINE & al. 2001, WEEKS & al. 2007, HEDGES & al. 2008, TEIXEIRA & al. 2008, KREMER & al. 2009, HOSOKAWA & al. 2010), reproductive manipulation is currently the most commonly documented effect of infection by *Wolbachia* from Sg-A and -B (WERREN & al. 2008). Yet at least two of the four known manipulations appear unlikely in ants. First, feminization of haploid male ants would yield sterile haploid females (WENSELEERS & al. 1998, VAN BORM & al. 2001) – clearly not an efficient strategy for maternally transmitted *Wolbachia*. On top of this, feminizing *Wolbachia* are only known from two different insects (a butterfly and a leafhopper), and both have a different sex-determination system than that employed by ants (HIROKI & al. 2002, NEGRI & al. 2006). Second, the induction of thelytokous parthenogenesis has also been ruled unlikely within the ants, based largely on theoretical grounds (WENSELEERS & al. 1998, SHOEMAKER & al. 2000). In particular, since the mechanism of parthenogenesis induction for *Wolbachia* involves duplication of the haploid complement of chromosomes in unfertilized eggs (STOUTHAMER 1997), *Wolbachia*-induced thelytoky would lead to the production of diploid ants that are homozygous at all loci. And given that the production of females requires heterozygosity at sex determining loci (CROZIER 1971), *Wolbachia*-induced chromosomal duplication would result in the production of sterile, diploid males in ants. In addition to this theoretical evidence, the several thelytokous ant species studied to date have all tested negative for *Wolbachia* infection (WENSELEERS & BILLEN 2000, HIMLER & al. 2009), further hinting that thelytoky is not a consequence of *Wolbachia* infection in ants.

Cytoplasmic incompatibility (CI) and male-killing comprise the two remaining varieties of reproductive manipulation. CI has been more typically proposed as a consequence of *Wolbachia* infection (WENSELEERS & al. 1998, SHOEMAKER & al. 2000, VAN BORM & al. 2001), based partly on the fact that it is the most common form of reproductive manipulation across the arthropods (WERREN & al. 2008). But the reality is that no study has performed the experiments required to demonstrate either phenomenon within the ants. The absence of such experimentation is, of course, no fault of any investigators. Instead, it derives from the difficulty of rearing and breeding ants in the laboratory, an obstacle that continues to obscure our views on how *Wolbachia* make their living in the Formicidae.

Beyond reproductive manipulation: a unique phenomenon in the ants. Given the lack of concrete evidence for reproductive manipulation, it is worth noting that a more definitive consequence of *Wolbachia* infection has been detected – reduced production of sexuals (WENSELEERS & al. 2002). This effect was seen for *Formica truncorum*, which showed polymorphic infections within colonies, exhibiting lower *Wolbachia* levels in adult workers compared to worker pupae, gynes, and males (45% vs. 87%, 94%, and 95%, respectively). The frequency of infection among adult workers varied between colonies, and colonies with lower *Wolbachia* levels produced a greater biomass of male and female sexuals on a per-worker basis.

Wrapped up in this result are two intriguing phenomena. The obvious one is that *Wolbachia* may exert a physio-logical cost. Although costs have been seen in other insects (FLEURY & al. 2000, HUIGENS & al. 2004b), this is the only piece of evidence supporting a detrimental effect in the ants. And it is interesting to note that the cost to *F. truncorum* only showed up from the perspective of sexual production (i.e., total sexual biomass), as there was no significant relationship between worker infection rate and worker biomass (WENSELEERS & al. 2002). So perhaps *Wolbachia* infection limits the abilities of workers to provide sufficient resources required for the development of sexual ants.

The second phenomenon of interest, apparent *Wolbachia* loss, is one that largely appears unique to ants. Loss in other arthropods is often driven by transmission failure; and the few cases in which individual arthropods are "cured" appear driven by diapause or exposure to extreme temperatures (STEVENS 1989, PERROT-MINNOT & al. 1996). In contrast, curing within *F. truncorum* is systematic, with symbionts apparently going extinct in almost half of their original worker hosts. Although it is possible that the diagnostic PCR procedure used to detect *Wolbachia* may have failed to detect low-titer infections (e.g., ARTHOFER & al. 2009), the bottom line is that curing (or dips in densities to below detectable levels) is a phenomenon seen only in the ants.

In most insects, symbiont disappearance from females would be a baffling and inviable strategy from *Wolbachia*'s perspective. Yet ants are eusocial insects that typically have a sterile worker caste, making queens the only transmitting members of most colonies. So barring a fitness benefit to the colony (e.g., if *Wolbachia* defend workers against natural enemies), *Wolbachia* persistence in non-transmitting workers may be under relaxed selection. In cases where these symbionts come with a cost – as for *F. truncorum* – both the host and the symbiont may be selected to terminate the relationship within the worker caste. Here, loss would promote increased colony performance, also enhancing *Wolbachia* fitness by favoring increased production of transmitting hosts (i.e., queens).

It is currently not clear whether loss is a result of clearance by ants or effective suicide by *Wolbachia*. Furthermore, in this latter case it is not known how the symbiont could determine that it is truly infecting a sterile host. Regardless, this unique and potentially adaptive phenotype does not appear limited to *Formica truncorum*. Indeed, *Wolbachia* loss from adult workers was originally suggested for adult *F. exsecta* workers, which harbor symbionts at a lower level than worker pupae from the same colonies (KELLER & al. 2001). Beyond this genus, a higher infection rate among *Acromyrmex echinatior* queens than adult workers suggests that loss also occurs within these leaf-cutter ants (VAN BORM & al. 2001). And furthermore, *Atta* and *Acromyrmex* workers from infected, monogynous colonies often show intra-colony polymorphism (VAN BORM & al. 2001, FROST & al. 2010), as one would expect given *Wolbachia* loss (or transmission failure). As some species show no evidence for loss (e.g., *Solenopsis invicta* workers and queens were argued to have similar infection levels according to SHOEMAKER & al. 2003), the breadth of this phenomenon across the Formicidae remains to be determined.

A synthesis and a road map for the future

In the fifteen years since *Wolbachia* were first detected in ants, a good deal of progress has been made in describing their prevalence and distributional patterns. These studies

have made it clear that ant-*Wolbachia* interactions exhibit several unique features, including symbiont loss, exceptional levels of multiple infection (Appendix 1; Tab. 1), and a potential effect of host mobility on both coevolution and levels of infection.

When we combine the observed trends of infection with our knowledge on the specificity of ant-*Wolbachia* interactions and the possible outcomes of infection, the patterns ascertained provide us with important clues about the biological attributes promoting *Wolbachia* infection. Below, I briefly reiterate the major findings reported above, weaving them into a series of hypotheses proposed to explain infection levels and the effects of *Wolbachia* on the ecology and evolution of their ant hosts.

Distributional patterns and their potential causes. As originally suggested by WENSELEERS & al. (1998), ants are common hosts of heritable *Wolbachia* symbionts. However, they are no longer the record holders when it comes to infection prevalence, with groups such as lice (Phthiraptera), crickets (Gryllidae), scorpions from the genus *Opistophthalmus*, and spiders from the Tetragnathidae exhibiting over two-fold greater infection rates (Fig. 1).

When further examining the trends of *Wolbachia* prevalence across the Formicidae, it is clear that the modestly high infection frequency for this family obscures notable variation among different tribes and genera (Fig. 1). For instance, species from the genera *Acromyrmex*, *Formica*, *Solenopsis*, and *Tetraponera* are commonly infected. In contrast, *Wolbachia* appear rare among *Dolichoderus* and *Leptogenys* species, whereas the heavily sampled genus *Pheidole* shows intermediate prevalence. Similar trends can be seen for other arthropods, thus indicating that generalizations about *Wolbachia* prevalence within higher-level taxa should be made with caution until extensive sampling has targeted a substantial fraction of the diversity that lies within.

Since *Wolbachia* rarely codiversify with their arthropod hosts, variation in prevalence suggests that their abilities to spread and persist differ among lower-level taxa. This could arise due to differences in the symbionts, their hosts, environmental factors, or a combination of the three. At least five different models invoking these factors can be hypothesized to explain this variation. First, arthropod taxa may differ in their frequency of opportunities to acquire *Wolbachia* symbionts. Second, host-specific symbionts may occasionally evolve key innovations enabling them to radiate across related hosts with favorable physiological, behavioral, reproductive, or ecological attributes. Third, phylogenetic differences among host taxa could influence the ease of spread of host-specific symbionts among related hosts. Under this scenario, clades with higher average relatedness (i.e., more recent times since common ancestry) among species and, presumably, greater physiological similarity, would favor the proliferation of host-specific *Wolbachia* symbionts (ENGELSTADTER & HURST 2006). Fourth, host taxa may vary in their susceptibility to invasion by reproductive manipulators. And fifth, pre-adapted symbionts could increase host fitness in some taxa, promoting their proliferation among related arthropods experiencing similar selective pressures. Keeping in mind the trends of infection across host taxa, we can begin to consider the relevance of the aforementioned causes within the ants and beyond.

(1) Opportunities for symbiont acquisition. This first possibility (differences in acquisition opportunities) has

been proposed to explain trends of multiple infection among social ant parasites (DEDEINE & al. 2005), which may be commonly exposed to hosts with different *Wolbachia* types. This suggests that a more formal assessment of prevalence across social parasites may prove fruitful. More generally speaking, parasitism has been commonly suggested as a route for *Wolbachia* transfer (HUIGENS & al. 2000, KITTAYAPONG & al. 2003), so it is interesting to note that the *Wolbachia* appear extremely rare among hymenopterans that parasitize rarely infected aphid hosts (WEST & al. 1998). However, in spite of their exceptional *Wolbachia* prevalence, lice are not known to be attacked by any mites or arthropod parasitoids (as pointed out in KYEI-POKU & al. 2005). This highlights the fact that we know little about the natural routes of horizontal transfer for the ants and the arthropods, in general. Until these are elucidated, we cannot fully assess the viability of the acquisition opportunity hypothesis as a major driver of frequency differences.

(2, 3) Specificity-based mechanisms. While the second and third scenarios (host specific symbionts, coupled with: (2) key symbiont innovations, or (3) host phylogenetic differences) are possible for the ants, it should be noted that their *Wolbachia* symbionts – even those from congeneric species – come from several different clades on the MLST phylogeny (RUSSELL & al. 2009a; Fig. 3). The current lack of genus- or tribe-specific clades suggests that these *Wolbachia* have a more generalized ability to infect ants. In light of this, it is currently difficult to invoke these candidate mechanisms, since their abilities to drive frequency differences among genera or tribes would require specificity at these taxonomic levels. Further assessment of these mechanisms will, therefore, benefit from extensive MLST typing and phylogenetics of *Wolbachia* from under-studied and highly infected genera, such as *Formica*, *Solenopsis*, and *Tetraponera*.

(4) Susceptibility to reproductive manipulators. Low susceptibility (part of scenario four) may play some role in explaining the rarity of *Wolbachia* in some arthropods, such as the aphids (Aphididae) and oak gall wasps (Cynipini). Both reproduce by cyclical parthenogenesis, meaning that their reliance upon asexual reproduction for much of the year should provide little opportunity for invasion by reproductive manipulators (WEST & al. 1998, ROKAS & al. 2002). So could differential susceptibility also explain infection-level variation across ant taxa? As argued in previous publications (WENSELEERS & al. 1998, SHOEMAKER & al. 2000), the ants should be impervious to invasion by feminizers and inducers of parthenogenesis, given their sex determination system and the known mechanisms of symbiont manipulation. Male-killing and CI remain viable alternatives, although neither has been formally demonstrated (see Appendix 4 for more on plausible phenotypes).

But assuming that CI and male-killing can occur, might their efficacy vary across species? While authors have addressed this topic for the ants and other haplodiploids (e.g., SHOEMAKER & al. 2000, VAVRE & al. 2000), the most promising possibility extends from the apparent correlation between *Wolbachia* infection (i.e., presence / absence for a given species) and dependent colony founding, especially budding and fission (see Fig. 2; Appendices 1 & 3; WENSELEERS & al. 1998). The logic behind this connection unfolds as follows. First, many dependent colony founders do not disperse on the wing, migrating to their new nest sites by foot (KELLER 1998). Such limited movement may

1. Does CI occur in the ants? Although this may be difficult to address through mating experiments, one could search for a cytological phenomenon indicative of CI. Specifically, researchers could search for uncondensed and fragmented chromosomes during the first mitotic division (BREEUWER & WERREN 1990, WERREN & al. 2008). To maximize the chances of detecting CI, eggs from uninfected, fertilized queens in the process of colony founding would be most appropriate targets. Furthermore, sampling from populations with intermediate *Wolbachia* prevalence would help to maximize the chance of matings between infected males and uninfected females.

2. Can *Wolbachia* defend ants against natural enemies? RNA viruses represent a leading candidate as a natural enemy that could be warded off by *Wolbachia* infection. Population-level and possibly manipulative, experimental studies could address the likelihood of defensive benefits.

3. Do ants serve as melting-pot hosts? *Wolbachia* genomes undergo frequent recombination (JIGGINS & al. 2001, WERREN & BARTOS 2001), a phenomenon relying upon the occurrence of co-infection (e.g., REUTER & KELLER 2003). Since ants are frequent hosts of multiple *Wolbachia* strains, future studies could address whether they serve as important arenas for genetic exchange. The effects of such recombination on the total *Wolbachia* gene pool will depend upon whether co-infecting strains from ants show the same degree of host specificity as strains from single infections. If so, then we can expect a smaller effect that is largely limited to ant-associated populations.

4. Is loss from workers a common phenomenon? Loss could represent an adaptive strategy to minimize *Wolbachia*-induced detriments on sexual production. It will be important to determine whether curing is under the control of the symbionts or the hosts and, thus, whether it has evolved on multiple occasions.

5. Does the mode of colony founding truly correlate with *Wolbachia* prevalence? Although other features are required to explain the full extent of variation (e.g., *Wolbachia* are prevalent in leaf-cutter ants, which engage in frequent independent colony founding), the rarity of *Wolbachia* in ants that utilize independent colony founding implicates population viscosity or some other correlate of colony founding mode as a determinant of *Wolbachia* invasion. Future screening endeavors should selectively target independent colony founders from across the phylogeny, to increase the statistical power required for a rigorous analysis of this relationship. Further studies on population structure in mtDNA genomes as well as assessments of physiological costs that could impede dispersal flights or successful colony establishment would also help to identify the responsible mechanisms.

6. What other heritable symbionts colonize the ants? Relatives of heritable *Arsenophonus* and *Spiroplasma* have been found in a limited number of ants (NOVÁKOVÁ & al. 2009, FUNARO & al. 2011), while *Bacillus* species were suggested to be vertically transmitted in *Solenopsis invicta* (see TUFTS & BEXTINE 2009). Aside from these microbes and from *Blochmannia* within the camponotines, we know little of the heritable symbionts that colonize these insects. Surveys for *Cardinium*, *Rickettsia*, and other widespread symbiont clades will help to elucidate the diversity of maternally transmitted microbes that utilize ants as hosts.

enable a neutral or slightly detrimental *Wolbachia* symbiont to increase in frequency within small local populations through genetic drift. Should these microbes actually induce CI, this could allow them to more readily exceed the threshold frequency for invasion (e.g., TURELLI 1994). In contrast, queens of independent founders typically engage in dispersal flights prior to colony founding, suggesting that these species should experience less population subdivision and genetic drift. All else being equal, this should make conditions for invasion by a CI-inducing *Wolbachia* more prohibitive (TURELLI 1994, XI & al. 2005), potentially explaining the greater rarity across independently founding species.

A higher incidence of *Wolbachia* across species with high population viscosity is among the leading candidates in our attempts to explain heterogeneous *Wolbachia* distributions across the Formicidae. However, much work remains to be done to more definitively address this hypothesis. First, it is not clear that *Wolbachia* of ants are deleterious inducers of CI and, thus, whether drift is typically required for their spread. Second, viscosity is not the only biological attribute that correlates with mode of colony founding: Independently founding queens expend a great deal of energy in the process of founding and raising young colonies. This expenditure is associated with evolved differences in physiological, morphological, and life-history traits between dependent vs. independent founders (KELLER & PASSERA 1989, 1990, STILLE 1996, HAHN & al. 2004). It stands to reason, then, that if *Wolbachia* impose even a slight physiological cost, successful colony establishment by independent founders could be drastically reduced. Along these lines, such costs could also make independently founding species less able to produce queens, given their known requirements for superior nutritional status (e.g., WHEELER 1986).

While the extent of the correlation between *Wolbachia* incidence and colony-founding mode awaits more definitive confirmation (see Appendix 1), additional details must be resolved to shed further light on the causative mechanisms. For instance, in species with mixed modes of colony founding, are *Wolbachia* enriched within dependently founding populations? Furthermore, is *Wolbachia* commonly found in species that utilize pleometrosis (co-founding by different queens), a type of dependent colony founding that often involves dispersal by flight? Clearly, increased sampling within species, further investigation of *Wolbachia*-induced phenotypes, and a more detailed characterization of colony founding modes and their correlated attributes are needed to untangle this interesting relationship.

(5) **The transport of beneficial phenotypes across species boundaries.** Finally, in attempting to explain why *Wolbachia* prevalence varies across ant (and other arthropod) taxa, we must consider the possibility that these symbionts may actually spread and proliferate because of fitness

benefits that are transportable across species boundaries (OLIVER & al. 2009). Invoking this mechanism to explain frequency differences requires that different groups of hosts experience different selective pressures and ecological challenges, and thus, that they differ in their capacities to benefit from *Wolbachia* infection.

Concrete benefits from *Wolbachia* have, indeed, been documented. For instance, it was recently shown that these symbionts provide their bedbug hosts with B-vitamins that are crucial for their development (HOSOKAWA & al. 2010). Interestingly, *Wolbachia* are common within several groups of arthropods that feed on blood throughout their development, including lice and fleas (infections are also known among kissing bugs and tsetse flies, though they are rare in ticks; Fig. 1; Appendix 2). Genes required for B-vitamin synthesis are encoded by *Wolbachia* from non-blood-feeding hosts (WU & al. 2004, KLASSON & al. 2009), suggesting that this metabolic capacity may be a pre-adaptation enabling *Wolbachia* proliferation in host taxa with vitamin-poor diets.

So what benefits might *Wolbachia* confer upon the ants? A recent series of publications suggests defense as one possibility. In particular, *Wolbachia* symbionts protect their *Drosophila* hosts against RNA viruses (HEDGES & al. 2008, TEIXEIRA & al. 2008), while those in mosquitoes promote upregulation of immunity-gene expression, reducing the loads of *Plasmodium* and fliarial nematodes (KAMBRIS & al. 2009, 2010). Similar studies on other bacteria have found defense to be a common theme for symbioses between arthropods and heritable bacteria (e.g., OLIVER & al. 2003, 2009, JAENIKE & al. 2010). Since ants are attacked by parasitoids and RNA viruses, among other natural enemies (VALLES & HASHIMOTO 2009, YANG & al. 2010), it is quite possible that *Wolbachia* could play defensive roles in these insects. So might *Wolbachia* proliferate among related hosts due to a capacity to defend against an array of shared, or related, natural enemies?

The *Solenopsis invicta* system offers a promising opportunity to examine this phenomenon, as the prevalence of both *Wolbachia* and virulent RNA viruses vary between populations (VALLES & al. 2009, YANG & al. 2010). While it is too soon to establish any correlations, early returns from these studies suggest that the Argentinian site with the highest prevalence of virulent RNA viruses (Santa Fe) also had the lowest *Wolbachia* frequency (VALLES & al. 2009, YANG & al. 2010). This certainly opens the door to the possibility that *Wolbachia* could be playing defensive roles in ants.

Conclusions

In summary, *Wolbachia* are likely the most common bacteria found across the ants, infecting over 34% of species from the Formicidae. Their prevalence varies considerably among different ant taxa, and it is possible that these differences extend from differential susceptibility to invasion. But proliferation due to mutualistic benefits, including defense against shared natural enemies, should be further considered as a consequence of *Wolbachia* infection and, thus, a possible driver of frequency differences.

Regardless of the causes, studies on ant associates have provided us with important and unique insights into the relationships between *Wolbachia* and their arthropod hosts. Future discoveries on their actual phenotypic effects, the

significance and causes of frequent multiple infection, and the generality of potentially adaptive loss from host workers (Box 2) will tell us much more about *Wolbachia*'s impacts and novelties, revealing their significance within this diverse, abundant, and influential group of eusocial insects.

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